AN INVESTIGATION OF PREGNANCY PROTEINS IN THE HORSE (Equus caballus)

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ABBREVIATIONS

AFP	α - feto protein
BPB	bromophenol blue
BSA	bovine serum albumin
DHT	dihydrotestosterone
eCG	equine chorionic gonadotrophin
EDTA	ethylenediamine tetraacetic acid
FSH	follicle stimulating hormone
hCG	human chorionic gonadotrophin
hPL	human placental lactogen
L.H.	Luteinizing Hormone
MPP 1	mare pregnancy protein 1
N.A.A.C.	negative antibody affinity chromatography
OPAA	ovine pregnancy associated antigen
OTP	ovine trophoblast protein
PAGE	polyacrylamide gel electrophoresis
PAPP A	pregnancy associated plasma protein A
PAPP B	pregnancy associated plasma protein B
PBS	phosphate buffered saline
PMS	pregnant mare serum
PZP	pregnancy zone protein
SDS	sodium dodecyl sulphate
SHBG	sex hormone binding globulin
TEMED	NNN'N' tetramethylethylene diamine

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SUMMARY

Proteins, either specific to, or associated with, pregnancy, have been reported in the circulation of various mammalian species, especially man, where most of them reach peak concentrations at term. However the variety of species studied is limited. Therefore the existence of similar proteins was investigated in the horse, a species which exhibits many unusual features of pregnancy. The investigation used similar immunological methods to those which had been successful in other species. However they failed to reveal any pregnancy specific or pregnancy associated proteins in the maternal circulation in late pregnancy. The existence of pregnancy proteins in the mare was further investigated using antisera to placental extracts and two dimensional electrophoresis of pregnancy serum. The former identified unique proteins in the equine placenta at term but these were absent from the maternal circulation. The latter technique confirmed the results of the immunological investigation on pregnancy serum that pregnancy specific proteins were absent in late gestation. It did however identify pregnancy specific proteins in the early stages of gestation. Investigation of two other parameters, serum alkaline phosphatase activity and sex hormone binding capacity, during pregnancy in the mare showed that although serum alkaline phosphatase activity varied it did not do so in a manner related to placental growth and that a protein similar to human sex

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sex hormone binding globulin was absent from equine serum.

On the basis of the results various theories could explain the lack of pregnancy proteins in the late PMS. Of these the most probable explanation was that the comparative placentation of the horse and of humans could cause the difference in the presence of these plasma proteins.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Pregnancy has been one of the most studied phases of mammalian life cycles but the means whereby the maternal/fetal inter-relationship is maintained are by no means elucidated. A feature of pregnancy in many species is the appearance of proteins in the maternal circulation which are not normally present. The first of these so called pregnancy proteins to be identified was in humans by Aschheim and Zondek (1927). Since then other such proteins have been discovered in a variety of species. In humans many pregnancy proteins have been discovered in the maternal circulation throughout pregnancy and much is known about their chemistry, biology and clinical significance.

Pregnancy proteins in laboratory animals, such as primates and rodents, have also been well documented in the hope of providing models for the study of human pregnancy proteins.

Although pregnancy proteins have been identified in domestic animals, such as sheep, horses, cows and swine, only the early stages of gestation have been thoroughly investigated. In a review of placental proteins Klopper (1980) states that these proteins are likely to "occur widely in mammals, which argues that they perform some essential function in pregnancy". That placental

placental pregnancy proteins similar to those in humans have not been identified in domestic animals possibly reflects the fact that they have not been investigated in these species. This research was therefore undertaken to investigate the proteins in late pregnancy serum from mares and to identify any pregnancy proteins present.

The following introduction has been divided into four sections. The first section deals with human pregnancy proteins and summarises the principal features of these proteins. The second section reviews the pregnancy proteins of primates and rodents and how they relate to the pregnancy proteins in humans. The third section demonstrates how research into pregnancy proteins in domestic animals has concentrated on the early stages of gestation and particularly on their involvement in one of the fundamental features of all pregnancies, the maternal recognition of the fertilised egg. Finally a summary of equine pregnancy is given, highlighting those features which are peculiar to the genus Equidae.

1.2 Human Pregnancy Proteins

The first pregnancy protein to be discovered in humans was chorionic gonadotrophin found by Aschheim and Zondek (1927). After a gap of some 50 years, two other pregnancy proteins were discovered. These were Pregnancy Zone Protein (PZP) (Smithies, 1959) and Placental Lactogen (hPL) (Josimovich and MacLaren, 1962). Only in the last 14 years,

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years, with the advancement of immunological techniques, have numerous other pregnancy associated proteins been identified.

The history of the new pregnancy proteins started with the publication by Tatarinov and Masyukevich (1970) who identified, immunologically, a globulin with β_1 electrophoretic mobility unique to the serum of pregnant women. Using an antiserum to placental extract Bohn (1971) identified the same protein which he named schwangershafts protein 1 (SP1). Four proteins were identified by Gall and Halbert (1972) in late pregnancy serum which could not be detected in non-pregnant subjects. Subsequent examination revealed that only two of these proteins were previously unreported and designated Pregnancy Associated Plasma Proteins A and B (PAPP A and B) with the other two being SP1 and hPL (Lin et al, 1974a). An immunological technique was used to identify these four proteins. An antiserum was raised in rabbits against late pregnancy serum and then adsorbed with non-pregnancy serum leaving only antibodies to those proteins peculiar to the pregnancy serum. These antibodies were then used to identify the proteins specific to pregnancy serum by immunological techniques such as crossed immunoelectrophoresis.

Continuing the work on human placental extract, Bohn (1972a) identified another protein unique to pregnancy. This protein, one of a host isolated from the placental extract and designated Placental Protein 5 (PP5), is a

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a soluble placental tissue protein (Sedlacek et al, 1976) which can be measured in the maternal circulation by radioimmunoassay (Klopper, 1980).

Other proteins exist which are pregnancy specific or have close associations with pregnancy and these include placental alkaline phosphatase, early pregnancy factor and sex hormone binding globulin. Recent work on immune complexes in pregnancy serum has revealed two new pregnancy associated proteins, namely pregnancy associated β_1 and α_2 macroglobulins, (Stimson and Farquharson, 1982) and perhaps, as techniques become more sophisticated, other pregnancy proteins will be identified in humans.

On the basis of their origin human pregnancy proteins may be divided into two groups:- proteins synthesized by the fetal/placental unit and those proteins which are synthesized by the mother. The principal proteins in the former group are hCG, hPL, SP1, PAAP A, PAPP B, PP5, placental alkaline phosphatase and α-fetoprotein while in the latter group the major proteins are PZP and SHBG.

Placental Proteins SP1

(a) Purification.

Various procedures exist for the isolation of SP1. The protein was purified from placental extract by Bohn (1976) using a combination of ammonium sulphate precipitation, immunoadsorption and hydroxyapatite chromatography. Although the

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TABLE 1.1 PHYSICO-CHEMICAL PROPERTIES OF HUMAN PREGNANCY PROTEINS

Protein	IEP Mobility	Estimated Mol. Wt.	pI	Post Partum Half Life	Peak in Gestation (µg/ml)
SP1	β_1	90,000* 110,000	3.8	1-2 days	Late Third Trimester (200)
PAPP A	α_2	750,000	4.4	2-4 days	Day of Delivery (400)
PAPP B	β_1	1,000,000	4.6 - 5.0	< 1 day	Third Trimester ?
hPL	α_2	20,000	5.7	20 mins	Mid Third Trimester (7)
PP5	β_1*	36,600*	4.6*	-	Third Trimester ≠ (0.045)
PZP	α_2	300,000 - 506,000	4.4 - 4.8	1-3 weeks	Third Trimester (850)

All information from Lin et al (1982) except SP1 and PP5*, Bohn, 1979 and PP5≠, Klopper, 1980

the process yielded 99% purity the procedure required a high degree of technical skill (Tatarinov, 1982). An alternative technique was employed by Sutcliffe (1976) involving late pregnancy serum applied to an affinity chromatography column containing immobilised antibodies to non-pregnancy serum. A novel and original method, protected by patent, exists which relies on the fact that SP1 binds to concanavalin A to give soluble complexes. Prior treatment to remove other con. A binding glycoproteins from the serum followed by affinity chromatography using a con. A sepharose carrier results in purification of SP1 (Tatarinov, 1982).

The main physico-chemical properties are given in Table 1. Although the protein has an isoelectric point of pH 4.15 (Bohn, 1976), a minor component of isoelectric point pH 6.0 has been observed (Lin et al, 1974b). This suggests some degree of microheterogeneity which may be due to carbohydrate content. In late pregnancy SP1 has been shown to be heterogeneous with a β_1 glycoprotein (SP1 β) and a high molecular weight α_2 glycoprotein (SP1 β) being identified (Teisner et al, 1978 and Westergaard et al, 1979). Immunoreactive SP1 can be detected in serum complexed with normal serum components (Hindersson et al, 1981) and in pregnancy urine (Bohn, 1979).

(b) Physiology.

The purification of SP1 has led to the production of high specificity antisera and their use in immunoassays to monitor the concentration of SP1 throughout pregnancy. Originally SP1 levels were measured using either radial immunodiffusion or Laurel-type rocket immunoelectrophoresis (Bohn, 1972b, Bruce and Klopper, 1978). However these techniques only allow levels of SP1 to be measured after the tenth week of pregnancy. For studies on SP1 in early pregnancy a radioimmunoassay was developed (Grudzinskas et al, 1977a). Using this assay SP1 can be detected in the circulation early in pregnancy, at 18 to 23 days after the L.H. surge which precedes ovulation (Grudzinskas et al, 1977b). This is slightly later than the β subunit of hCG used for pregnancy diagnosis. However it is possible that the proteins are produced together by the pre-implantation embryo, the difference being due to the greater sensitivity of the h C G β subunit assay (Klopper, 1980).

The increase in concentration of SP1 in the first 10 weeks is rapid which probably reflects the proliferation of the trophoblast (Grudzinskas et al, 1977, Klopper, 1980). During the second trimester the increase in serum concentration is much slower followed by a more rapid rise in the third trimester before reaching a plateau at the

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the 37th week. This pattern is similar to the growth curve of the placenta and it may be that SP1 concentrations are related to the mass of functioning trophoblast at any stage of gestation (Klopper, 1980). The peak concentration at 37 weeks is 200 µg/ml. and the half life, calculated from its rate of disappearance after delivery, is approximately 22 hours (Klopper, Buchan and Wilson, 1978).

Using radioimmunoassay SP1 has been demonstrated in males and non-pregnant females at concentrations up to 0.5 mg/100 ml. (Wurz, 1979 and Rutanen and Seppala, 1980). This extra placental SP1 is believed to originate from fibroblast cells (Rosen et al, 1979 and Engvall et al, 1979).

The role of SP1 in pregnancy remains unknown. However several suggestions based on experimental observations have been made but these lack definitive proof. A possible role of SP1 in carbohydrate metabolism has been suggested based on the relationship between SP1 concentrations and maternal glucose levels (Tatra et al, 1976). The possibility that SP1 is an oestrogen binding protein has also been considered (Bohn, 1973).

The prevention of immunological rejection of the foetus by the mother is a phenomenon which has not

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not been elucidated and SP1 has been suggested as a possible immunosuppressive agent. Suppression of mitogen stimulated lymphocytes by physiological concentrations has been demonstrated (Tatarinov et al, 1980, and Golovistikov et al, 1980). However, Stimson (1980) showed that removal of this protein did not significantly alter the immunosuppressive properties of pregnancy serum.

Pregnancy Associated Plasma Proteins A and B

The studies on PAPP A and B have followed a similar pattern to those of SP1. The proteins have been isolated in a number of ways but most are based on a combination of protein separation techniques (Sutcliffe et al, 1979, Bischof, 1979, Lin et al, 1982, Davey and Teisner, 1982). PAPP A (Table 1) is believed to be a dimer with subunits of molecular weight 400,000 (Bischof, 1979). Although some similarity between PAPP A and α_2 macroglobulin has been noted (Bischof, 1979) on peptide mapping, the proteins proved to be very different (Sutcliffe et al, 1980).

(a) Physiology of PAPP A and B.

PAPP A can be detected at 5 weeks post fertilisation and reaches a concentration of 0.5-4 mg/100 ml. at term (Bischof et al, 1981). During early pregnancy the concentration of PAPP A in the maternal circulation follows the placental weight curve. Typically the concentration increases steeply in early

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early pregnancy, slowly in the second trimester and more rapidly in the third. However at 37 weeks when placental weight reaches a plateau concentrations of PAPP A continue to rise (Klopper, 1982). It has been claimed that there is a further rise preceding the onset of labour (Smith et al, 1981). The fact that PAPP A concentrations rise after placental growth has ceased suggests some extra-placental source of this protein and it is probable that the decidua produces PAPP A (Bischof et al, 1982).

PAPP B (Table 1) is a large glycoprotein which is eluted in the IgM fraction during gel filtration of pregnancy serum on Sepharose 4B (Halbert and Lin, 1979). It can be separated from PAPP A by its insolubility in 30% ammonium sulphate, PAPP A being soluble (Halbert and Lin, 1979).

Due to the insensitivity of the immunoelectrophoretic assay for PAPP B its presence in the maternal circulation cannot be detected until the second trimester. The concentration rises gradually in the second trimester, steeply in the third before reaching a plateau about the 37th week. The kinetics are similar to those of SP1 and correspond to the placental weight curve (Lin et al, 1982). It has a post partum half life of less than one day.

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The functions of PAPP A and B are as yet unknown although various suggestions have been made. In preliminary studies both PAPP A and B could bind steroid hormones but the binding was too weak to be specific (Lin et al, 1982). Both anti-plasmin and anti-urokinase activity have been reported for PAPP A as well as inhibition of complement mediated haemolysis using purified PAPP A (Bischof, 1981). Both PAPP A and B demonstrate some degree of in vitro immunosuppression (Kiefer, 1977). However, although both proteins suppressed the stimulation of lymphocytes by phyto-haemagglutinin the degree of immunosuppression was unable to account for that exerted by term pregnancy serum.

Placental Protein 5

One further protein has been added to these pregnancy specific proteins, Placental Protein 5. Isolated immunologically from protein fractions of placental extract with a host of other tissue antigens, this protein was found to be unique to the placenta. The other placental proteins were found in other adult and fetal tissues (Bohn, 1979). Although PP5 may be a tissue specific protein, it can be detected in the maternal circulation at 8-10 weeks of gestation by radioimmunoassay (Seppala et al, 1979, and Obiekwe et al, 1979). The concentration of PP5 increases with pregnancy reaching a plateau of 4.5 µg per 100 ml in

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in the last 4 weeks of pregnancy (Seppala et al, 1979, and Obiekwe et al, 1979). The physico-chemical properties of PP5 are given in Table 1.

The fact that PP5 levels are higher in pregnancy serum than in pregnancy plasma gives some insight into its possible function (Obiekwe et al, 1979). The reason for this is that PP5 forms large molecular weight complexes with heparin which can be reversed by protamin (Salem et al, 1980). The fact that PP5 also has anti-plasmin and anti-trypsin activities (Bohn and Winckler, 1977) and that it interacts with thrombin (Salem et al, 1981) has led to the hypothesis that PP5 plays a part as a natural blood coagulation inhibitor at the placental site.

Placental Lactogen

Human placental lactogen (hPL) is a small protein of molecular weight 21,000 (Table 1) and no carbohydrate content (Niall et al, 1973). The normal rate of synthesis of hPL in late pregnancy has been estimated at 1-2 g/day (Beck et al, 1965, and Kaplan et al, 1968) and it has a biological half life of 10-20 mins. (Chard, 1982a). The maternal concentration of hPL shows a similar pattern to SP1 and PAPP B and the levels correspond to the placental weight curve (Chard, 1982a).

A large number of biological activities have been

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been proposed for hPL including lactogenesis, growth promotion, stimulation of the corpus luteum, erythropoiesis, inhibition of fibrinolysis, an effect on carbohydrate and lipid metabolism and immunosuppression (Chard, 1982a). Although these activities have been demonstrated in experimental systems the evidence that hPL has specific and essential functions in normal pregnancy is very limited (Letchworth, 1976, and Chard, 1982a).

Chorionic Gonadotrophin

Human chorionic gonadotrophin (hCG) is a trophoblastic glycoprotein hormone with a molecular weight of 38,000 and a carbohydrate content 30%. The protein consists of two dissimilar subunits designated α and β (Tojo et al, 1982). The use of hCG in pregnancy diagnosis stems from the fact that it can be detected in the maternal circulation 9 or 10 days post fertilisation. Thereafter the concentration rises rapidly reaching a peak at the 8th to 10th week of pregnancy before declining to 10% of its original value, at which it remains, until term. In contrast free hCG α levels rise continuously until term whereas the free β subunit concentration remains low (Ashitaka et al, 1974).

The strong luteotrophic function of hCG in the human female plays an important role in maintaining the function of the corpus luteum in early pregnancy (Ashitaka et al, 1973).

Placental Alkaline Phosphatase

Another protein which can be detected in the maternal circulation during pregnancy is the placental alkaline phosphatase. The placental form of this enzyme is readily distinguishable from other serum isoenzymes by its remarkable thermostability (Neale et al, 1965). It can also be distinguished immunochemically from other serum isoenzymes (Boyer, 1963, and Sussman et al, 1968). The placental isoenzyme appears to be a late evolutionary event since similar analogues have only been identified in chimpanzee and orangutan (Doellgast and Benirschke, 1979). The placental isoenzymes which have been identified in other mammalian species have properties similar to the liver isoenzyme (Goldstein and Harris, 1979).

In the normal healthy adult basal levels of the placental alkaline phosphatase (2 ng/ml) have been detected by a highly sensitive radioimmunoassay (Wada et al, 1979). During pregnancy the levels of placental alkaline phosphatase in the maternal circulation rise to reach a value of 150 to 400 ng/ml by the third trimester (Holmgren et al, 1978). The increased rate of production correlates with the growth of the placenta (Fishman et al, 1968).

Production of Placental Proteins

Direct evidence for the placental production of the various pregnancy proteins has only been

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been produced for hPL. Using a radioactive gene probe, mRNA for hPL was identified exclusively in the syncytiotrophoblast (McWilliams and Boime, 1980). The evidence, that the other proteins mentioned are placental in origin, stems from immunofluorescent studies which have shown SP1 (Bohn, 1972a, Horne et al, 1976, Lin and Halbert, 1976, and Tatarinov et al, 1976), PAPP A (Lin and Halbert, 1976), PP5 (Bohn, 1976), hCG (Dreskin et al, 1970) and placental alkaline phosphatase (Dempo et al, 1980, and Kameya et al, 1973) localised within the trophoblast mainly in the syncytiotrophoblast. The evidence for the placental production of PAPP B is circumstantial and relies upon the fact that, like SP1, hPL and PAPP A, it can be isolated from placenta in concentrations significantly higher than could be accounted for by its content in maternal serum (Halbert and Lin, 1979).

There is now considerable evidence that the placental proteins are produced in normal non-pregnant adults. SP1, hPL, hCG and PP5 can all be detected in seminal plasma by radioimmunoassay (Lee et al, 1983). The reason for their presence in seminal fluid is unknown but they are not produced by testis or sperm since the level in vasectomised and non-vasectomised males are similar (Chard, 1982b).

The factors which control the placental production of these proteins are unknown. However a novel idea,

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idea, proposed by Chard (1982b) states that the levels of pregnancy proteins in the maternal circulation are a product of the mass of functioning trophoblast and the maternal blood flow at the placental/maternal interface, the syncytiotrophoblast. An increase in blood flow at the placental interface increases the rate of removal of the proteins and stimulates their rate of synthesis. However this hypothesis does not explain, in the case of SP1, why the concentration is higher in the maternal peripheral circulation than in the retroplacental blood (Klopper et al, 1979). This observation could be explained by the fact that rather than direct secretion into the maternal blood by the chorionic villi the point of entry for SP1 is disintegrating migrating trophoblast (Klopper et al, 1979).

A further observation is that no definite function has been proven for any placental protein in vivo and it is possible that "they are by-products of a more fundamental process concerned with the basic functioning and maintenance of the placenta as an individual and independent organism" (Gordon and Chard, 1979). Combining these two ideas it is possible to arrive at the conclusion that placental proteins perform their function in the placenta and that their presence in the maternal circulation is unnecessary for pregnancy. The observations that an almost complete deficiency of hPL and SP1 may be associated

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associated with a normal pregnancy perhaps adds some weight to this hypothesis (Gaede et al, 1978, and Grudzinskas et al, 1979).

Clinical Use of Placental Proteins

Despite the failure to elucidate any in vivo function for the placental proteins, their clinical implications as monitors of pathological pregnancies are numerous. On clinical use and suggested function, the placental proteins can be divided into two groups (Chard, 1982b). Group 1 consists of proteins with possible hormonal/ enzymatic functions such as placental alkaline phosphatase, hCG, hPL and SP1. The circulating levels of these proteins are reduced in the presence of fetal pathologies such as growth retardation and acute fetal distress. Measurement of hPL and SP1 as obstetric parameters for identifying fetal risk has been well established (Gordon et al, 1978 and Grudzinskas et al, 1981) and may be of real value in reducing perinatal mortality (Spellacy et al, 1975).

Group 2 proteins consists of placental proteins with a probable local function such as PP5 and PAPP A. Their concentration in the maternal circulation is raised in the presence of placental pathologies such as placental abruption and pre-eclampsia. However more research is required into the relationship of these proteins with placental pathologies before they are accepted for routine obstetric practice.

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Placental Proteins as Tumour Markers

Pregnancy proteins have been used as tumour markers for both trophoblastic and non-trophoblastic cancers. Tatarinov et al (1974) were the first to show the presence of SP1 in 30% of patients with chorionepithelioma and other trophoblastic tumours using an immunodiffusion technique. Since then pregnancy proteins have been detected in patients suffering from a number of malignant tumours, including those of breast, lung, bowel, ovarian (epithelial), testis and melanoma (Sheth et al, 1974, Vaitukaitis et al, 1976, Rosen et al, 1975, Stone et al, 1977, Tatarinov and Sokolov, 1977, Samaan et al, 1976, and Grudzinskas et al, 1980).

Pregnancy Proteins of Non-Placental Origin

(a) Pregnancy Zone Protein.

The pregnancy zone protein (PZP) was first identified in the sera from pregnant women by starch gel electrophoresis (Smithies, 1959). Since then this protein has been identified independently by various groups and has therefore collected numerous synonyms, including pregnancy associated macroglobulin, pregnancy associated α_2 glycoprotein, pregnancy associated globulin, serum factor Xh and α_2 pregnoglobulin. The physico-chemical properties of PZP are given in Table 1. Although PZP is found in both men and women, during pregnancy the concentration rises

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rises dramatically to exceed all other pregnancy protein concentrations (von Schoultz and Stigbrand, 1982). During pregnancy, levels begin to rise probably around the time of implantation. In the first trimester the increase is exponetial with the rate of increase slowing down during the second and third trimester (von Schoultz, 1974). There is considerable variation between individuals in the concentrations of this protein at term, the range being 20-400 mg/ 100 ml. with a mean value of 100-200 mg/100 ml. (von Schoultz and Stigbrand, 1982). The post partum concentration decreases slowly and reaches pre-pregnancy concentrations at about 6 weeks (von Schoultz, 1974).

The site of production of PZP is not the feto-placental unit but the mother. In tissue culture PZP has been produced by leucocytes (Stimson and Blackstock, 1975) and by macrophages from liver, spleen and reticulo-endothelial tissue (Lundgren et al, 1979). Immunofluorescent studies have provided strong evidence that hepatocytes produce PZP and it is most likely that the liver is the principal source of PZP (von Schoultz and Stigbrand, 1982).

The function of PZP remains unknown although it has been shown to be immunosuppressive (von Schoultz et al, 1973, Damber et al, 1975, and Stimson, 1976).

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Although PZP concentrations increase during oestrogen therapy it is unlikely that it acts as a transport protein for steroid hormones since it binds them with low affinity (von Schoultz and Stigbrand, 1982). An interesting suggestion is that it may act as a feeder protein providing the fetus with amino acids by its degradation in the placenta (von Schoultz and Stigbrand, 1982).

Sex Hormone Binding Globulin

Sex hormone binding globulin is a normal serum protein which binds reversibly and with high affinity the main biologically active androgen, testosterone and somewhat less well the active oestrogen, oestradiol (Anderson, 1974). The protein is a β-globulin and from the various molecular weight estimates, 52,000 (Mercier-Bodard et al, 1970) and 95,000-115,000 (Van Baelen et al, 1968, Rosner et al, 1969, Corvol et al, 1971, and Hansson et al, 1972) it appears to exist as a dimer. Assays for SHBG are usually based on tritiated 5α dihydrotestosterone since it has higher affinity for this steroid than for testosterone and DHT does not bind to the corticosteroid binding globulins (Anderson, 1974). The production of SHBG by the liver is controlled by the hormones it binds, i.e. oestrogens stimulate its production and androgens inhibit (Pearlman et al, 1967, and Vermeulen et al, 1969). Levels of SHBG rise markedly in pregnancy to some 5-10 times their original value and remain

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remain high until term and thereafter fall to their original value with a half life of 5 days (Anderson, 1974).

1.3 Pregnancy Proteins in Laboratory Animals

Although the majority of research into pregnancy proteins has been performed in humans, researchers have investigated proteins of a similar nature in laboratory animals such as primates and rodents. The aim has been to provide models, based on laboratory animals, to assist in the study of pregnancy proteins in humans.

Primates

Proteins immunochemically related to SP1 have been identified in the placentae and plasma of non-human primates. The analogues identified in apes and monkeys show only partial immunological identity with human SP1. The proteins in apes and monkeys are however trophoblast - specific since they cannot be detected in the non-pregnant state (Bohn and Ronneberger, 1973, Bohn et al, 1976, Stevens et al, 1976).

Using an immunoadsorption technique the proteins antigenically related to SP1 have been extracted from the placentae of apes and monkeys. The proteins isolated from chimpanzee, rhesus and cynomolgus monkeys had faster electrophoretic mobilities than

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TABLE 1.2 COMPARISON OF HUMAN AND PRIMATE SP1

Physical Properties	Rhesus SP1	Human SP1
Sed. Coeff	3.85	4.55
Mol. Wt.	80,000	90,000
pI	3.8	4.1
IEP mobility	$\beta_1 - \alpha_2$	β_1

from Bohn, 1979

than human SP1 on polyacrylamide gels and were more acidic (Bohn et al, 1976). A comparison of the properties of human SP1 and rhesus SP1 is given on Table 2.

Since SP1 is produced around the time of implantation there is the possibility that it could be an appropriate target for immunological destruction of an early pregnancy. Experiments using active or passive immunisation with human SP1 in cynomolgus monkeys have demonstrated an anti-fertility effect (Bohn and Weinmann, 1974 and 1976).

The cross reactivity of anti-human PAPP A and B antisera has also been tested in subhuman primates (Lin and Halbert, 1978). Cross reactivity was detected in pregnant chimpanzee, orangutan and old World monkeys which was undetectable in non-pregnant animals. The cross reactivity obtained with pregnant chimpanzee and orangutan showed immunological identity with human PAPP A and B while that of old World monkeys was only partial. Similar reactions were observed with antisera to SP1 and placental lactogen. No cross reactivity was obtained using antisera to human PAPP A, B, SP1 and hPL in the sera of pregnant new World (squirrel) monkeys.

Using an antiserum to human pregnancy zone protein von Schoultz et al (1976) demonstrated cross reactivity with serum from pregnant Rhesus monkeys. A PZP

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PZP analogue was also demonstrated in various other old World monkey species. However in some species there was a decrease in concentration during pregnancy (Lin et al, 1976a).

Pregnancy Proteins in Rodents

Using a hyperimmune antiserum raised against late pregnant rat serum and adsorbed with non-pregnant rat serum, Lin et al (1974c) were able to demonstrate four pregnancy associated plasma proteins. The antisera to human pregnancy associated plasma proteins did not cross react with pregnant rat serum and the antiserum to rat pregnancy proteins did not cross react with human pregnancy serum. An interesting point is that the pregnancy protein in the rat were undetectable in pseudopregnant rats (Lin et al, 1974c).

By the same technique, four murine pregnancy associated proteins were identified (Lin et al, 1974c). The antisera to the murine pregnancy proteins did not cross react with any proteins in rat serum.

Independently four murine pregnancy proteins have been identified and characterised (Hau et al, 1978, Hau et al, 1980, and Hau, 1982). The relationship between these four proteins and those identified by Lin et al (1974c) is unknown (Waites and Bell, 1984). Owing to the similarity in placentation between humans and mice (hemochorial) and because the fecundity of mice is high an attempt has been made to devise a model for the study of human pregnancy proteins based on mice (Hau,

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TABLE 1.3 PROPERTIES OF MURINE PREGNANCY PROTEINS

Protein	Cross Reacts With	Mol. Wt.	pI	IEP Mobility	Half-Life
PAMP 1	human PZP	150,000	4.2	α_1	26 hrs
PAMP 2	human SP1	80,000	4.0	α_2	13 hrs
PAMP 3	-	62,000	4.1	Alb	-
PAMP	human AFP	70,000	4.4	α_1	15 hrs

(Hau, 1982). The proteins have been termed pregnancy associated murine proteins 1, 2, 3 and 4 (PAMP 1-4) and three of these appear to be analogues of human pregnancy proteins. Using antisera raised in rabbits, only PAMP-4 showed immunological cross reactivity with human AFP (Hau et al, 1981a). However when antisera against murine and human pregnancy proteins raised in chickens was used, immunological cross reactivity was found between human PZP and PAMP-1 (Hau et al, 1981b) and between human SP1 and PAMP-2 (Hau et al, 1980). Some of the properties of PAMP 1-4 are given in Table 3. Apart from the discrepancies between the molecular weight of PAMP-1 and that of hPZP and the concanavalin binding properties between AFP and PAMP-4 the physico-chemical properties of the analogues are similar (Hau, 1982). The levels of PAMP 1 and hPZP are similar during the first half of pregnancy but in the second half hPZP levels remain high while those of PAMP-1 fall (Hau et al, 1981b). Both proteins show large variations between individuals and both are present in non-pregnancy serum at low levels (Hau, 1982). The levels of SP1 and PAMP-2 are similar throughout pregnancy (Hau et al, 1980). PAMP-4 and AFP levels in the maternal circulation both show a high degree of correlation with the fetal weight throughout pregnancy (Hau et al, 1981a). At present, no human analogue to PAMP-3 has been identified although there is the possibility that it is similar to human sex hormone binding globulin (Hau, 1982).

1.4 Pregnancy Proteins in Domestic Animals

The majority of research into pregnancy proteins in domestic animals has tended to concentrate, for agricultural reasons, on the successful establishment of pregnancy. Only the early stages of gestation in the cow, sheep, horse and pig have been thoroughly investigated, there being little documentation on the later stages.

Studies on pregnancy associated proteins have revealed tissue antigens associated with pregnancy in a number of animals but apart from hormones of placental origin few proteins comparable to the human series have been found.

Sheep

Using an antiserum raised against day 14 sheep embryos Cerini et al (1976) were able to demonstrate pregnancy associated antigens in the corpus luteum, uterus and embyronic membranes by immunofluorescence. The antigens were also detected in plasma and erythrocytes by haemagglutination at day 6. The antigens were undetectable in non-pregnant sheep. Continuing this work with calf antisera to day 16 sheep embryos, Staples et al (1978) identified an ovine pregnancy associated antigen (oPAA). The antigen was detected in all samples of trophoblast and uterine tissue and in 94% of uterine flushings from day 14-19 pregnant ewes but not in serum. Attempts to isolate the antigen revealed a high molecular weight fraction and a low molecular weight fraction of 42,600 and 17,400 daltons respectively indicating that the molecule is probably dimeric (Staples,

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(Staples, 1980). The antigen can be detected sufficiently early in pregnancy to be implicated in the prevention of luteal regression and could therefore be partly responsible for the maternal recognition of pregnancy. However the agent responsible for luteal maintenance has disappeared by day 24 (Rowson and Moor, 1967) whereas oPAA can be detected in late pregnancy (Staples et al, 1978).

In the sheep there is little evidence to suggest the presence of a chorionic gonadotrophin with similar properties to those of hCG (Heap et al, 1979) although an hCG like substance has been detected in the early trophoblast tissue of sheep by radioreceptor assay (Wintenberger-Torres, 1978). A placental lactogen has been detected in plasma by day 60 (Kelly et al, 1974) and rises to a concentration of 1000-2000 ng/ml. at term. However the assay used in this case was a mammary gland binding assay utilising ^{125}I labelled ovine prolactin and ovine prolactin. A more recently developed homologous radioimmunoassay (Handwerger et al, 1977) detected oPL at 41-50 days of gestation and the concentration increased progressively to 2547 ng/ml. at 121-130 days gestation in sheep with singleton gestations. Levels in twin gestations were significantly higher. Concentrations of oPL decreased just before partuition (Kelly et al, 1974, and Handwerger et al, 1977). Compared with humans high levels of oPL were detected in cord plasma suggesting

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suggesting that oPL may have a direct action in the fetus during pregnancy. The half life, calculated by surgical removal of the placenta, was 29 mins., similar to hPL (Handwerger et al, 1977).

Work with day 14-16 sheep embryo homogenates and their ability to maintain corpus luteum function in sheep indicated the presence of an antiluteolytic component in the embryos. The fact that the factor is thermolabile and susceptible to protease activity suggests that it is protein in nature. The anti-luteolytic activity had disappeared by day 21-23 since homogenates of this age failed to prevent luteal regression (Martal et al, 1979). Working with in vitro culture products of day 16 sheep embryos, Godkin et al (1984) identified a protein, ovine trophoblast protein (oTP-1), which was capable of maintaining corpus luteum activity. This protein was the major product of the cultured embryos between days 13 and 21 of pregnancy, the period during which the maternal recognition of pregnancy in the sheep takes place. The protein was undetectable in serum and whether or not it is the sole reagent responsible for maintaining corpus luteum activity in early pregnancy is unknown. However, the fact that a non-serum trophoblastic protein appears responsible for luteal maintenance in the sheep (Godkin et al, 1984) is in contrast to primates where a serum chorionic gonadotrophin is implicated in luteal maintenance (Niswender et al, 1972).

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Using cross reacting antisera, von Schoultz et al (1976) reported the detection of an analogue to human PZP in sheep. Antisera to human PZP cross reacted with pregnant dog and monkey serum but not with sheep. However an antiserum to dog PZP showed strong cross reactivity with serum from pregnant sheep.

Apart from oPL and oPZP no other serum pregnancy proteins have been reported in the later stages of gestation in the sheep.

Cow

In the cow Roberts and Parker (1976) detected three proteins which appeared in uterine flushings at day 7 of gestation. At day 15 post fertilisation a pregnancy specific protein with a molecular weight between 50,000 and 60,000 can be detected in the bovine uterus (Laster, 1977).

An antiserum to bovine placental membranes from day 20-45 embryos revealed two pregnancy specific proteins. The proteins, designated A & B, were both located in the endometrium and placenta whereas only A could be detected in amniotic fluid. The proteins were undetectable in the uterine endometrium of non-pregnant cows. The proteins were detected in the placenta from day 25 to day 270 of pregnancy but not in placental membranes from 280 day conceptuses. Partial purification of the proteins revealed that protein

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protein A had a molecular weight of 65-70,000 and an isoelectric point of pH 4.6-4.8 and on Ouchterlony double diffusion showed a reaction of identity with bovine α_1 fetoprotein. Protein B had a molecular weight of 47-53,000 and an isoelectric point of pH 4.0-4.4 but its identity remains unknown. Neither protein could be detected in plasma or on erythrocytes by haemagglutination (Butler et al, 1982).

A bovine placental lactogen (bpL) has been identified and isolated by several research groups (Bolander and Fellows, 1976, and Hayden and Forsyth, 1979). Using a radioreceptor assay, Hayden and Forsyth (1979) monitored placental and plasma levels of bPL throughout pregnancy in dairy cows. Levels in bovine placental cotyledons rose from day 60 to day 200 before falling by day 250. In contrast to this total lactogenic activity in bovine plasma remained below 150 ng/ml. throughout gestation confirming earlier results obtained using a bioassay (Buttle and Forsyth, 1976). The bPL was partially purified and had a molecular weight of 45,000 by gel filtration and an isoelectric point of pH 5.3 (Hayden and Forsyth, 1979). The bPL was found to be equipotent to ovine prolactin in stimulating secretory activity of mouse mammary gland. These findings are in agreement with those of Roy et al (1977) who reported a preparation of bPL with a similar lactogenic potency and which circulated in late pregnancy with a concentration of less than 100 ng/ml. as measured

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measured by a specific radioimmunoassay.

In contrast to this the bPL isolated and purified by Bolander and Fellows (1976) from bovine placenta had a molecular weight of 22,150 by gel filtration. However in SDS gel electrophoresis bPL migrated as if it had a molecular weight of between 59,000 and 62,000. Two bands were obtained when the bPL was subjected to isoelectric focusing, a major band of pI 5.86 and a minor band of pI 6.1. Two peaks of bPL activity were obtained on DEAE cellulose chromatography, bPL1 and bPL2, although no significant difference in amino acid composition was detected between the two forms. The bPL had poor lactogenic potency as measured by radioreceptor assay. Levels of bPL as measured by radioimmunoassay were low for the first two trimesters and then rose sharply to a plateau of 1 μg/ml in the third trimester (Bolander et al, 1976). Higher levels of bPL were detected in animals bearing twins. Levels in dairy cows were greater than those in beef cows and high yielding dairy cows tended to have greater than average serum levels of bPL (Bolander et al, 1976).

There is no explanation available as to the difference between the placental lactogen of Bolander and Fellows (1976) and that of Hayden and Forsyth (1979).

Using an antiserum to dog PZP, von Schoultz et al (1976) were able to demonstrate the existence of a bovine pregnancy zone protein in pregnant cow serum. As with

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with sheep PZP there was no cross reactivity with anti-human PZP.

There are no reports of any other pregnancy associated plasma proteins in late gestation in the cow.

Pig

An analogue to human pregnancy zone protein has been detected in pigs. Originally von Schoultz et al (1976) reported little or no cross reactivity between the antiserum to dog PZP, which cross reacted with pregnant cow and sheep serum, and pregnant sow serum. However in a later study immunoprecipitates obtained between anti-dog PZP and pregnant sow serum were used to raise an antiserum to swine PZP. The anti-swine PZP showed a reaction of identity between dog PZP and swine PZP. However, the levels of PZP were higher in both castrate males (80 Kg body weight) and non-pregnant sows than in pregnant sows 1-2 weeks before farrowing. The significance of this is unknown (Martinsson and Carlstrom, 1977).

Goats

Using an in vitro bioassay high levels of lactogenic activity, immunologically distinct from pituitary prolactin, were detected in pregnant goats. Co-culture of placental and mammary gland tissue indicated the placental origin of this activity which was referred to as caprine placental lactogen (cPL). Using a radio-receptor assay for lactogenic and growth hormone

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hormone activity levels of cPL were measured throughout gestation. The concentration of cPL was low until day 50 (< 50 ng/ml) and thereafter rose to reach a peak in the last trimester of pregnancy (1000 ng/ml). As in the sheep, levels decreased in the last 12 days of pregnancy. The approximate molecular weight of the cPL was 20,000 as measured by gel filtration (Currie et al, 1977). This is the only pregnancy associated plasma protein which has been demonstrated in the goat.

1.5 The Early Pregnancy Factor

A subject which has attracted much interest, both in the human and domestic animal field, is the discovery by Morton et al (1977) of an "early pregnancy factor" (EPF). Using a rosette inhibition assay, which measures the capacity of antilymphocyte serum to inhibit rosette formation between T cells and heterologous erythrocytes (Bach et al, 1969), Morton et al (1974) showed that the rosette inhibition titre (RIT) was dependent on the lymphocytes used. If lymphocytes from pregnant animals or lymphocytes incubated in the serum from pregnant animals were used, the RIT was greater. This observation led to the discovery of the early pregnancy factor. Using the rosette inhibition assay, EPF can be detected in the serum of mice, humans and sheep from 6 to 24 hours (Morton et al, 1976, 1977 and 1979) and in cattle 4 days (Nancarrow et al, 1981) after fertilisation. Partial characterisation of the EPF in sheep has led to the discovery of multiple forms at

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at different stages of gestation (Clarke et al, 1980). Soon after fertilisation a high molecular weight form (250,000) exists which slowly disappears as gestation proceeds to be replaced by a smaller 50,000 form which is prevalent in mid gestation. This situation is similar to that in mice (Clarke et al, 1978). In contrast, however, a transient 20,000 form can be detected in sheep in very early pregnancy. The 250,000 form consists of a carrier component present in normal serum and the 50,000 form. All three forms can be made by two components A and B prepared from early pregnancy serum. Both A and B are required for an increase in RIT (Clarke et al, 1980). EPF levels are high in early pregnancy and steadily decline for the first two-thirds of pregnancy and may thereafter become undetectable (Morton et al, 1977, 1979 and 1982). Component A is synthesized by the oviduct and is dependent on oestrus and pregnancy whereas B is produced by the ovary and is pregnancy dependent. Production of EPF B is the result of the combined action of signals from the pituitary and the zygote (Cavanagh et al, 1982).

A porcine EPF has recently been demonstrated which, although it displays the same characteristic components of other EPFs, its levels during gestation remain high (Morton et al, 1983).

Several reports have been published detailing failure to detect the EPF using lymphocytes from pregnant women or

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or lymphocytes incubated in pregnancy serum (Thomson et al, 1980, and Cooper and Aitken, 1981). However, this failure is possibly due to the inherent complexity of the assay rather than evidence against the existence of the EPF.

At present, the function of the EPF is unknown but it has been shown to be immunosuppressive (Noonan et al, 1979) and may be involved in the acceptance of the fetus by the mother (Morton et al, 1976).

1.6 Equine Pregnancy

In the mare the fertilised egg enters the uterus 5 to 6 days after ovulation. An unusual feature of equine pregnancy is the fact that only fertilised eggs enter the uterus while unfertilised eggs remain in the fallopian tube where they slowly degenerate (van Niekerk and Gerneke, 1966, Betteridge and Mitchell, 1975). Development of the allanto-chorionic placenta is not completed until day 40 post ovulation and the conceptus does not become firmly implanted until day 45-50 with the formation of the allanto-chorionic villi which interdigitate with corresponding crypts in the endometrium. During this time the conceptus lies free in the uterine lumen and may even migrate from one uterine horn to the other (Amoroso, 1952, Bain and Howey, 1975, Ewart, 1897, and Hawkins et al, 1979). The free embryo relies upon the uterus for most, if not all, of its metabolic needs and uterine secretions have been

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been shown to contain proteins which may serve as enzymes and carrier molecules for hormones, vitamins and minerals (Bazer et al, 1978). A number of unique proteins which are not of serum origin are present in the uterus until day 20 of pregnancy (Zavy et al, 1982). One of these proteins has been identified as equine uteroferritin and may be involved in the transport of iron to the embryo (McDowell et al, 1982).

It is during the time that the embryo lies free that one of the most unusual events in equine pregnancy commences. Around day 36 of pregnancy there appears, within the gravid horn of the uterus, densely packed decidual-like cells which form the endometrial cups (Schauder, 1912). The endometrial cups encircle the embryo and steadily enlarge for the next 20 to 30 days. This growth causes them to become raised above the endometrium until day 70 when the cups begin to degenerate and are eventually sloughed off the surface of the endometrium. The mature cup consists of a well demarcated and densely packed mass of very large epithoid decidual-like cells. During their development the cups become surrounded by leucocytes which eventually assist in the degeneration and destruction of the cups by day 160 (Allen, 1980).

Originally the progenitor cells of the endometrial cups were thought to be maternal in origin (Clegg et al, 1954, and Amoroso, 1955) and the conceptus provided the stimulus

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stimulus which transformed endometrial cells into cup cells (Clegg et al, 1954, and Allen, 1970). However recent evidence indicates that the cells are fetal, originating from the chorionic girdle, which rapidly invade and phagocytose the endometrial epithelium and migrate into the endometrial glands and uterine stroma. Here they differentiate into large sessile endometrial cup cells (Moor et al, 1975). The chorionic girdle is formed on the embryo at the interface of the developing allantois and the regressing yolk sac where the outer chorionic cells rapidly divide and become piled on top of one another forming a thickened and plainly visible chorionic girdle (Allen, 1980).

The placenta of the mare is epitheliochorial with six layers of tissue separating the maternal and fetal blood (Grosser, 1909). This is the simplest form of placentation and consists of the apposition of the tissue of the placentas of each organism. The layers are endothelium of blood vessels, connective tissue, epithelium of uterus, trophoblast, connective tissue and endothelium of blood vessels.

The fetal origin of the endometrial cups and the presence of paternal histocompatibility antigens explains why the leucocytes surround the cup in a manner reminiscent of host rejection of a skin allograft (Allen, 1975).

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With the formation of the cups there appears, in the maternal circulation, a glycoprotein hormone, equine chorionic gonadotrophin (eCG), formerly pregnant mare serum gonadotrophin, which the cup cells produce during their lifespan (Allen and Moor, 1972). eCG is a high molecular weight glycoprotein, 53,000 daltons (Gospodarowicz, 1972), which expresses both FSH and LH like biological activities (Stewart et al, 1977). Dissociation of eCG into two subunits has been demonstrated (Papkoff, 1974). Biological activity of the individual subunits was low but on recombination both FSH and LH like activities returned. The isoelectric point of eCG is pH 1.8 (Bourrillon and Got, 1957) and the carbohydrate content is nearly 50% (Bourrillon et al, 1959).

eCG was discovered by its ability to increase the weight of immature rat ovary (Cole and Hart, 1930) and this has formed the basis of a bioassay to quantify eCG (Cole and Ewary, 1941). The sensitivity of the assay is approximately 2 i.u. of eCG. More recently immunological assays have been devised which are more sensitive, including haemagglutination inhibition assays and radioimmunoassay based on equine LH (Allen, 1969, Nett et al, 1975). Levels of eCG are detectable about days 35-40 and rise steeply to reach a peak between days 55-65 before declining to a minimum by day 120 (Allen, 1969). Peak values are variable, i.e. between 11 i.u./ml and 112 i.u./ml (Allen, 1969) and levels have been shown to vary with size, parity, number of fetuses present and fetal genotype (Cole,

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(Cole, 1938, Rowlands, 1963 and Clegg et al, 1962).

The detection of eCG in the maternal circulation corresponds with the appearance of a "crop" of secondary or accessory luteal structures. Plasma progesterone levels, which decline steadily from day 12-14 after ovulation, rise sharply with the appearance of these accessory luteal structures. The fact that the appearance of the accessory luteal structures and their secretion of progesterone coincides with the beginning of endometrial cup formation and their secretion of eCG led to the assumption that eCG was solely responsible for stimulating the luteal structures and maintaining their secretory activity (Amoroso et al, 1948). However isolated experiments and observations have cast some doubt as to whether eCG is solely responsible for maintaining luteal function during pregnancy. In some cases, despite surgical removal or abortion of the embryo, the endometrial cups remain and secrete eCG for up to 100 days. Despite this primary and secondary corpora lutea may regress within 20 days (Allen, 1980). Another observation against the direct luteotrophic action of eCG comes from progesterone levels in twin pregnancies and interspecific mule conceptuses. In the former case, the eCG levels may be very high compared with the norm and in the latter they may be very low due to increased immunological activity against the endometrial cups. In spite of these wide variations in eCG concentrations the plasma progesterone levels are similar to normal intraspecific pregnancies

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pregnancies (Allen, 1975, Day and Rowlands, 1947).

Considerable follicular growth can be detected in the ovaries of 17-25 day pregnant mares, well before the appearance of eCG (Bain, 1967). The 10-12 day waves of FSH that occur during the oestrus cycle in the mare continue into early pregnancy (Evans and Irvine, 1975) and this coincides with the observations of waves of follicular growth during early pregnancy (van Rensburg and van Niekerk, 1968). Despite this follicular growth secondary ovulations do not normally occur until the appearance of eCG (van Niekerk, Thesis). Therefore pituitary FSH must stimulate follicular growth and the LH activity of eCG is necessary to ovulate or luteinise the follicles to form the accessory corpora The action of eCG may help to maintain the secondary corpora it helped to produce but some other unknown fetal/pituitary factor is involved since loss of the fetus leads to regression of the luteal structures (Allen, 1980).

Investigations by Gidley-Baird et al (1983) identified a new protein specific to pregnancy in the mare, Mare Pregnancy Protein I (MPP I). The protein was detected at day 30 of pregnancy and was also demonstrated at days 55 and 68 of pregnancy. The protein was undetectable in non-pregnant mares and stallions. The function of MPP I and its origin are unknown although it cannot be produced by the endometrial cups which are unformed at day 30.

have been investigated during pregnancy in the mare and these include steroid hormones, prolactin and lactogenic activity.

As mentioned previously, plasma progesterone levels decrease 12-14 days post ovulation before rising sharply at day 40 to reach a peak at day 60. Thereafter they decline to a low level by day 110 at which they remain until shortly before term. Prior to partuition there is an increase in progesterone levels (Terblanche and Maree, 1981). Following the demise of the luteal structures, it is assumed that the placenta, which contains high levels of progesterone, supplies sufficient levels of this hormone to maintain the pregnancy (Short, 1958, and Allen, 1984).

At approximately 100 day of pregnancy the fetal gonads undergo tremendous enlargement so that by days 230-250 they are often larger than the maternal ovaries (Cole et al, 1933, and Pashen and Allen, 1979). This growth is paralleled by a sharp rise in conjugated oestrogens in the maternal blood and urine. The principal oestrogens are oestrone and two unique to Equidae, equilin and equilenin (Cox, 1975). Levels of these oestrogens rise rapidly between days 120 and 210. After this the levels fall slowly to partuition and take a precipitous post-partum fall (Nett et al, 1975). Fetal gonadectomy results in a fall in circulating oestrogens which supports the concept of a feto-placental unit for oestrogen synthesis in the mare. The oestrogens appear to play a major role in

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in fetal development since gonadectomy resulted in retarded fetal growth (Pashen and Allen, 1979). The retarded fetal growth may be the result of an alteration in utero-placental blood flow, since studies in sheep indicate that oestrogens and their precursors affect both the distribution and rate of blood flow through uterine and placental blood vessels (Resnik et al, 1974, and Rosenfeld and Worley, 1978).

Attempts to identify an equine placental lactogen have been unsuccessful. Using a co-culture of equine placenta and mouse or rabbit mammary gland, Forsyth et al (1975) were unable to demonstrate the stimulation of the mammary gland by products of the equine placenta. Also using an in vitro bioassay, which had been successful in women (Forsyth and Edwards, 1972) and goats (Buttle et al, 1972), they failed to obtain positive results with equine plasma at any stage of gestation. Partially purified horse prolactin produced responses in the assay indicating that the negative results were not due to the lack of response from the mammary gland. Little variation in prolactin concentration throughout gestation in the mare has been shown by Nett et al (1975).

Using the antiserum to swine PZP, Martinsson and Carlstrom (1977) demonstrated an equine PZP but no further reference has been made to this analogue. Apart from eCG, MPP I and ePZP, no other pregnancy associated serum

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serum proteins have been identified in the mare.

1.7 Aim of Work

The purpose of the following research was to investigate pregnant mare serum and identify any pregnancy associated proteins present. Initially late pregnant mare serum was investigated immunologically following preparation of antisera to whole and fractionated pregnant mare serum. Fractionation was performed using gel filtration and negative antibody affinity chromatography.

Since the source of most pregnancy proteins in humans is the placenta, an equine placental extract was prepared and antisera raised against the whole or fractionated extract. The antisera were used to investigate equine placental extract and late pregnant mare serum for proteins particularly associated with pregnancy.

Serum, from throughout gestation, was then subjected to two dimensional polyacrylamide gel electrophoresis as a further means of detecting the appearance of pregnancy associated proteins in equine serum. Finally two other proteins, (Alkaline phosphatase and sex hormone binding globulin) known to increase in concentration throughout gestation in humans, were investigated in pregnant mares by estimation of their activity in equine serum.

CHAPTER 2

ANTISERA TO PREGNANT MARE SERUM

2.1 Introduction

The identification of the "new" pregnancy proteins in humans has relied upon immunological techniques requiring antisera which are specific to the proteins. The fact that the pregnancy proteins are either only found in, or present in, higher concentrations than normal in the maternal circulation has formed the basis for generating these specific antisera. Using third trimester pregnancy serum (or plasma), where the proteins are at their highest concentration, hyperimmune antisera are raised in a suitable host. These antisera can then be adsorbed exhaustively with normal male or non-pregnant female serum. This removes all the antibodies from the antisera which cross react with antigens common to both non-pregnant and pregnant serum. The antibodies remaining are those directed against antigens which are present only in pregnancy serum. These antibodies can then be used to identify the proteins in the pregnancy serum using standard immunological techniques such as crossed immunoelectrophoresis or Ouchterlony double diffusion. This adsorption technique clearly identified pregnancy proteins in humans (Gall and Halbert, 1972) and was therefore chosen as the principal technique for identifying pregnancy proteins in late gestation in the mare.

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Three methods were chosen in order to obtain antisera for adsorption. The first method was based on that of Gall and Halbert (1972) and used a hyper-immune antiserum to late pregnant mare serum. The other two methods used prior fractionation of the pregnant mare serum before obtaining an antiserum. Fractionation was performed using gel filtration in the first case or negative antibody affinity chromatography. All methods had been successfully applied in humans (Gall and Halbert, 1972, Stimson, pers. com. and Sutcliffe, 1976).

2.2 Materials and Methods

2.2.1 Materials

Serum was obtained from four pregnant mares in the last month of gestation and used to prepare a pool of pregnant mare serum. Pools of stallion serum and non-pregnant mare serum were also prepared from serum obtained from two stallions and two non-pregnant mares. All sera were stored at -20^{o}C until required.

Gel Bond was obtained from FMC Corporation, Marine Colloids Division, Bio-Products, Rockland, Maine. The immersible CX10 units for ultrafiltration were purchased from Millipore Corporation, Bedford, Mass. Sepharose 4B and Sephacryl S300 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, as were the proteins thyroglobulin, catalase and aldolase for calibration of the gel filtration column. The other proteins used for calibration, bovine serum albumin,

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albumin, α chymotrypsinogen and myoglobin were purchased from Sigma Chemical Company Limited, Fancy Road, Poole, Dorset, England, as were the cyanogen bromide, high molecular weight polyethylene glycol and Freund's complete and incomplete adjuvant. Agarose was obtained from Serva, Feinbiochemica, Heidelberg. The MIP-tests for the detection of eCG were provided by Carter-Wallace Inc., Cranbury, New Jersey. All other chemicals and reagents were of analar grade where possible.

2.2.2 General Methods

Ouchterlony double diffusion precipitation tests were performed using a modification of the method of Ouchterlonly (1958). Gels were formed by pouring 10 ml of molten agarose, 1%(w/v) in 0.9% (w/v) saline, onto the hydrophilic side of an 8 cm x 8 cm sheet of Gel Bond on a horizontal surface. Wells are cut in the solidified agarose to take 20 µl (maximum) of antiserum or sample and after addition of these the plates were incubated for 24 hrs at room temperature or 48-72 hrs at $4^{o}C$ in a sealed humid box. Following incubation gels were pressed and washed three times in 0.9% (w/v) saline and three times in distilled water, air dried and stained. The staining solution consisted of 250 mg Crocein scarlet and 15 mg Coomassie brilliant blue in 100 ml of 5% acetic acid and 3% trichloroacetic acid. Destaining was in 3% acetic acid (Crowle and Cline, 1977).

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Immunoelectrophoresis

The method of Grabar and Williams (1953) was used for immunoelectrophoresis (IEP). Gels of 1% (w/v) agarose in electrophoresis buffer, 20 mM sodium barbitone, containing 4.3 mM diethyl barbituric acid at pH 8.6, were cast by pouring 10 ml of agarose on a 5 cm x 9 cm slide of Gel Bond, hydrophilic side up. Serum (10 µl) was added to wells, 0.4 cm diameter, along with 1µl of 0.1% aqueous solution of bromo-phenol-blue dye (BPB). Electrophoresis was performed at 12V/cm until the dye front had moved 4 cm. 200 µl of antiserum was added to troughs cut alongside the wells. The gel was then incubated for 24 hrs at room temperature or 48 hrs at $4^{o}C$ in a sealed humid box. Washing and staining were carried out as described for the double diffusion method.

Crossed Immunoelectrophoresis (XIE)

Crossed immunoelectrophoresis on agarose gels was performed using the method of Eckersall and Beeley (1980). Molten agarose (10 ml) was cast on 8 cm x 8 cm plates of Gel Bond on the hydrophilic side. The agarose was 1% (w/v) made up in electrophoresis buffer. Serum (10 µl) was applied to a 0.4 cm diameter well cut 1 cm from the left hand edge and 0.5 cm from the base. 1 µl of 0.1% (w/v) aqueous solution of BPB was also added. Electrophoresis was carried out at 12V/cm at $4^{o}C$ until the dye front was 6 cm across the gel. After the first dimensional electrophoresis the upper 7 cm of the gel were removed and replaced with 8 ml of agarose

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agarose to which at 56°C, 500-1000 µl of antiserum were mixed. The gel was turned through 90° and electrophoresed for 18 hrs at 4V/cm. Gels were washed and stained as described previously.

Antiserum Adsorption

Two methods were employed to adsorb antisera. In the first method the adsorption was performed overnight at 4°C in an end over end mixer. The second technique involved the antiserum having to stand at 4°C for 48 hrs with occasional mixing. The antibody/antigen precipitates were removed by centrifugation at 13,000 g for 10 minutes. All antisera were adsorbed with either stallion or non-pregnant mare serum (i.e. normal equine serum). The adsorptions were performed in a sequential pattern starting at 5% and ending when the antiserum was fully adsorbed for normal equine serum, i.e. the point at which no precipitin lines were detected between the adsorbed antiserum and normal equine serum by Ouchterlony double diffusion and immunoelectrophoresis. This fully adsorbed antiserum was then used to test pregnant mare serum.

Antibody Enrichment

Fully adsorbed antisera were enriched for immunoglobulins using a modification of the method described by Kekwick (1940). To the antiserum 18% $Na_2 SO_4$ was added and the precipitate obtained by centrifugation. The precipitate was then suspended in as little volume of phosphate buffered saline (5mM potassium phosphate,

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phosphate, 0.16 NaCl at pH 7.4) as possible and dialysed against PBS overnight. This solution was designated antibody enriched and used in Ouchterlony double diffusion, immunoelectrophoresis and crossed immunoelectrophoresis in a similar fashion to the whole antiserum.

Radial Immunodiffusion

Radial immunodiffusion was performed according to the method of Mancini et al (1964). Agarose, 5 ml of 1% (w/v) in 0.9% saline (w/v), was melted and cooled to 56°C. At this temperature 100 µl of antibody was added and the agarose poured onto the hydrophilic surface of a 5 cm square of Gel Bond. After cooling wells, 0.4 cm diameter, were cut in the agarose a minimum of 2 cm apart. Samples to be analysed were applied to the wells (10 µl) and left overnight at room temperature in a sealed humid box. Gels were washed and stained as described in Ouchterlony double diffusion.

SDS Polyacrylamide Gel Electrophoresis

A modification of the method described by Laemmli (1970) was used for SDS polyacrylamide gel electrophoresis (SDS PAGE). Gels were either 8 cm x 8 cm with 1 mm spacers or 20 cm x 15 cm with 0.8 mm spacers. The stock solutions and solutions for gel preparation are given in the appendix. The lower $^{3}/_{4}$ of the gel consists of the resolving gel. This was poured between the plates, overlayed with dist H_2O and

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and left to polymerise. Once polymerisation was complete the water was poured off and the surface of the gel rinsed once with a small volume of stacking gel buffer. Stacking gel was then poured to the top of the plates. Wells were formed in the stacking gel by means of a plastic comb.

Prior to electrophoresis samples were boiled for 2 minutes in sealed containers in an equal volume of solubilizing buffer. Samples (5-15 µl) were then applied to the wells in the stacking gel. Upon dilution of the electrophoresis buffer SDS was added to a concentration of 0.1% w/v. Gels were run at either 15 mA constant current (8 cm x 8 cm gels) or 25 mA constant current (20 cm x 15 cm gels). Electrophoresis was stopped when the BPB dye front was 1-3 cm from the end of the gel. Staining was performed in 0.1% (w/v) Coomassie Brilliant Blue R250 dissolved in a methanol : acetic acid : H_2O (5 : 1 : 5) solution and destained in 25% methanol, 10% acetic acid and 65% H_2O.

Protein Estimation

Protein estimations were performed using the method of Sedmak and Grossberg (1977). The assay solution consisted of 0.06% (w/v) Coomassie G250 dissolved in 2.2% (w/v) HCl. The absorbance of this solution was between 1.3 and 1.5 at 465 nm. A standard curve was prepared using BSA in 0.9% saline over a concentration range of 1-100 µg/ml. 0.5ml of assay solution and

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and 0.5 ml of standard were added and the absorbance at 620 nm determined immediately. An identical procedure was used for samples and the protein concentration determined by intrapolation of the absorbance on the standard curve. If necessary the sample was diluted so that absorbance lay in the linear region of the curve, i.e. 1-60 µg/ml.

2.2.3 Antisera to Whole Pregnant Mare Serum (wPMS)

Each of three rabbits was injected with a total volume of 1 ml of a 1:1 emulsion of serum in Freunds in complete adjuvant at four subcutaneous sites along the back. The serum was either from pregnant mares during the last month of gestation or from stallions. The injections were repeated at monthly intervals for one year. Blood was obtained from the rabbits 10-12 days after injection and serum separated and stored at $-20^{o}C$ until required.

The antiserum to stallion serum obtained after one year was adsorbed at various concentrations of stallion serum to determine the percentage of normal equine serum required to remove all antibodies to normal serum antigens from the antiserum. Once this was found the rabbit anti-wPMS was adsorbed at this percentage and tested against PMS and stallion serum by Ouchterlony double diffusion, immunoelectrophoresis and crossed immunoelectrophoresis.

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An antibody enrichment step was performed on the fully adsorbed antiserum to wPMS. The antibody enriched solution was then used to test for cross reactions against PMS and stallion serum using Ouchterlonly double diffusion and immunoelectrophoresis.

2.2.4 Antisera to Fractionated Pregnant Mare Serum (fPMS)

Antisera to subfractions of pregnant mare serum were prepared following gel filtration of whole PMS.

Gel filtration was performed using sephacryl S300 in a column 95 cm x 2.8 cm diameter. The eluting buffer was 5 mM potassium phosphate pH 7.4 with 0.5 M NaCl. In total 100 ml of late pregnant mare serum was fractionated in 2.5 ml aliquots. The flow rate through the column was 40 ml/hr and 3.5 ml fractions were collected. Protein peaks were detected by their absorbance at 280 nm and concentrated using ultrafiltration with Millipore CX10 immersible units. The column was calibrated using the following proteins, thyroglobulin, catalase, aldolase, bovine serum albumin, α chymotrypsinogen and equine myoglobin. Four protein peaks were obtained from each run of the PMS and each peak was pooled and concentrated to 15 mg/ml in the case of the first two peaks and 60 mg/ml in the case of the third and fourth peaks.

Two injection protocols (Stimson, W.H., personal

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personal communication) were used to raise antisera to the pooled fractions. The fractions from 50 ml of late pregnant mare serum were injected by each protocol.

(A) Rabbits were injected with the PMS fractions as follows:

Day 1 - 1 ml of 1:1 emulsion of the fraction in Freund's complete adjuvant given intra muscularly.

Day 14 - A repeat of Day 1.

Day 24 - 1 ml of the fraction alone given intra peritoneally.

Day 34 - Rabbits were bled, serum separated and stored at -20^{o}C until required.

Two rabbits were injected per fraction.

(B) Alum adjuvant.

In this procedure fractions 1 and 2 were amalgamated in order to increase the protein content for injection and 2 rabbits were used per fraction. To 10 ml of each fraction, 32 ml of water and 36 ml of 10% (w/v) $KAl\ SO_4\ 12H_2O$ were added. The pH was adjusted to pH 6.5 using 5N NaOH which gave a heavy precipitate. After

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After centrifugation the precipitate was washed twice in 0.9% (w/v) saline before being suspended in a final volume of 40 ml H_2O. This preparation was stable for 14 days.

Rabbits were injected as follows:

Day 1 - 5 ml of alum precipitate in each hind leg muscle.

Day 14 - A repeat of Day 1.

Day 24 - 1 ml of untreated fraction given intra peritoneally.

The rabbits were bled on Day 34 and the serum separated and stored at -20^{o}C.

Both sets of antisera were then adsorbed with stallion serum at a variety of concentrations between 5 and 50% and the resultant adsorbed antisera were then tested against PMS and stallion serum by Ouchterlony double diffusion and immunoelectrophoresis.

Partial purification of IgG was carried out as described in antibody enrichment (Section 2.2.2). IgG concentrates were then used to test PMS and stallion serum by Ouchterlony double diffusion and immunoelectrophoresis.

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2.2.5 Antiserum to fPMS - Negative Antibody Affinity Chromatography

The method used for this chromatographic process was that of Sutcliffe (1976). Antiserum to stallion serum was raised in goats by intramuscular injections at 4 weekly intervals of a 1:1 emulsion of stallion serum and Freund's incomplete adjuvant, 1 ml in each leg. After the fifth and sixth injections 1 litre of blood was obtained and the serum separated.

Preparation of IgG

A two step procedure for the purification of IgG was employed and both steps were monitored using radial immunodiffusion and SDS PAGE.

IgG was initially partially purified using precipitation by 18% Na_2SO_4 (Kekwick, 1940). The precipitated immunoglobulins were then suspended in 40% of their original serum volume. The precipitate was dissolved in a phosphate buffer pH 6.5 made by mixing 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4 to the desired pH and making the volume up to 100 ml with distilled water.

QAE sephadex was then suspended in the same phosphate buffer and left to swell overnight before being packed into a column 45 cm x 2.8 cm diameter. The column was equilibrated at pH 6.5 using the phosphate buffer. The crude IgG solution was dialysed against the phosphate buffer pH 6.5 prior to chromatography.

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The crude IgG solution was divided into 50 ml aliquots and passed through the QAE sephadex column. The flow rate was 40 ml per hour and 10 ml fractions were collected. Protein peaks were determined by their absorbance at 280 nm. Bound protein was eluted from the column using the phosphate buffer pH 6.5 containing 0.5 M NaCl. The column was then re-equilibrated with phosphate buffer, pH 6.5, prior to another run. The first peaks were pooled and concentrated by dialysis against high molecular weight polyethylene glycol and tested for the presence of IgG using radial immunodiffusion. The purity of the first peak IgG was tested using SDS PAGE and its reactivity to stallion serum tested using immunoelectrophoresis and crossed immunoelectrophoresis.

CNBr activated sepharose was prepared and the protein coupled to it according to the method of Porath et al (1967). 1.5 litres of Sepharose 4B were washed overnight in distilled water to remove preservatives before being suspended in 1042 ml distilled H_2O. All procedures involving CNBr were performed in a fume cupboard. CNBr (125 g) was dissolved in 2084 ml of distilled H_2O. The Sepharose 4B and the CNBr were then mixed and during the course of the reaction the pH was maintained at pH 11 by pumping 2 M NaOH via a variable speed peristaltic pump. The NaOH was required to maintain a high pH to prevent the release of cyanide gas during the reaction. The NaOH was

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was added rapidly to begin with but the rate of addition gradually decreased until the pH remained constant and the NaOH addition was stopped. The activated sepharose was washed for 1 hour in distilled H_2O and suspended in the coupling buffer 0.1 M $NaHCO_3$. The IgG, in coupling buffer, was added at a concentration of 5 mg/ml and gently agitated at room temperature for 2 hours. Binding to the activated sepharose was checked by comparing the IgG content of the supernatant before and after mixing using radial diffusion with a donkey anti-goat IgG. Any remaining active groups were blocked by washing with 1 M ethanolamine pH 8.0, for 1 hour. Non covalently bound protein was removed by three washing cycles of high and low pH buffers (0.1 M Acetate Buffer, pH4 and 0.1 M Borate Buffer pH 8) in 1 M NaCl. The sepharose was then packed into a column and equilibrated with phosphate buffered saline.

5 ml aliquots of late pregnant mare serum were passed through the column. The column was maintained at $4^{o}C$. The flow rate was 25 ml/hr and 10 ml fractions were collected. The first peak, detected by its absorbance at 280 nm, was collected, concentrated by ultrafiltration and stored at $-20^{o}C$. The second peak was eluted using 100 ml of 0.5 M acetic acid after which the column was re-equilibrated with phosphate buffered saline prior to more PMS being applied. 5 ml aliquots of stallion serum and non-pregnant mare serum

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serum were also applied and the first peaks retained and treated similarly. In total 200 ml of PMS and 50 ml of stallion and non-pregnant mare sera were passed through the column.

The first peak concentrates from 100 ml of the PMS were used to raise an antiserum in two rabbits. The concentrate was emulsified 1:1 with Freund's incomplete adjuvant and 1 ml injected at four subcutaneous sites on the back. Rabbits were bled after four monthly injections and the serum stored at -20^{o}C.

For immunological analysis the first peak PMS was divided into an early and late region on the basis of elution profile. The early and late regions were tested against the antiserum to the first peak PMS using Ouchterlony double diffusion and crossed immunoelectrophoresis and compared with the first peaks from stallion and non-pregnant mares. The antiserum was then adsorbed with 30% stallion serum and again tested against the first peak PMS and the first peak stallion and non-pregnant mare sera using Ouchterlony double diffusion and crossed immunoelectrophoresis.

The effectiveness of the affinity column was assessed by running serum known to contain eCG (taken from a day 55 days pregnant mare). 5 ml of this serum was passed through the column and both the first peak and

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FIGURE 2.1

RESULTS OF ANTISERUM TO WHOLE PREGNANT MARE SERUM

Gel (a)
1. 10 µl whole pregnant mare serum.
2. 10 µl stallion serum.
As 200 µl rabbit anti-pregnant mare serum in the trough.

Gel (b)
1. 10 µl whole pregnant mare serum.
2. 10 µl stallion serum.
As 200 µl rabbit anti-pregnant mare serum adsorbed with 30% stallion serum in the trough.

For gels (a) and (b) immunoelectrophoresis was performed at 12v/cm on gels of 1% (w/v) agarose in 20 mM sodium barbitone and 4.3 mM diethyl barbituric acid at pH 8.6 as described in Section 2.2.2.

Gel (c) p.s. 10 µl whole pregnant mare serum.
Antiserum in second dimension was 500 µl of rabbit anti-pregnant mare serum.

Gel (d) p.s. 10 µl of whole pregnant mare serum.
Antiserum in second dimension was 500 µl of rabbit anti-pregnant mare serum adsorbed with 30% stallion serum.

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For gels (c) and (d) crossed immunoelectrophoresis was performed on 1% (w/v) agarose in 20 mM sodium barbitone and 4.3 mM diethyl barbituric acid, pH 8.6 as described in Section 2.2.2.

Gel (e) 1 & 4. 10 µl of pregnant mare serum.
2 & 5. 10 µl of stallion serum.
3 & 6. 10 µl of non-pregnant mare serum.
As rabbit anti-pregnant mare serum in centre well.

Gel (f) 1 & 4. 10 µl of pregnant mare serum.
2 & 5. 10 µl of stallion serum.
3 & 6. 10 µl of non-pregnant mare serum.
As rabbit anti-pregnant mare serum adsorbed with 30% stallion serum in centre well.

For gels (e) and (f) Ouchterlony double diffusion was performed on 1% (w/v) agarose in 0.9% (w/v) saline as described in Section 2.2.2.

Fig.2.1 a

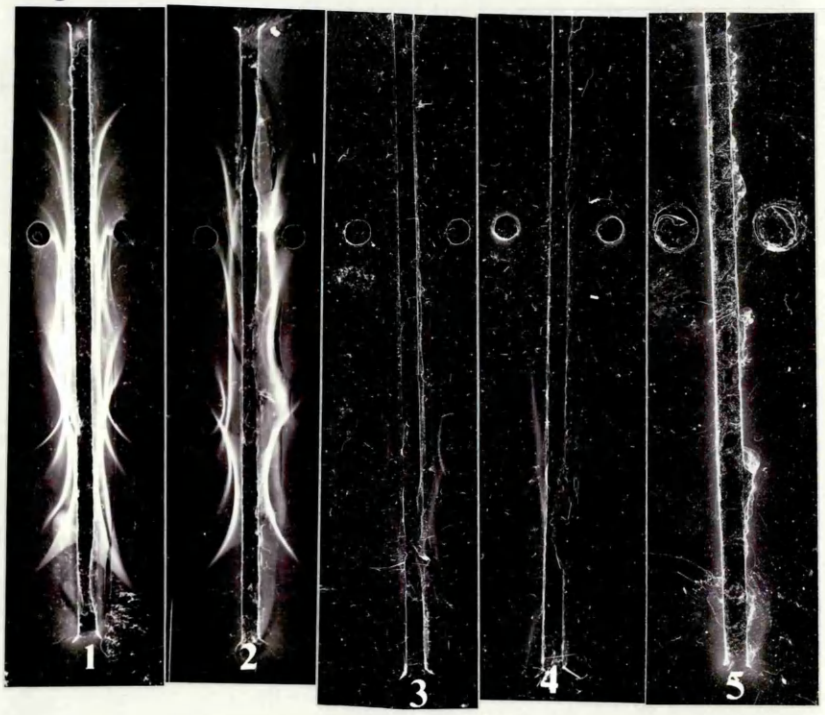

Fig. 2.1.a

Adsorption of rabbit antistallion serum

The wells contained 10 µl of stallion serum

The troughs contained 200 µl of rabbit antistallion serum adsorbed at the following percentages:-

1	0%	stallion serum
2	5%	stallion serum
3	10%	stallion serum
4	20%	stallion serum
5	30%	stallion serum

Immunoelectrophoresis was performed at 12V/cm on gels of 1% (W/V) agarose as described in Section 2.2.2 (p.45)

For antiserum production see p.49 and for results see p.57

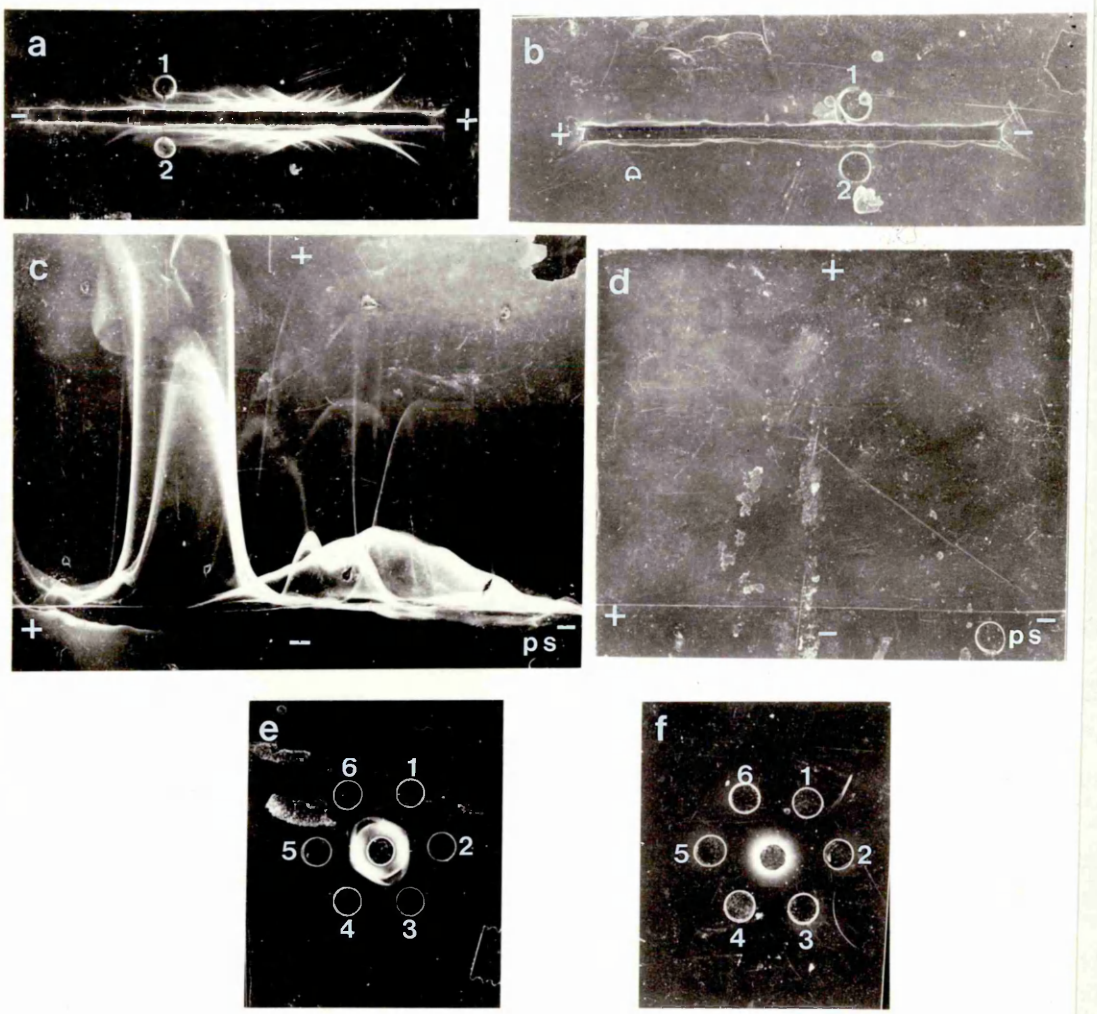

Fig. 2.1 Results of Antisera to Whole P.M.S.

For methods used see p.49, for results see p.57 and for discussion see pp.63-65.

and the second collected, concentrated by ultrafiltration and tested for eCG using a commercial haemagglutination test (MIP-test).

However the result obtained from this test is only qualitative, i.e. whether eCG is present or not.

2.3 Results

2.3.1 Antiserum to Whole PMS

The antiserum to whole PMS produced cross reactions with pregnant mare and stallion sera on Ouchterlony double diffusion, immunoelectrophoresis and crossed immunoelectrophoresis. With the antiserum to stallion serum the minimum concentration required to totally adsorb cross reacting antibody from the antiserum was 30%. This concentration of stallion serum was then used to adsorb the antiserum to whole PMS from each of the rabbits immunised. The adsorbed antisera were then tested against PMS and stallion serum by Ouchertlony double diffusion, immuno-electrophoresis and crossed immunoelectrophoresis (Fig. 2.1). No antibody/antigen precipitates were detected between the adsorbed antiserum from any rabbit and the pregnant mare serum.

The three antisera adsorbed with 30% stallion serum were pooled, subjected to antibody enrichment, and tested, using Ouchterlony double diffusion and immunoelectrophoresis, against stallion and pregnant mare sera. A precipitin line was detected between the enriched adsorbed antisera and PMS on Ouchterlony

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FIGURE 2.2

DIAGRAMMATIC REPRESENTATION OF AN OUCHTERLONY DOUBLE DIFFUSION GEL CONSISTING OF:

1, 4.	10 µl of pregnant mare serum.
2, 5.	10 µl of stallion serum.
3, 6.	10 µl of non-pregnant mare serum.
As	Pooled antisera against whole pregnant mare serum, fully adsorbed with 30% stallion serum and antibody enriched as described in Section 2.2.2.

Ouchterlony double diffusion was performed on 1% (w/v) agarose in 0.9% (w/v) saline as described in Section 2.2.2.

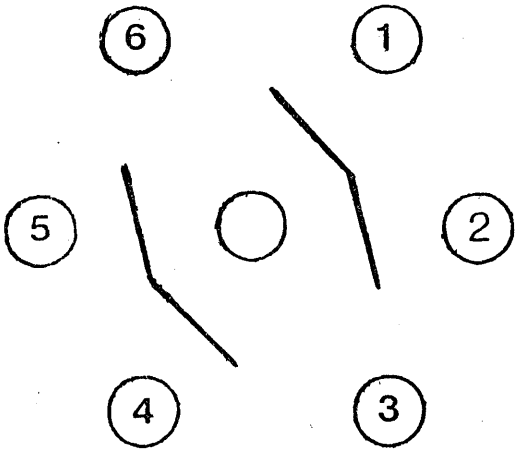


Fig. 2.2 Diagrammatic Representation of An Ouchterlony Double Diffusion Gel.

The results are given on pp.57-58 and discussed on p.64.

FIGURE 2.3

GEL FILTRATION CALIBRATION CURVE

The sephacryl S 300 column (95 cm x 2.8 cm diameter) was calibrated with the following proteins:-

		Molecular Weight
1.	thyroglobulin	669,000
2.	catalase	232,000
3.	aldolase	158,000
4.	bovine serum albumin	67,000
5.	α chymotrypsinogen	25,000
6.	equine myoglobin	18,000

The void volume, as indicated by the arrow on the graph, was obtained using blue dextran 2000.

For calibration two duplicate runs were made with three proteins in each run. The first run consisted of thyroglobulin, aldolase and α chymotrypsinogen and the second consisted of catalase, bovine serum albumin and equine myoglobin. For each run 50 mg of the respective proteins were dissolved in 2.5 ml of 5 mM potassium phosphate, pH 7.4, with 0.5 M NaCl and 10% glycerol and applied to the column. Gel filtration was performed at $4^{o}C$ as described in Section 2.2.4. The mean elution volume for each protein was calculated and graphed against the natural logarithm of the molecular weight.

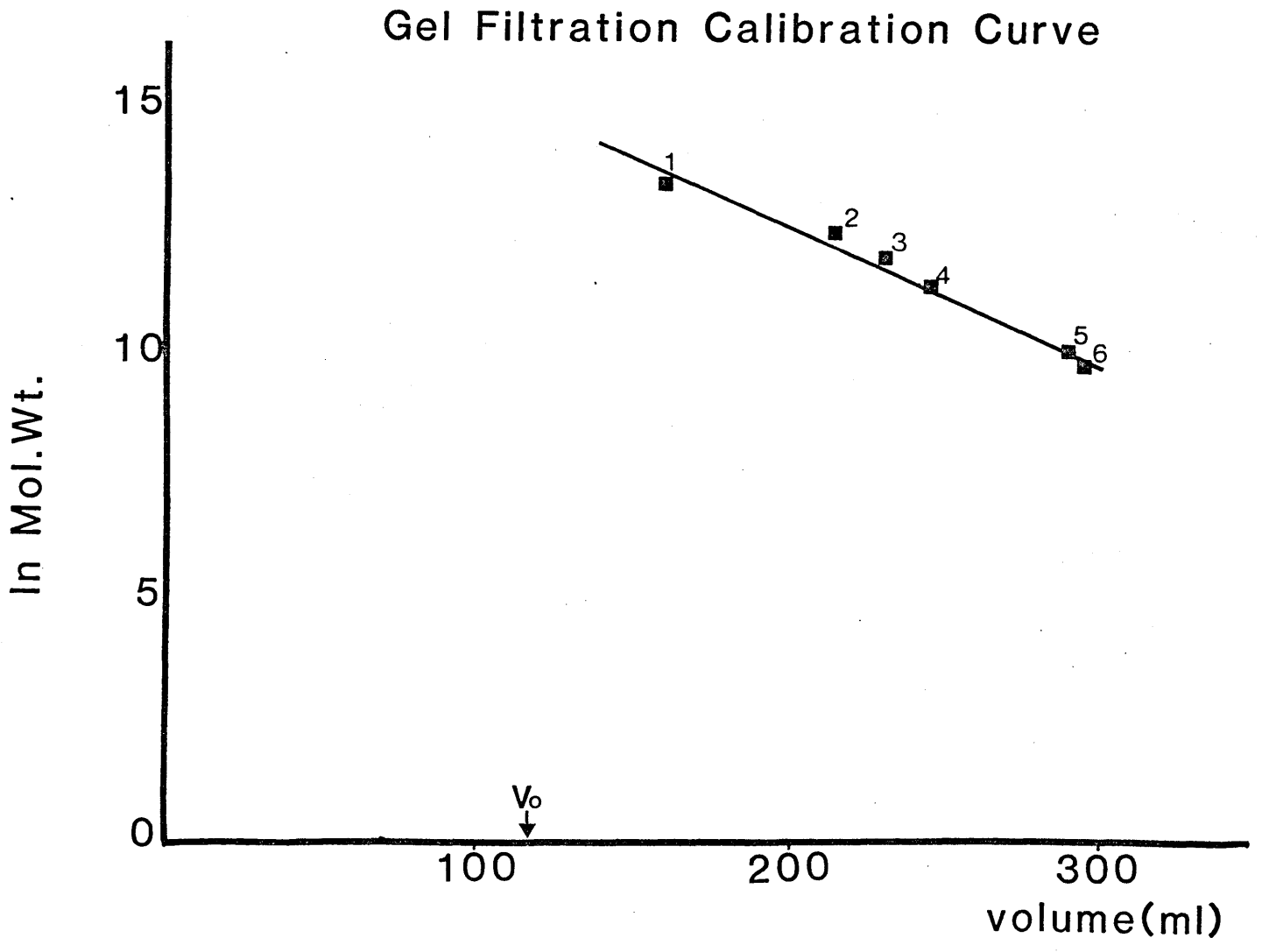
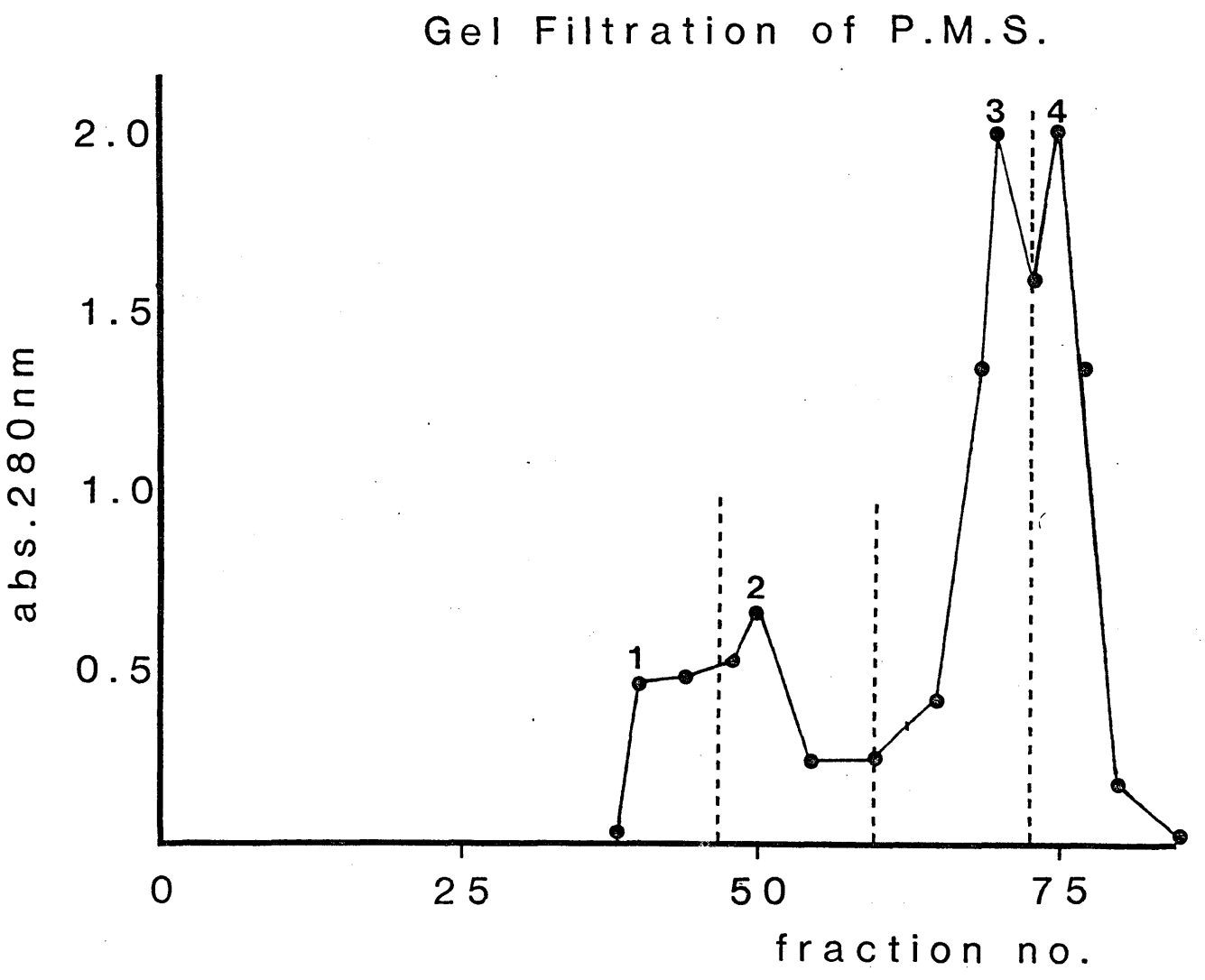

Gel Filtration Calibration Curve
15
10
5
0
ln Mol.Wt.
1
2
3
4
5
6
Vo
100
200
300
volume(ml)

FIGURE 2.4

GEL FILTRATION OF PREGNANT MARE SERUM

Gel filtration of pregnant mare serum was performed at 4°C on a sephacryl S 300 column, 95 cm x 2.8 cm diameter, as described in Section 2.2.4.

A 2.5 ml aliquot of pregnant mare serum with 10% glycerol was applied per run. The four peaks which were obtained and pooled for antiserum production are indicated by the dotted lines on the graph. Protein peaks were detected by their absorbance at 280 nm.


Gel Filtration of P.M.S.
2.0
1.5
1.0
0.5
abs.280nm
1
2
3
4
0
25
50
75
fraction no.

Ouchterlony double diffusion but this showed a reaction of identity with a line produced by stallion serum (Fig. 2.2). No reactions were detected by immunoelectrophoresis.

2.3.2 Antisera to PMS Fractionated by Gel Filtration

The gel filtration column was calibrated using thyroglobulin, catalase, aldolase, bovine serum albumin, α chymotrysinogen and equine myoglobin and their elution volumes graphed against the ln of their molecular weight (Fig. 2.3). Fractionation of pregnant mare serum gave four peaks of molecular weight ranges 1,800,000 - 1,300,000, 1,300,000 - 660,000, 660,000 - 120,500 and 120,500 - 45,000 (Fig. 2.4).

Results for Injection Protocol 1

Antisera raised to fractions 1-4 produced very strong cross reactivity with pregnant mare serum and stallion serum. The antisera were adsorbed by stallion serum at a variety of concentrations. At 50% stallion serum there were no antibody/antigen precipitates detected between the adsorbed antisera and normal equine serum. These totally adsorbed antisera when tested against late PMS by Ouchterlony double diffusion and immunoelectrophoresis revealed no precipitin lines. At the other concentrations of stallion serum, 5%, 10%, 20% and 30%, although precipitates were visible on Ouchterlony and immunoelectrophoretic gels between the adsorbed antisera and PMS similar precipitin

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FIGURE 2.5

ANTISERA RAISED TO FRACTIONATED PMS

Gel (1)
1. 10 µl of pregnant mare serum.
2. 10 µl of stallion serum.

From right to left the troughs contain the following:

Rabbit antiserum to first peak gel filtration PMS.

Rabbit antiserum to second peak gel filtration PMS.

Rabbit antiserum to third peak gel filtration PMS.

Rabbit antiserum to fourth peak gel filtration PMS.

Gel (2)
1. 10 µl of pregnant mare serum.
2. 10 µl of stallion serum.

The trough contains rabbit antiserum to first peak gel filtration PMS adsorbed with 5% stallion serum.

Gel (3)
1. 10 µl of pregnant mare serum.
2. 10 µl of stallion serum.

The trough contains rabbit antiserum to first peak gel filtration PMS adsorbed with 10% stallion serum.

Gel /

Gel (4)

1. 10 µl of pregnant mare serum.
2. 10 µl of stallion serum.

The trough contains rabbit antiserum to first peak gel filtration PMS adsorbed with 20% stallion serum.

Gel (5)

1. 10 µl of pregnant mare serum.
2. 10 µl of stallion serum.

The trough contains rabbit antiserum to first peak gel filtration PMS adsorbed with 30% stallion serum.

For all gels, immunoelectrophoresis was performed at 12v/cm on gels of 1% (w/v) agarose in 20 mM sodium barbitone and 4.3 mM diethyl barbituric acid at pH 8.6 as described in Section 2.2.2.

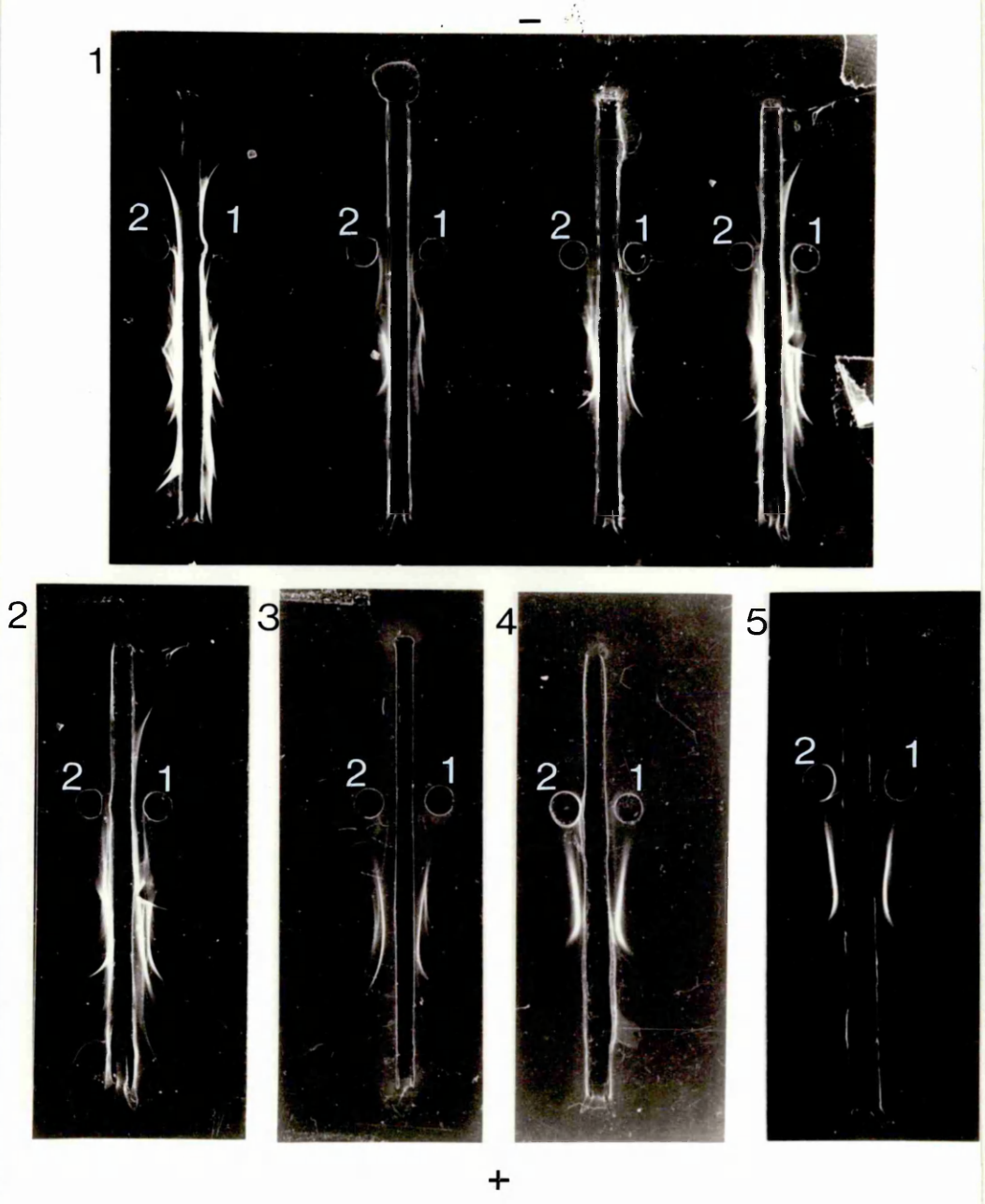

Fig. 2.5 Antisera to Fractionated P.M.S.

The methods used for antisera production are given on pp.50-52, the results are given on pp.58-59 and discussed on pp.65-67.

TABLE 2.1 Estimated CROSS REACTIVITY REMAINING AFTER ADSORPTION OF ANTISERA TO GEL FILTRATION FRACTIONS (PROTOCOL 1) WITH VARIOUS CONCENTRATIONS OF STALLION SERUM

Antiserum Against	% adsorbing stallion serum				
	0	10	20	30	50
Fraction 1	+++	++	+	+	-
Fraction 2	+++	++	+	+	-
Fraction 3	+++	++	+	-	-
Fraction 4	+++	++	++	+	-

The cross reactivity between the gel filtration fractions and the corresponding antiserum adsorbed with various percentages of stallion serum was estimated from the number of arcs remaining and the intensity of cross reactivity as observed on immunoelectrophoretic gels

+++ high cross reactivity

++ medium

\+ low

\- no cross reactivity

FIGURE 2.6

IMMUNOELECTROPHORESIS USING ANTISERA RAISED TO FRACTIONATED PMS (ALUM ADJUVANT)

1. 10 µl of pregnant mare serum.
2. 10 µl of stallion serum.

From left to right the troughs contain the following:-

Rabbit antiserum to peaks 1 and 2 from gel filtration of PMS.

Rabbit antiserum to third peak gel filtration PMS.

Rabbit antiserum to fourth peak gel filtration PMS.

Immunoelectrophoresis was performed at 12v/cm on gel of 1% (w/v) agarose in 20 mM sodium barbitone and 4.3 mM diethyl barbituric acid at pH 8.6 as described in Section 2.2.2.

Fig. 2.6.a Immunoelectrophoresis using Antisera Raised to Fractionated P.M.S. (Alum Adjuvant).

1. 10 µl of pregnant mare serum
2. 10 µl of stallion serum

In gels A and B the troughs contain, from left to right,

200 µl rabbit anti 1/2 fraction
200 µl rabbit anti 3rd fraction
200 µl rabbit anti 4th fraction

In gel A the antisera are adsorbed with 10% stallion serum and in gel B the antisera were adsorbed with 50% stallion serum.

Immunoelectrophoresis was performed at 12V/cm on 1% (W/V) agarose as described in Section 2.2.2.

Antisera were raised as described in methods pp.50-52, the results obtained are given on pp.58-59 and discussed on pp.65-67.

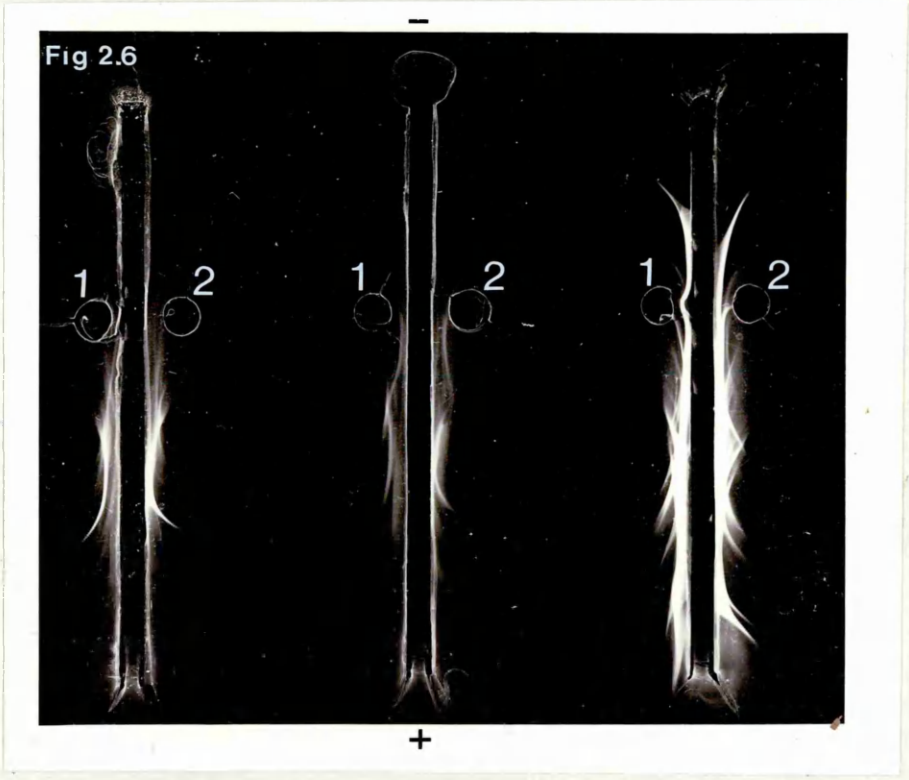

Fig.2.6.a

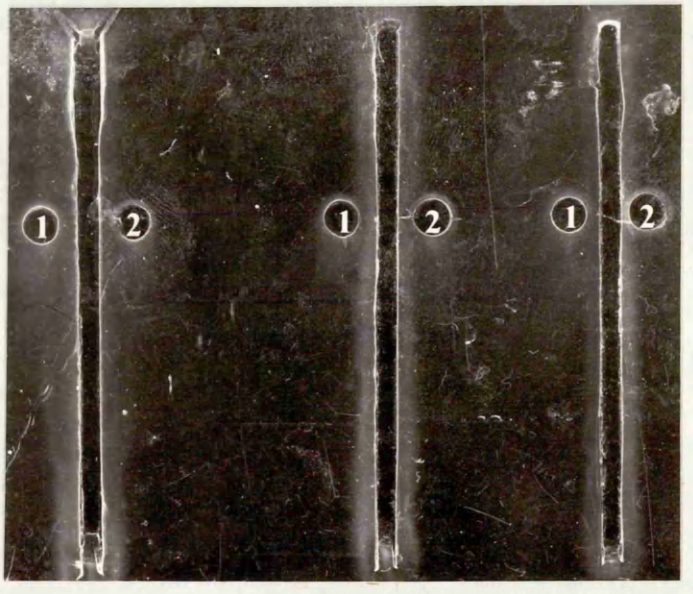

TABLE 2.2 Estimated CROSS REACTIVITY REMAINING AFTER ADSORPTION OF ANTISERA TO GEL FILTRATION FRACTIONS (PROTOCOL 2) WITH VARIOUS CONCENTRATIONS OF STALLION SERUM

Antiserum Against	% of adsorbing stallion serum				
	0	10	20	30	50
Fraction 1/2	+++	++	+	+	-
Fraction 3	+++	++	++	+	-
Fraction 4	+++	++	+	+	-

The cross reactivity between the gel filtration fractions and the corresponding antiserum adsorbed with various percentages of stallion serum was estimated from the number of arcs remaining and the intensity of cross reactivity as observed on immunoelectrophoretic gels

+++ high cross reactivity

++ medium

\+ low

\- no cross reactivity

precipitin lines were also detected in stallion serum (Fig. 2.5 and Table 2.1).

After several booster injections of 1 ml peak material given intraperitoneally a pool of antisera to each peak was obtained. After adsorption at 50% stallion serum the immunoglobulins were precipitated as described previously. The enriched preparation obtained was then used on Ouchterlony double diffusion and immunoelectrophoresis to test PMS and stallion serum. A precipitin line was observed on the Ouchterlony gels between the enriched antibody to the first and second peaks and the stallion serum. The gel filtration fractions were also tested but failed to reveal any unique reactions.

Protocol 2

Again the antisera raised by this injection protocol showed strong cross reactivity with stallion and late pregnant mare sera (Fig. 2.6). The antisera to fractions 1-4 were treated similarly to those from protocol 1. Complete adsorption of the antisera was obtained by 50% stallion serum with no cross reactivity between the adsorbed antisera and PMS. Cross reactivities were obtained at the intervening stallion serum concentrations but these were common to both PMS and stallion serum (Table 2.2).

With the enriched IgG preparations from the 50%

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FIGURE 2.7

PURIFICATION OF IgG FROM GOAT ANTISERUM TO STALLION SERUM

1. a. 5 µl supernatant from goat anti-stallion serum after addition of 18% (w/v) Na_2SO_4.

   b. 5 µl precipitate from goat anti-stallion serum after addition of 18% (w/v) Na_2SO_4.

The Mancini gels consisted of 100 µl of donkey antiserum to goat IgG added, at 56°C, to 5 ml of 1% (w/v) agarose in 0.9% (w/v). The gel was incubated at room temperature for 24 hours (Section 2.2.2).

2.a,b,c. 10 µl first peak from QAE sephadex chromatography of precipitate from 18% Na_2SO_4 treatment of goat antiserum to stallion serum.

e,f,g. 10 µl second peak from QAE sephadex chromatography of precipitate from 18% Na_2SO_4 treatment of goat antiserum to stallion serum.

The purity of the IgG after these procedures was investigated using SDS polyacrylamide gel electrophoresis.

3. /

3. 1. supernatant from 18% Na_2SO_4 treatment of goat antiserum to stallion serum.

2. precipitation from 18% Na_2SO_4 treatment of goat antiserum to stallion serum.

3. first peak from QAE sephadex chromatography of precipitate from 18% Na_2SO_4 treatment of goat antiserum to stallion serum.

4. pure swine IgG.

5. standards:- α chymotrypsinogen and equine myoglobin.

6. ovalbumin.

7. bovine serum albumin.

The polyacrylamide gel electrophoresis was performed under denaturing conditions as described in Section 2.2.2. All protein concentrations were approximately 2 mg/ml except the standards and swine IgG which were applied at 50 µg/ml. 10 µl was added per well.

4. s.s. stallion serum (10 µl)

The antiserum in the second dimension was 200 µl of the goat anti-stallion serum IgG. Crossed immunoelectrophoresis was performed on 1% (w/v) agarose in 20 mM sodium barbitone and 4.3 mM diethyl barbituric acid, pH 8.6, as described in Section 2.2.2.

5. a. 10 µl supernatant before 2 hours mixing with CNBr sepharose.

b. 10 µl supernatant after 2 hours mixing with CNBr sepharose.

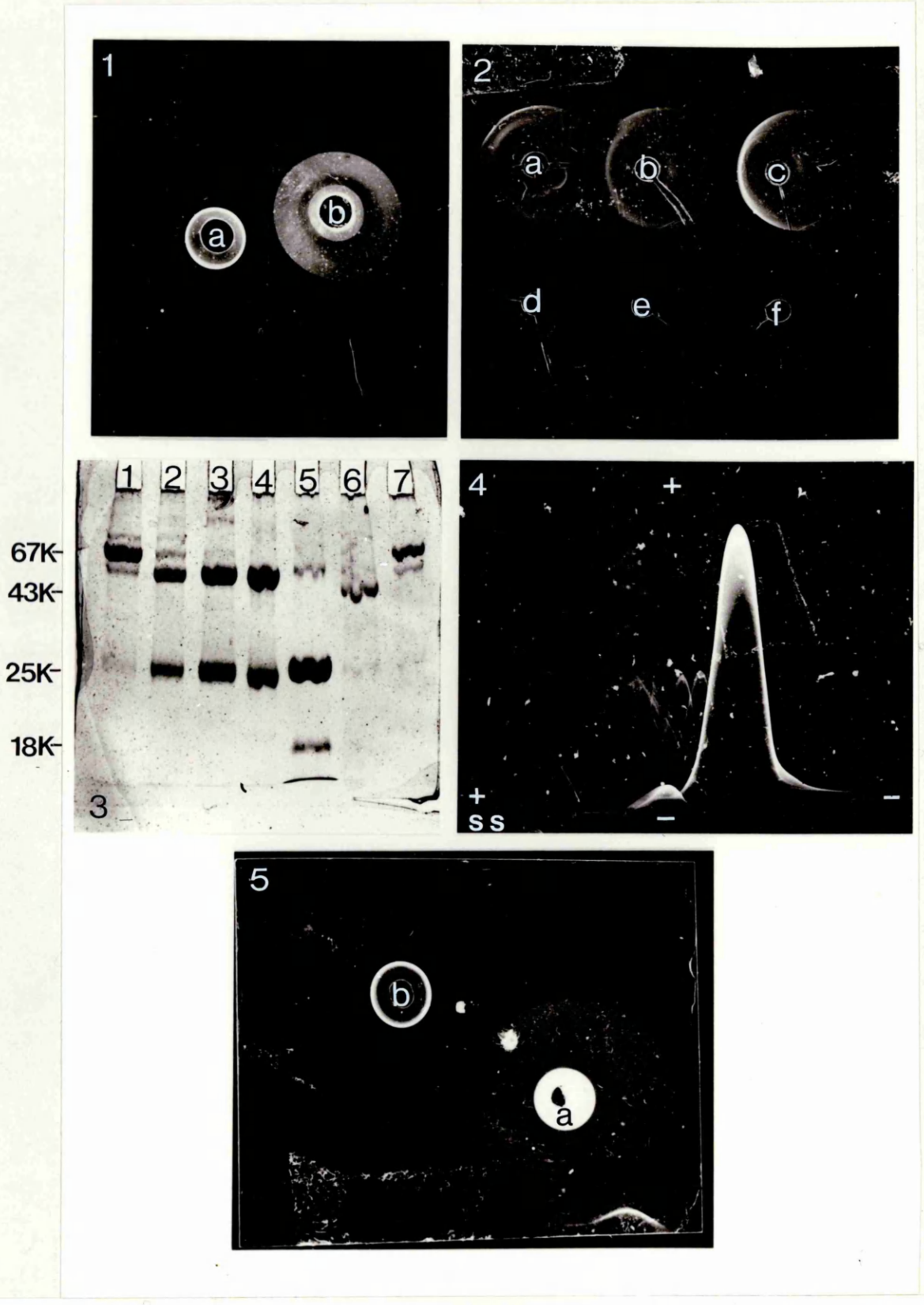

Fig. 2.7 Purification of IgG From Goat Antiserum To Stallion Serum.

For methods see pp.53-55, results pp.60-61 and discussion p.68.

50% adsorbed antisera a precipitin line was detected between the antiserum to fractions 1 and 2 and stallion serum on Ouchterlony double diffusion. All other samples, including the gel filtration fraction concentrates, failed to show any precipitation reactions.

2.3.3 Antisera to PMS Fractionated by Negative Antibody Affinity Chromatography (NAAC)

The efficiency of the purification procedure for IgG is demonstrated by Figure 2.7. The radial immunodiffusion gel indicates that the 18% Na_2SO_4 precipitated most of the immunoglobulins from the serum (Fig. 2.7.1). However the precipitate still contained some serum proteins as seen in the SDS PAGE gel (Fig. 2.7.3). The first peak from the QAE sephadex column contained all of the IgG (Fig. 2.7.2) and this was shown to have only two major bands corresponding to molecular weights 25,000 and 50,000 as calculated from the standards on SDS PAGE (Fig. 2.7.3). The bands corresponded with the two bands obtained from pure swine IgG. The reactivity of this preparation of IgG was tested against stallion serum by immunoelectrophoresis and crossed immunoelectrophoresis and was shown to be poly specific (Fig. 2.7.4). From the two litres of goats' blood, 25 g of pure IgG were isolated. Most of this IgG was successfully coupled to the activated sepharose as very little

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FIGURE 2.8

NEGATIVE ANTIBODY AFFINITY CHROMATOGRAPHY OF PREGNANT MARE SERUM

The negative antibody affinity chromatography column consisted of 25 g of goat anti-stallion IgG coupled to 1.5 litres of CNBr activated sepharose 4B. A 5 ml sample of pregnant mare serum was applied to the column, at 4^{o}C, and the first peak eluted as described in Section 2.2.5. The second peak was eluted using 100 ml of 0.5 M acetic acid which was added to the column on elution of the first peak (point indicated by arrow on graph). The peaks were detected by their absorbance at 280 nm. The first peak was divided into an early and late region as indicated by the line on the graph.

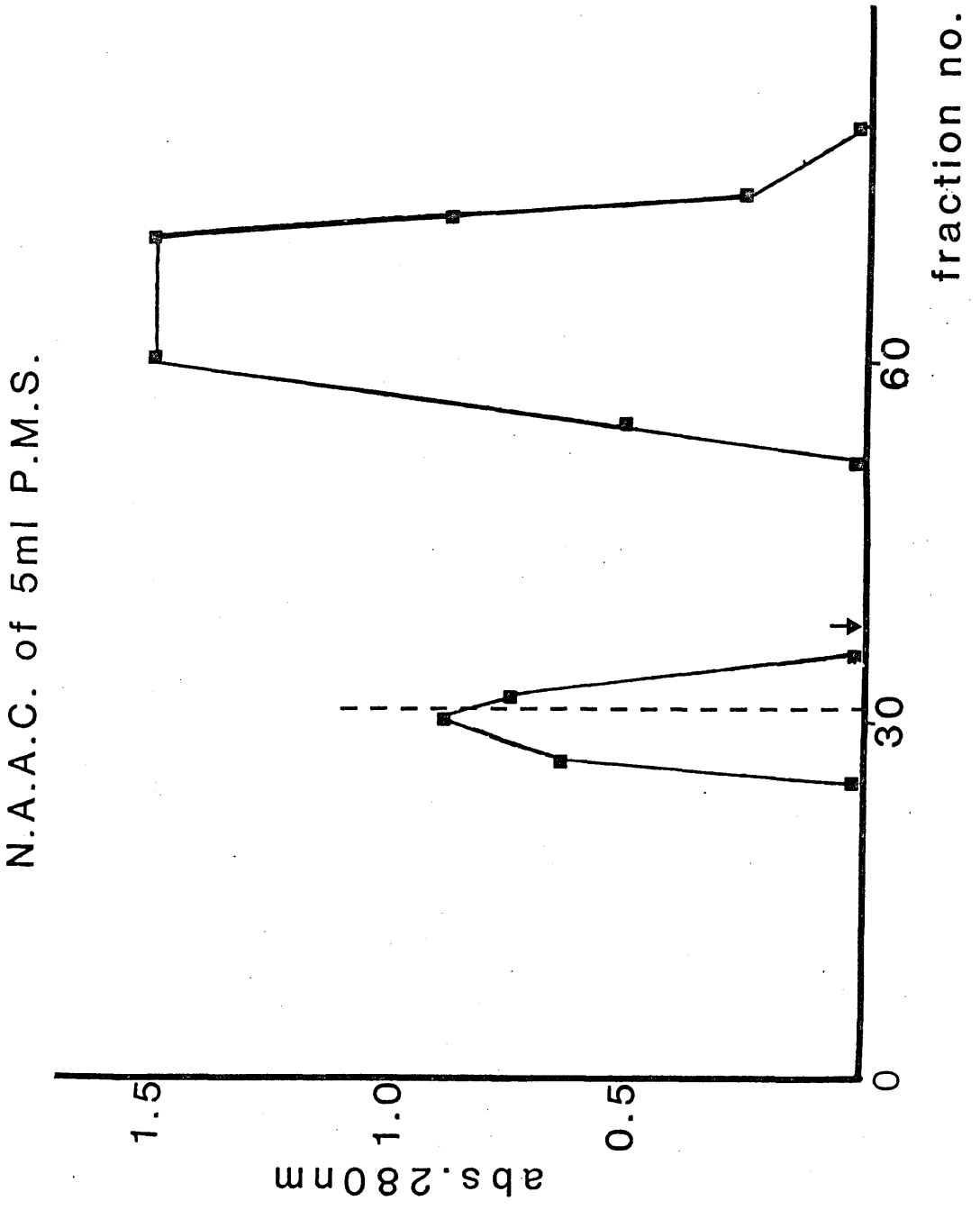

Fig. 2.8

For methods see pp.55-57, results pp.61-62 and discussion pp.68-70.

The binding capacity was calculated from the

$$\frac{\text{total protein eluted in 2nd peak}}{\text{total protein applied}} \times 100\%$$

See results p.61 and discussion p.67-70

TABLE 2.3 PERCENTAGE BINDING TO NEGATIVE ANTIBODY AFFINITY CHROMATOGRAPHY COLUMN

Vol. of Serum Applied	% Binding to Column
3.0 ml	80%
5.0 ml	75%
7.5 ml	67%

Table 2.3a

Binding capacity for N.A.A.C. for every 10 runs of 5 ml equine serum.

No. of Runs	Binding Capacity
10	75%
20	74.8%
30	73%
40	73%
50	71%
60	62.5%

little IgG was present in the supernatant after 2 hours incubation as shown by radial immunodiffusion (Fig. 2.7.5).

A 5 ml aliquot of pregnant mare serum applied to the negative antibody affinity chromatography column gave a small breakthrough peak and a larger second peak eluted with 100 ml of 0.5 M acetic acid (Fig. 2.8). The recovery of protein from the column was calculated from the total protein applied and the total protein eluted and this was found to be 95%. For a 5 ml aliquot the column bound 75% of the protein (Table 2.3). This figure was similar for stallion and non-pregnant mare sera. A 3 ml sample gave a binding of 80% whereas a 7.5 ml sample gave a binding of 67% (Table 2.3). The volume of 5 ml of serum was chosen by virtue of its binding capacity and the ease of detection of the first peak. The binding capacity of the column was continuously monitored and after 6 months the capacity began to decline and the column was discarded. By this time sufficient first peak material had been obtained. Of the 200 ml of PMS applied to the columnthe first peaks from 100 ml were pooled, concentrated and used for antiserum production in three rabbits. The peaks from the other 100 ml were divided into an early and late region on the basis of elution profile, pooled, concentrated and used for immunological analysis. 50 ml of normal equine serum was also applied to the column and the

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FIGURE 2.9

POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE FIRST PEAK N.A.A.C. OF PREGNANT MARE SERUM

Track No.	1	equine α and β globulins*
	2	equine albumin (Cohn fraction V)≠
	3	standards:- ovalbumin, α chymotrypsinogen and myoglobin.
	4	stallion serum.
	5	late pregnant mare serum.
	6	first peak N.A.A.C. of pregnant mare serum, early region.
	7	first peak N.A.A.C. of pregnant mare serum, late region.

Polyacrylamide gel electrophoresis was performed in a 10% acrylamide gel under denaturing conditions as described in Section 2.2.2. All protein concentrations were approximately 2 mg/ml except the standards which were at concentrations of 50 μg/ml.

* obtained from Pentex Incorporated, Kankakee, Illinois.

≠ obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England.

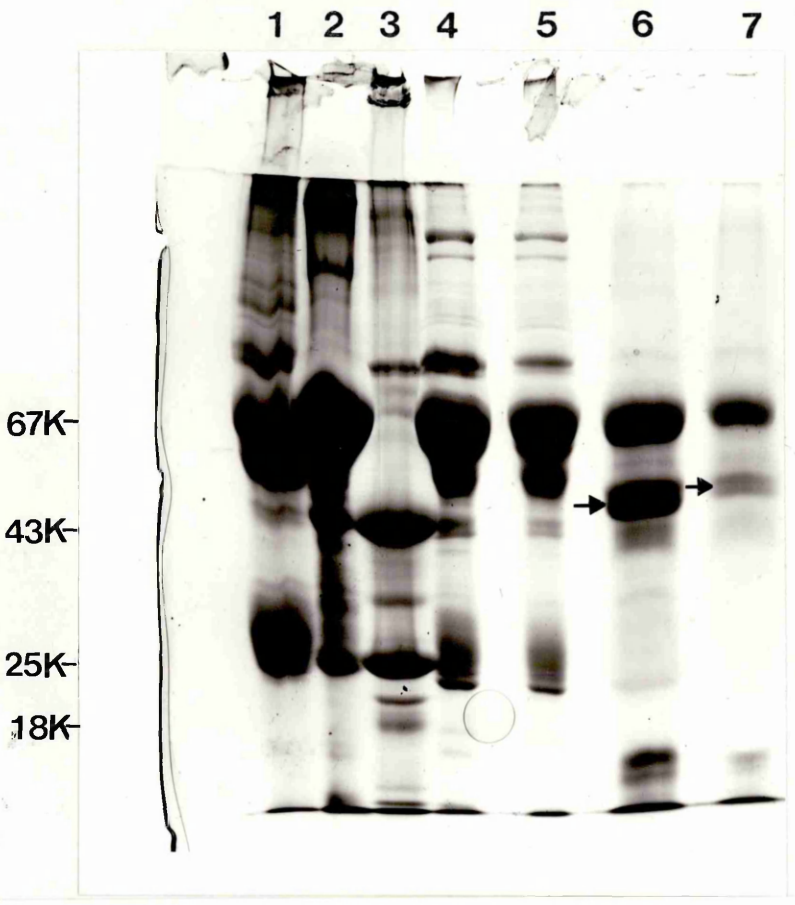

Fig. 2.9 PAGE of The First Peak N.A.A.C. of Pregnant Mare Serum.

The bands in lanes 6 and 7, marked with the arrow, are probably due to proteolytic degradation of albumin occurring during storage. The results are presented on p.62 and discussed on pp.68-69.

FIGURE 2.10

DIAGRAMMATIC REPRESENTATION OF CROSSED IMMUNOELECTROPHORETIC GEL

Using the antiserum to the first peak N.A.A.C. of pregnant mare serum -

s.s. 10 µl of stallion serum.

p.s. 10 µl of pregnant mare serum.

500 µl of the rabbit anti first peak N.A.A.C. of pregnant mare serum was used in the second dimension.

Crossed immunoelectrophoresis was performed using 1% (w/v) agarose in 20 mM sodium barbitone and 4.3 mM diethyl barbituric acid, pH 8.6, as described in Section 2.2.2.

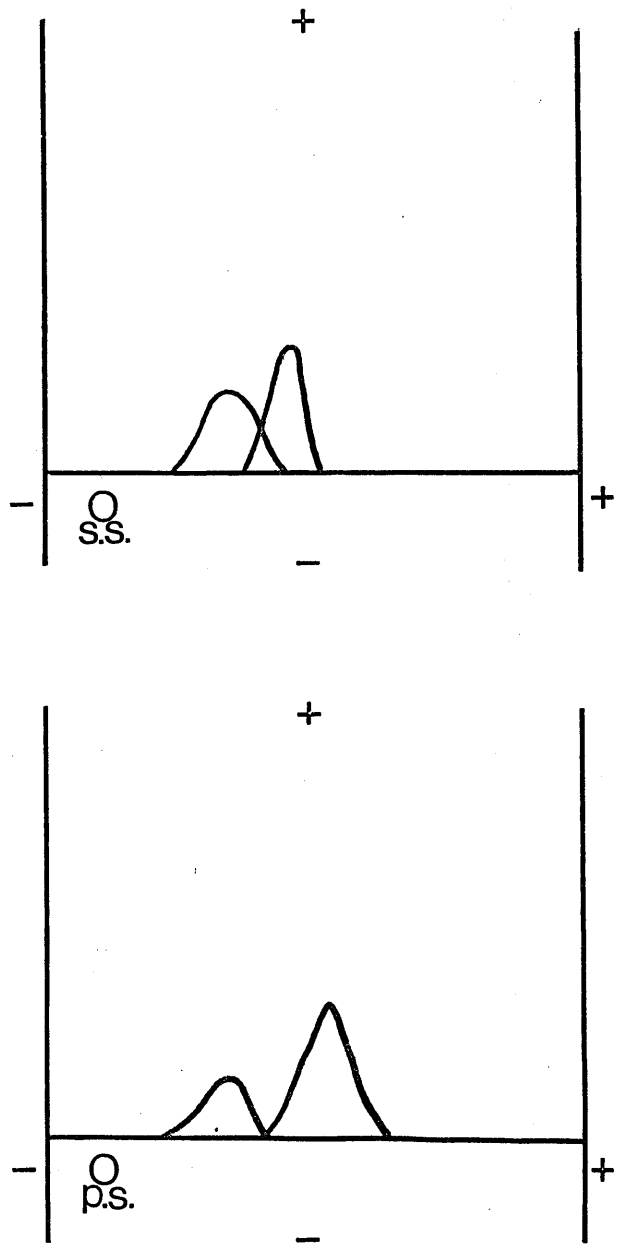

Fig. 2.10 Crossed Immunoelectrophoresis using the Antiserum to the First Peak N.A.A.C. of P.M.S.

The antiserum was raised by the method given on p.56.

For results see p.62 and discussion on pp.68-69.

+However owing to the limitations of the MIP test quantitation was not attempted and the enhancement of the eCG concentration in the first peak could not be calculated.

the first peaks pooled and concentrated and used for comparison with the first peaks PMS in the immunological analysis.

The first peak eluting when the eCG positive serum was applied to the column was concentrated to its original volume (5 ml) and was eCG positive. The second peak contained no eCG^{+}.

SDS PAGE of both the early and late regions of the first peak showed that they contained only proteins which were present in normal equine serum (Fig. 2.9). The major protein present was equine albumin. Attempts to reduce contamination of the first peak by albumin using a pre-chromatographic run on Affi-gel blue failed since the first peak from the NAAC was undetectable when dealbuminised serum was applied despite a large volume being used. A first peak was also undetectable upon rechromatography of the concentrated first peak material obtained from 20 ml PMS.

The antiserum obtained to the first peak reacted with two proteins in both pregnant mare and stallion sera on crossed immunoelectrophoresis (Fig. 2.10). The pattern obtained on the gels with pregnant mare and stallion sera was similar. No peaks were detected after the antiserum was adsorbed with 30% stallion serum. Similar results were obtained on Ouchterlony double diffusion. Using the early and

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FIGURE 2.11

OUCHTERLONY DOUBLE DIFFUSION OF ANTISERUM TO FIRST PEAK N.A.A.C. OF PREGNANT MARE SERUM

1. 10 µl of first peak N.A.A.C. of pregnant mare serum (early region).
2. 10 µl of stallion serum.
3. 10 µl of first peak N.A.A.C. of pregnant mare serum (late region).
4. 10 µl of pregnant mare serum.

As 20 µl of rabbit anti first peak N.A.A.C. of pregnant mare serum.

Ouchterlony double diffusion was performed on 1% (w/v) agarose in 0.9% (w/v) saline as described in Section 2.2.2.

Fig. 2.11.a

Ouchterlony Double Diffusion of Antiserum to First Peak N.A.A.C. of P.M.S.

1, 2, 3, 4 are as given in Fig. 2.11

5 First peak from the normal equine serum

In gel A the antiserum in the centre well is to the lst peak N.A.A.C. of P.M.S.

In gel B the antiserum in the centre well is to the lst peak N.A.A.C. of P.M.S. adsorbed with 30% stallion serum.

Ouchterlony double diffusion was performed as described in Section 2.2.2.

For antiserum production and sample preparation see pp.55-56, for results see pp.61-63 and for discussion see pp.68-69.

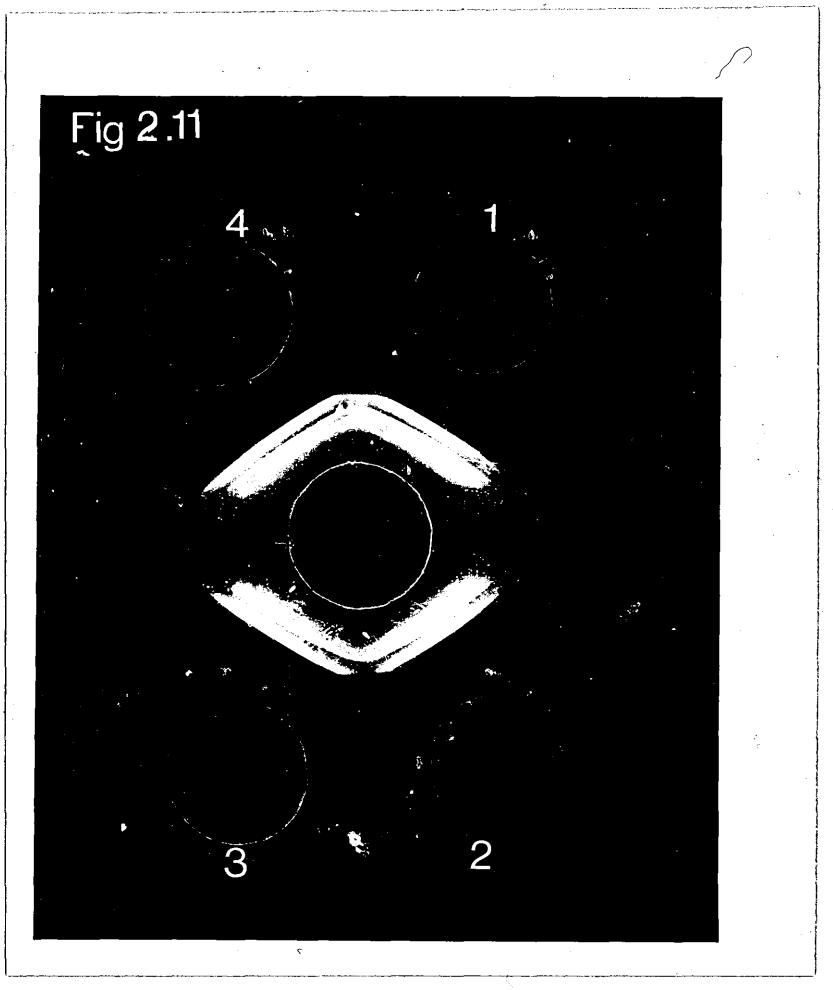
Fig 2.11
4
1
3
2

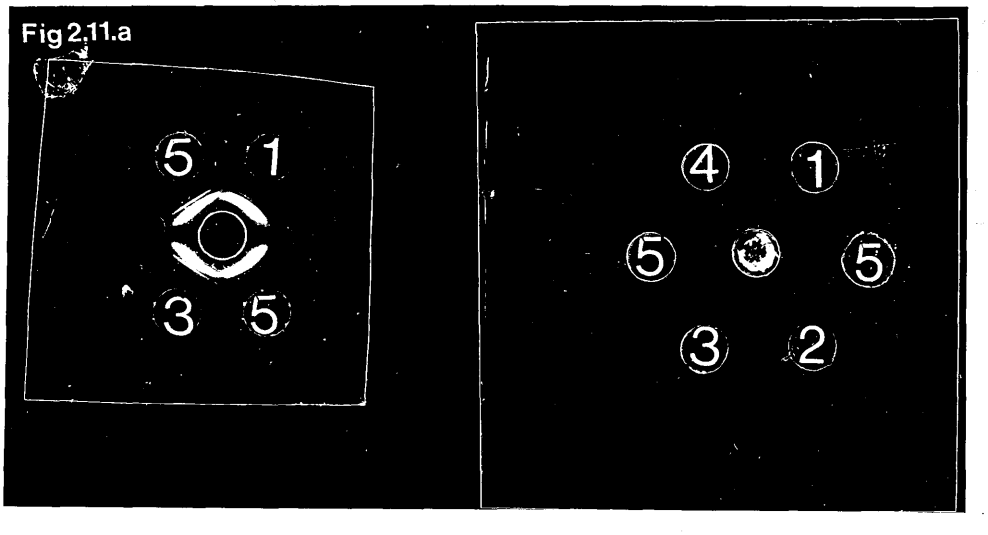
Fig 2.11.a
5
1
3
5
4
1
5
5
3
2

and late regions of the PMS and the first peak from normal equine serum reactions were obtained but the precipitate lines were identical. All reactions were removed when the antiserum was adsorbed with 30% stallion serum (Fig. 2.11).

2.4 Discussion

All the immunological methods described in this chapter have been successfully used in identifying pregnancy proteins in the late pregnancy serum of humans and laboratory animals However when applied to mares the methods consistently failed to reveal any proteins peculiar to the late stages of pregnancy.

The first method, the antiserum to whole pregnancy serum, has proved very successful in identifying pregnancy associated proteins in humans. In a review of placental proteins Klopper (1980) states that "we owe our awareness of the existence of these proteins" to the first method. The pioneering work of Gall and Halbert (1972) in identifying four pregnancy associated plasma proteins in humans, relied upon raising an antiserum to whole pregnancy plasma (36 weeks gestation) and adsorbing it with normal human plasma (120 mg lyophilized plasma/ml antiserum). The hyperimmune antisera were raised in rabbits by three weekly injections of pregnancy plasma emulsified in an equal volume of Freund's complete adjuvant. Using this method at least two of the antigens were readily detectable by

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by immunoelectrophoresis and were visible prior to staining. However the fact that only two of the antigens were readily visible, the other two required very potent antisera and longer staining, is perhaps indicative that concentration differences had to be considered. In subsequent publications (Lin et al, 1974a and 1974b) their antisera to pregnancy plasma were fully adsorbed then the immunoglobulins precipitated using 50% $(NH_4)_2SO_4$. The immunoglobulins were then re-suspended in as small a volume as possible and this preparation consistently revealed three antigens on immunoelectrophoresis and four on crossed immunoelectrophoresis. In fact, these pregnancy proteins were still detectable by immunoelectrophoresis even after the pregnancy plasma was diluted 8 fold. An identical procedure was used by Lin et al (1974c) to investigate pregnancy proteins in rats and mice and successfully identified four such proteins.

Antisera to pregnant mare serum were raised in three rabbits and a pooled antiserum was totally adsorbed with normal equine serum. Precipitation of the immunoglobulins and subsequent immunological investigation failed to reveal any proteins unique to the pregnant mare serum. The reactivity of the immunoglobulins after precipitation was preserved as indicated by the fact that a precipitin line, common to pregnant mare and stallion sera, was observed on Ouchterlony double diffusion. It is possible that this protein was albumin although it was not specifically identified as such.

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Therefore this straightforward technique failed to reveal antigens peculiar to pregnancy in the later stages of gestation in the mare. As the technique has been successfully applied to identify pregnancy specific proteins in humans and other mammalian species, it was surprising that it failed to identify any pregnancy specific proteins in late pregnant mare serum. Indeed the technique has been used to identify pregnancy proteins in the mare during early pregnancy. An antiserum to day 55 pregnant mare serum, adsorbed with 100% stallion serum, revealed two proteins unique to pregnancy. One was eCG, the other was a new protein, MPP 1 (Gidley-Baird et al, 1983).

A possible explanation of the failure of the antiserum to whole PMS to reveal proteins unique to late pregnancy is that the antisera obtained did not contain antibodies to pregnancy proteins because these proteins were present in the late PMS at low concentrations. The response to these proteins could be masked by the much higher concentrations of other serum proteins. The subsequent methods employed attempted to reduce such masking by fractionating the late PMS prior to antisera production.

The method of fractionating the PMS by gel filtration and the two injection protocols have successfully been applied to identifying pregnancy proteins in humans (W.H. Stimson, personal communication). The combination of fractionation and concentration should have been sufficient to amplify the levels of all serum proteins to

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to such an extent that they would elicit a response from the rabbit immune system. Also the variation in the route of administration of the immunogen should have increased the likelihood of obtaining a suitable antiserum. The poly specific nature of the antisera obtained is evident from the immunoelectrophoretic gels. However when the antisera were fully adsorbed with normal equine serum no antigens unique to the late PMS were identified. Even the enriched immunoglobulins preparations from the antisera failed to reveal any antigens unique to the late pregnant mare serum.

Use of gel filtration prior to antiserum production has been successfully used in identifying and investigating murine pregnancy proteins (Hau, 1982). The method consisted of pooled pregnant mice sera fractionated by gel filtration and the three peaks collected, concentrated and injected into rabbits (Hau et al, 1978). Two pregnancy associated murine proteins were initially identified by this technique (Hau et al, 1978) but further analysis has identified four murine pregnancy associated proteins (Hau, 1982). The antisera obtained to the gel filtration fractions required adsorption with a 10-30% volume of a pool of male mice sera to identify two of the proteins (Hau et al, 1978). No immunoglobulin concentrates were required.

The use of gel filtration and an injection protocol similar to protocol A was used by Waites and Bell (1984) to identify two murine pregnancy proteins, one of which

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which was shown to be a female specific acute-phase reactant. The production of the antisera to murine pregnancy proteins by the gel filtration technique has made the mouse a reasonable candidate for a model for the study of pregnancy proteins in humans (Hau, 1982).

Failure to identify pregnancy proteins in different fractions of late PMS confirms the findings of the antiserum to whole PMS.

One final immunological method was employed to produce antisera against pregnant mare serum. An affinity column was prepared to remove all the normal serum proteins from PMS and allow any unique to pregnancy to pass straight through. The column consisted of antibodies to stallion serum proteins immobilised on Sepharose 4B. In a sample of PMS applied to the column the normal serum components would bind while any peculiar to the PMS would elute in a first peak. This strategy was applied by Sutcliffe (1976) and the subtractive or negative approach was termed negative antibody affinity chromatography (NAAC). Using immunoglobins from a sheep antisera against adult male human serum coupled to Sepharose 4B umbilical cord serum and second trimester fetal serum were applied to the column. Antisera were raised against the first peaks and from the cord serum three proteins were identified, AFP and two pregnancy associated proteins. Three proteins were identified in the fetal serum and a minimum of six in amniotic fluid. All antisera contained antibodies to

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to normal serum proteins but these were removed by adsorption with normal serum. The usefulness of the technique is perhaps indicated by the number of antigens identified in amniotic fluid since previous work had failed to show such a wide range of antigens (Sutcliffe, 1975, Sutcliffe and Brock, 1973). The technique was used to investigate further the proteins in human amniotic fluid (Sutcliffe et al, 1978).

This method was applied to pregnant mares using a poly specific antiserum to stallion serum raised in goats from which 25 g of IgG was isolated. All of these immunoglobulins were coupled to the activated sepharose giving the affinity column a high capacity for normal serum proteins. SDS PAGE of the first peak demonstrated that it contained only normal serum antigens, the major of which was albumin. The antiserum to the first peak cross reacted with several proteins in PMS and stallion serum but this cross reactivity was removed by adsorption of the antiserum with normal serum. Therefore it can be concluded that all the proteins eluting in the first peak are normal serum components and their elution is probably due to saturation of the binding sites on the column. These proteins are probably at high concentrations in the serum. Attempts to reduce contamination by albumin using an albumin affinity column failed since, when the dealbuminised sample was applied to the column, the first peak was undetectable. Re-chromatography of first peak material also failed to resolve a first peak. This is further evidence that the

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the proteins eluting in the first peak are common to pregnant mare and stallion sera.

This technique has several advantages over standard protein separation techniques in that the average degree of purification of minor proteins is proportional to the number of fractions obtained and each fraction has to be analysed by raising a corresponding antiserum (Sutcliffe, 1976). The technique has been successfully used to remove adult contaminants from partially purified rabbit AFP (Pikho et al, 1971) and in the purification of crude tissue extracts. (De Carvalho et al, 1964, and Anderson et al, 1974). However the process does have some disadvantages, namely non-specific interactions between the column matrix and proteins and the fact that different proteins may share common antigenic determinants. This might have meant that unique proteins in the PMS could have been removed as the concentration of normal serum proteins was reduced to non-competitive levels. The 5 ml sample of PMS applied to the column was a small volume compared with the column. It could have been possible that these non-specific interactions were removing proteins unique to the PMS. This problem was overcome by Sutcliffe (1976) and Sutcliffe et al (1978) by passing a large volume of material through the column until only a small percentage of the starting material was eluting. In this method a small volume was chosen (5 ml) which gave a reasonably sized first peak indicating that the column was saturated for normal serum antigens and that non-specific interactions would be minimised. Thus although

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although the first peak contained normal serum protein contaminants it would hopefully be enriched with those proteins unique to PMS. Evidence that this was indeed the case is given in the experiment performed with eCG positive serum. At day 55 eCG may be regarded, in concentration terms, as a minor serum component. The fact that eCG eluted in the first peak indicates that non-specific interactions are minimal and it would be reasonable to assume that any pregnancy proteins in late PMS would behave likewise. Indeed it is possible that this technique could be used to purify eCG and MPP 1. The failure of this method to detect proteins specific to late pregnancy in the mare corroborates the immunological evidence obtained in the previous experiments.

On the basis of the evidence obtained from the immunochemical analyses described, there appears to be a major difference between the components of late pregnant mare serum and late pregnancy serum from humans, other primates and rodents. In the latter case, proteins specific to pregnancy are readily demonstrable by methods which have failed to yield results in mares. There are two possible explanations as to why these immunological methods have failed in the mare. It is possible that the proteins are present in the maternal circulation but are either at a low concentration or are poor immunogens in the rabbit. Therefore despite their presence in the maternal circulation during pregnancy, they are not recognised by the rabbit immune system and no antibodies

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antibodies are produced against them.

The alternative and perhaps more likely explanation is that there are no pregnancy specific proteins in late pregnant mare serum. The principal source of pregnancy proteins in most species during late gestation is the placenta. Therefore this lack of pregnancy proteins in the mare may result from inter-species differences in placentation. Possibly the equine placenta does not have any specific proteins or alternatively pregnancy proteins could be present within the placenta but are not secreted into the maternal circulation. Equine placenta was therefore investigated immunologically to identify any placental specific pregnancy proteins.

CHAPTER 3

ANTISERA TO PLACENTAL EXTRACT

3.1 Introduction

Pregnancy proteins in humans can be divided into two types:-

(1) proteins which are specific to pregnancy, and

(2) proteins which are not specific to pregnancy but nevertheless have strong associations with it.

Proteins in the latter group are synthesized by the mother but increase in response to pregnancy. It is now generally assumed that the principal source of proteins peculiar to pregnancy in humans is the syncytiotrophoblast of the placenta (Klopper, 1980, and Chard, 1982b) which is the layer of placental tissue in contact with the maternal circulation. This assumption is mainly supported by immunohistochemical studies, but further evidence comes from the fact that these proteins are present in placental extracts in higher concentrations than could be accounted for by their content in the maternal serum. For instance, Lin et al (1976b) were able to demonstrate that the placental content of hPL, PAPP A and B and SP_1 was too high to be derived from the maternal circulation. In contrast, the levels of

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of pregnancy zone protein, which is most probably of maternal liver origin (von Schoultz and Stigbrand, 1982), in the placental extract, could have been due to PZP in the maternal serum.

Owing to the levels of the pregnancy proteins in the placenta, this tissue has been used as a source of antisera to human pregnancy proteins. Four different proteins were originally identified in the sera of pregnant women using antisera to protein fractions from placentae (Bohn, 1971). Antisera obtained from rabbits, immunised with protein fractions from human placenta and adsorbed with normal adult serum, revealed four antigens on Ouchterlony double diffusion gels. One of the proteins was hPL and the others were designated SP_1 SP_2 and SP_3. Further studies revealed that SP_1 was a placental protein specific to pregnancy, whereas SP_2 and SP_3 were pregnancy associated, and not of placental origin, SP_2 being sex hormone binding globulin and SP_3 being pregnancy zone protein (Bohn, 1976). Since 1971 further work by Bohn has revealed a large number of placental proteins using antisera to placental extracts (Bohn, 1979 and Bohn et al, 1982).

Pregnancy proteins have also been identified in the placentae of various monkey species using antisera to protein fractions of placental extracts (Behrman et al, 1974).

In the sheep, day 14-19 embryo homogenates have been used to raise antisera to pregnancy associated antigens

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antigens (Cerini et al, 1976, Stapes et al, 1978) which could be detected in plasma. Similar work in the cow identified two pregnancy specific proteins using an antiserum to placental membranes from day 20-45 embryos. There antigens were localized in the trophoblast and placenta but not in plasma (Butler et al, 1982). Experiments performed with antisera to early equine and porcine embryos also failed to reveal any antigens in plasma, although they succeeded in identifying trophoblast specific antigens (Findlay et al, 1979). In late pregnancy, ovine and bovine placentae have been used as a source of placental lactogens (Fellows et al, 1976) and once purified they have been used in developing homologous radioimmunoassays (Robertson et al, 1980, Bolander et al, 1976) which have been used to monitor this protein throughout gestation in these species.

Hence in both humans and domestic animals, the embryonic membranes have been used as a source of antisera to proteins specific to pregnancy. However in domestic animals the emphasis has again been placed on the early stages of gestation. Therefore in this study antisera were raised to extracts of delivered equine placenta. It was hoped that the antisera would identify pregnancy specific substances in the placental extract and the antisera could be used to investigate whether such antigens were present in late pregnant mare serum. If pregnancy specific proteins were present in late pregnant mare serum then the placenta would be the most probable source and they would be present in this tissue at higher concentration

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concentration than in the maternal circulation. Investigations were therefore performed using antisera to whole placental extract and also with placental extract which had been fractionated by gel filtration and ammonium sulphate precipitation.

3.2 Materials and Methods

3.2.1 Materials

The placentae were obtained from the following mares, one pony, one thoroughbred (2) and one Clydesdale, as soon after delivery as possible. Pools were prepared from pregnant mares (4) and stallions (2) sera. Liver and kidney were obtained fresh from a slaughtered colt.

Materials for immunological methods and gel filtration were identical to those given in Chapter 2.

All other reagents were of analar grade where possible.

3.2.2 Methods

The equine placentae, obtained after delivery, were extensively washed with 0.9% saline to remove blood. Small sections of placental tissue (approx. 1 cm^2) were frozen and thawed several times prior to mixing with an equal volume of phosphate buffered saline and homogenised using a Sorvall blender.

Equal volumes from each extract were pooled and further

further homogenisation was performed using a ground glass homogeniser. Particulate material was removed by centrifugation at 15,000g and the supernatant retained and stored at -20°C until required. Samples of equine liver and kidney were treated likewise.

Gel filtration of placental extract was performed on the same column as described for equine serum (Section 2.2.3) 5 ml aliquots of placental extract were passed through the column. The flow rate and eluting buffer were as described previously. The fraction size collected was 6.0 ml. Three pooled fractions were obtained and these were concentrated and stored at -20°C until required.

Ammonium sulphate precipitation was performed at 4°C on 100 ml of placental extract as follows.
Saturated ammonium sulphate was added to a concentration of 50% and the solution allowed to stand for 30 minutes. The precipitate was obtained by centrifugation and washed with 50% $(NH_4)_2SO_4$ before being suspended in as small a volume of phosphate buffered saline as possible. The remaining supernatant was dialysed against two changes of phosphate buffered saline over a 24 hour period and concentrated by dialysis against high molecular weight polyethylene glycol. The solution obtained from the 50% $(NH_4)_2SO_4$ precipitate was also dialysed for 24 hours against PBS. Both solutions, termed ppt 50 and spt 50, were stored at -20°C until

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until required.

Antisera to the whole placental extract were raised in three rabbits. The rabbits were given an initial subcutaneous injection at four sites on the back of a 1:1 emulsion of Freund's complete adjuvant and whole placental extract. A total volume of 1 ml was administered. The injections of whole placental extract were repeated at monthly intervals for six months but emulsified with Freund's incomplete adjuvant. Blood was obtained 10 days after the final injection and serum separated and stored at -20°C. These three rabbits were then used to raise antisera to the pooled gel filtration fractions. Each of the rabbits received an initial injection of fractions 1, 2 or 3 emulsified with Freund's complete adjuvant. The injections were given subcutaneously with a total volume of 1 ml injected per rabbit. This was repeated with Freund's incomplete adjuvant twice, at monthly intervals, before the antisera were obtained 10 days after the final injection.

Two new rabbits were injected with the 50% $(NH_4)_2SO_4$ supernatant and precipitate. The initial injection of 1 ml of a 1:1 emulsion with Freund's complete adjuvant wasfollowed by three injections at monthly interval given in Freund's incomplete adjuvant. Serum was obtained 10 days after the final injection and stored at -20°C until required. These rabbits were then

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Table 3.1

Protein concentration (mg/ml) injected for production of antisera to placental extracts.

Antigen	Protein Conc. (mg/ml)
Whole placental extract	6
1st peak gel filtration	2
2nd peak gel filtration	2
3rd peak gel filtration	2
$(NH_4)_2SO_4$ precipitate	8
$(NH_4)_2SO_4$ supernatant	16

then given a final injection of 1 ml whole placental extract in Freund's complete adjuvant, the antisera being obtained 10 days after injection.

Ouchterlony double diffusion, immunoelectrophoresis and crossed immunoelectrophoresis were performed as described in the general methods section (Section 2.2) as were SDS polyacrylamide gel electrophoresis and protein estimation.

Adsorption of all antisera was performed using stallion serum and liver and kidney homogenates at various concentrations until no cross reactivity was detected between the antisera and stallion serum or the tissue extract. The adsorbed antisera were then used to examine the whole or fractionated placental extract using Ouchterlony double diffusion, immunoelectrophoresis and crossed immunoelectrophoresis.

Immunoblotting was performed by Dr. J.G. Lindsay at the Department of Biochemistry, University of Glasgow. The antiserum used was a pool obtained from the rabbits injected with the 50% $(NH_4)_2SO_4$ ppt and spt after they received the final injection of whole placental extract. The method used for immunoblotting is given in the Appendix.

The protein conc. (mg/ml) for each placental extract preparation injected is given in table 3.1

FIGURE 3.1

CROSSED IMMUNOELECTROPHORESIS USING ANTI-WHOLE PLACENTAL EXTRACT

1. 10 µl of stallion serum.
2. 10 µl of whole placental extract.

500 µl of rabbit anti-whole equine placental extract was used as the antiserum in the second dimension.

Crossed immunoelectrophoresis was performed on 1% (w/v) agarose in 20 mM sodium barbitone and 4.3 mM diethyl barbituric acid, pH 8.6, as described in Section 2.2.2.

In immunoelectrophoresis the whole placental extract did not run very well. This was probably due to residual particulate material in the extract.

Fig. 3.1 Crossed Immunoelectrophoresis using Anti Whole Placental Extract.

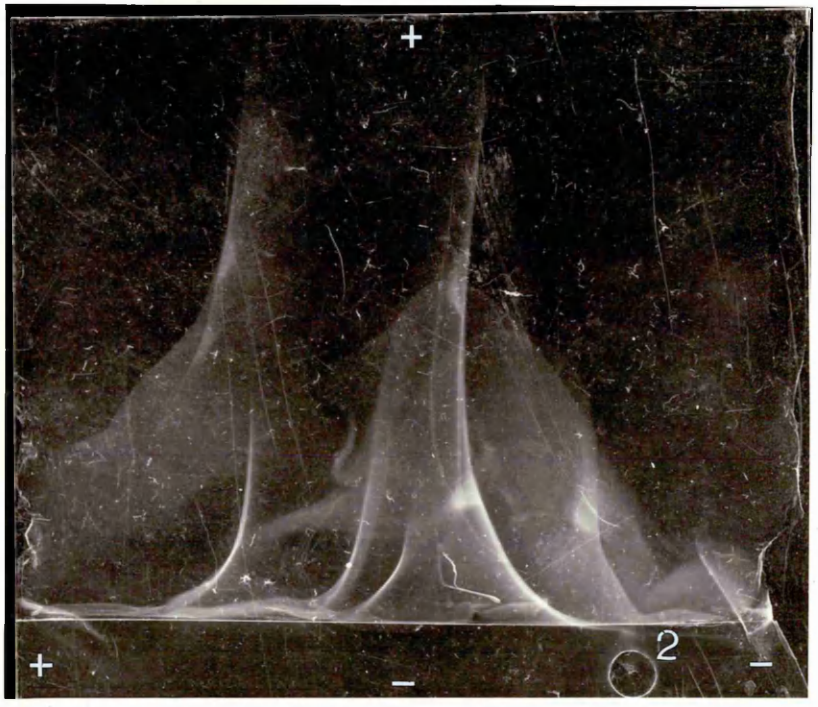
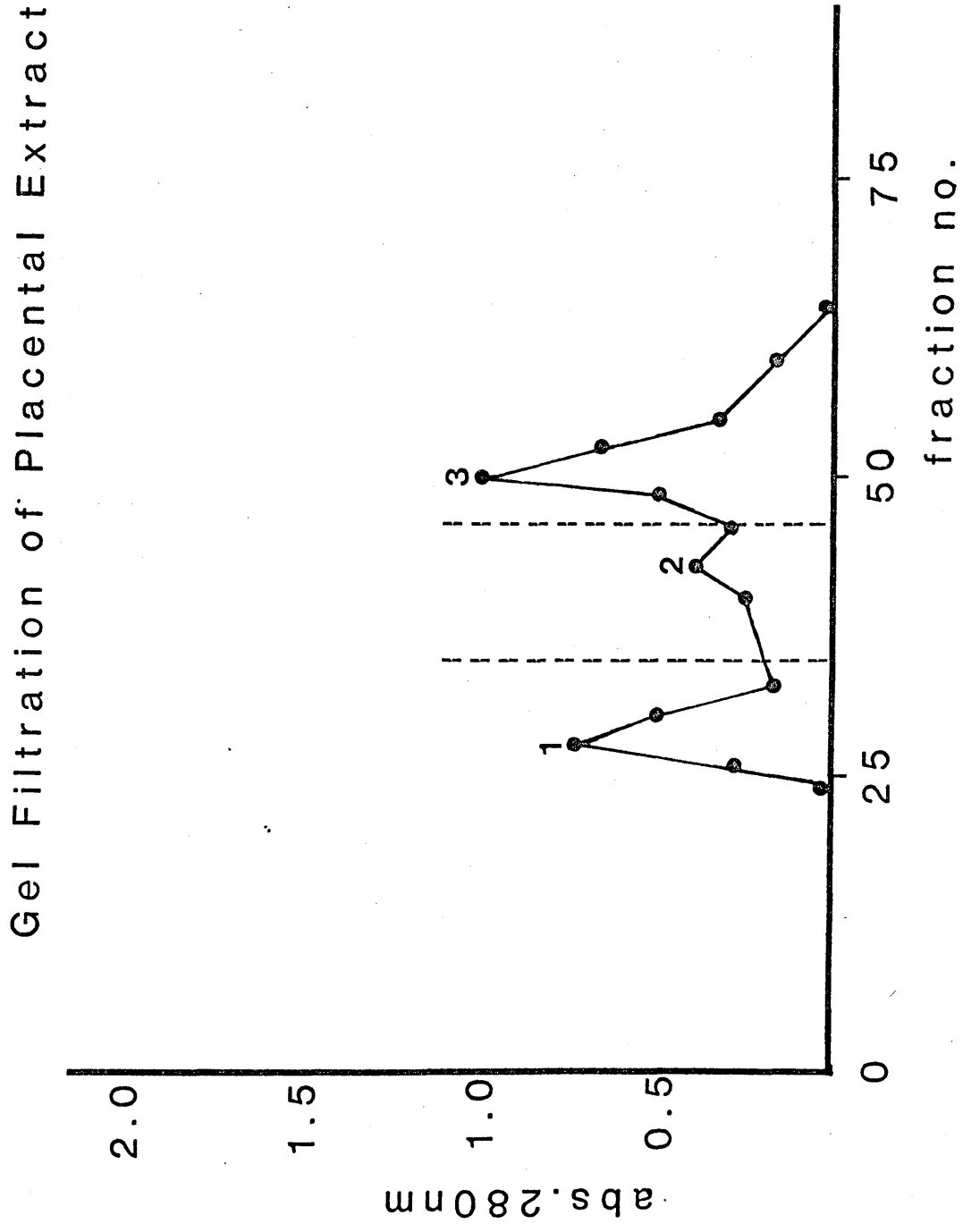


FIGURE 3.2

GEL FILTRATION OF EQUINE PLACENTAL EXTRACT

Gel filtration of the placental extract was performed at $4^{o}C$ on a sephacryl S300 column, 95 cm x 2.8 cm diameter. The eluting buffer and flow rate were as described in Section 2.2.4. 6.0 ml fractions were collected. A 5 ml aliquot of whole placental extract with 10% glycerol was applied per run. The three peaks which were obtained and pooled for antiserum production are indicated by the dotted lines on the graph. Proteins peaks were detected by their absorbance at 280 nm.



The molecular weight ranges for peaks 1, 2 and 3 are 800,000 - 150,000, 150,000 - 54,000 and 54,000 - 10,000

For results see p.79 and for discussion see p.83.

FIGURE 3.3

OUCHTERLONY DOUBLE DIFFUSION USING ANTI-WHOLE PLACENTAL EXTRACT against gel filtration fractions of whole placental extract.

1, 4. 10 µl of pregnant mare serum.

2, 5. 10 µl of stallion serum.

3, 6. 10 µl of third peak from gel filtration of whole placental extract.

Antiserum in well:- A 10 µl rabbit anti-whole placental extract.

B 10 µl rabbit anti-whole placental extract adsorbed with 10% stallion serum.

C 10 µl rabbit anti-whole placental extract adsorbed with 10% stallion serum and 10% liver and kidney extract.

Ouchterlony double diffusion was performed on 1% (w/v) agarose in 0.9% (w/v) saline as described in Section 2.2.2.

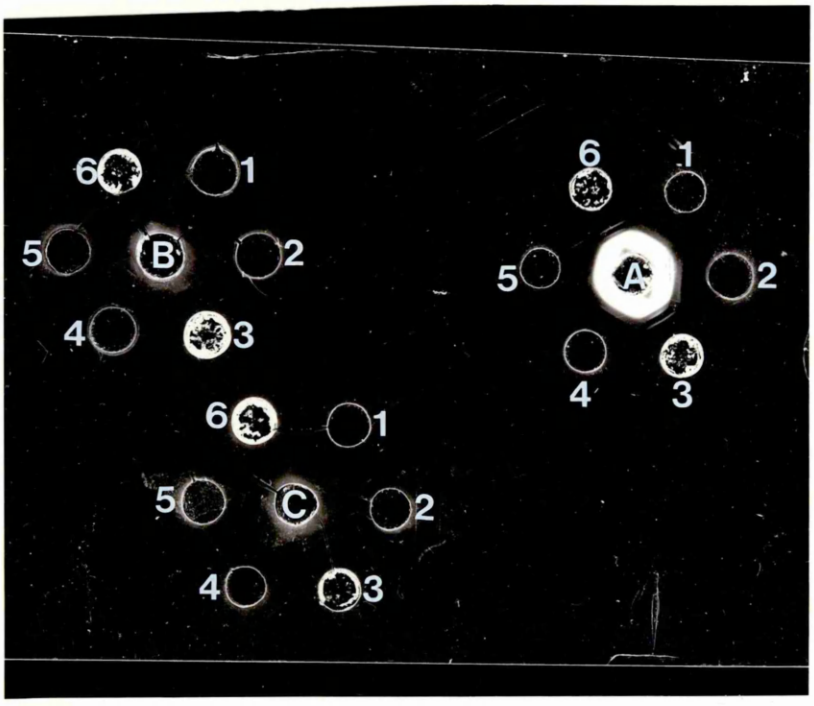


Fig. 3.3 Ouchterlony Double Diffusion using Anti Whole Placental Extract.

The antiserum was obtained as described on p.77. For results see p.79 and for discussion see p.82-83.

3.3 Results

The antiserum to whole placental extract, cross reacted with the placental extract on Ouchterlony double diffusion and crossed immunoelectrophoresis. It also cross reacted with pregnant mare and stallion serum. The reactions on Ouchterlony double diffusion between the placental extract, PMS and stallion serum, were of identity and the patterns obtained, with the placental extract and pregnant mare and stallion sera on crossed immunoelectrophoresis, were similar (Fig. 3.1). However all cross reactivity between the antiserum and placental extract was removed by 10% stallion serum. This percentage stallion serum also removed all cross reactivity from pregnant mare and stallion sera.

Fractionation of the placental extract by gel filtration gives three distinct peaks (Fig. 3.2). Molecular weights were calculated using the calibration curve for the column (Fig. 2.3). The peaks corresponded to molecular weights 730,000, 80,000 and 18,000. Peaks 1 and 2 were colourless, whereas peak 3 was a deep red colour.

The peaks were analysed, using the antiserum to whole placental extract, by Ouchterlony double diffusion and immunoelectrophoresis against pregnant mare and stallion sera. Peak 3 contained an antigen which appeared unique to the placental extract since the precipitin line remained even after the antiserum was adsorbed with stallion serum and liver and kidney extract (Fig. 3.3).

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FIGURE 3.4

OUCHTERLONY DOUBLE DIFFUSION USING ANTISERA TO FRACTIONATED PLACENTAL EXTRACT (GEL FILTRATION) to identify specific antigens in the peaks obtained by gel filtration of whole placental extract.

A 1, 3. 10 µl third peak from gel filtration of whole placental extract.
2. 10 µl equine myoglobin (50 µg/ml)
4. 10 µl equine haemoglobin (50 µg/ml)

Antiserum in well A was 10 µl of rabbit antiserum to third peak by gel filtration of placental extract.

B a, c. 10 µl of second peak from gel filtration of whole placental extract.
b, d. 10 µl of equine albumin (50 µg/ml)

Antiserum in well B was 10 µl of rabbit antiserum to second peak from gel filtration of placental extract.

Ouchterlony double diffusion was performed on 1% (w/v) agarose in 0.9% (w/v) saline as described in Section 2.2.2.

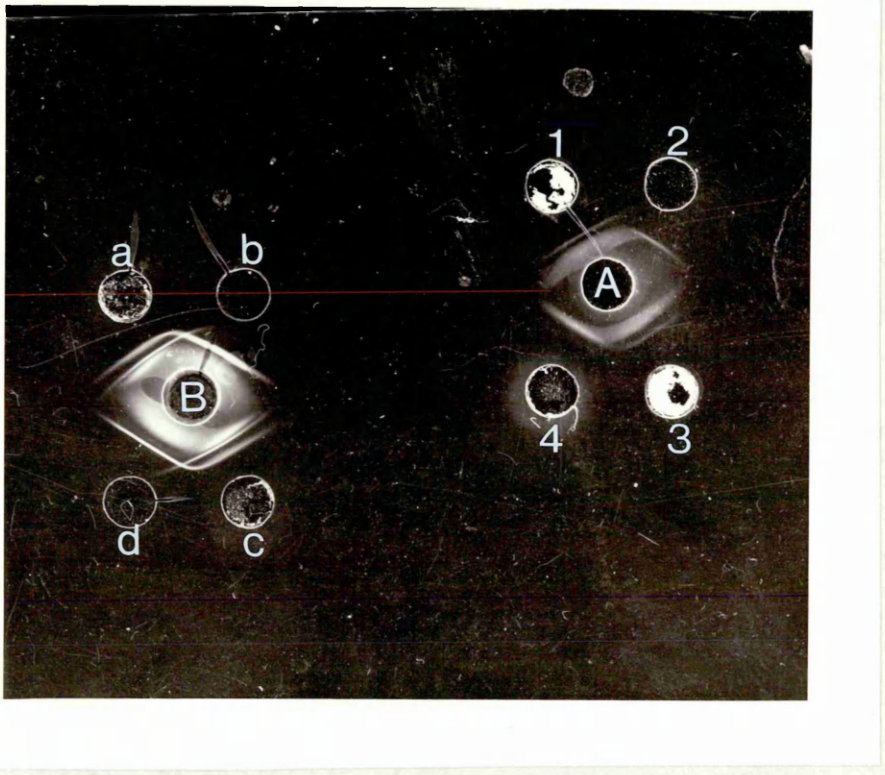

Fig. 3.4 Ouchterlony Double Diffusion using Antisera to Fractionated Placental Extract (Gel Filtration).

For results see p.80.

FIGURE 3.5

IMMUNOELECTROPHORESIS OF PLACENTAL EXTRACT FRACTIONS FROM GEL FILTRATION against the antisera to the placental extract gel filtration fractions.

1. 10 µl of first peak gel filtration of whole placental extract.
2. 10 µl of second peak gel filtration of whole placental extract.
3. 10 µl of third peak gel filtration of whole placental extract.
4. 10 µl of stallion serum.

From right to left the antiserum in the trough is as follows:-

200 µl of rabbit anti first peak placental extract fractionated by gel filtration.
200 µl of rabbit anti second peak placental extract fractionated by gel filtration.
200 µl of rabbit anti third peak placental extract fractionated by gel filtration.

Immunoelectrophoresis was performed at 12v/cm on 1% (w/v) agarose in 20 mM sodium barbitone and 4.3 mM diethyl barbituric acid, pH 8.6, as described in Section 2.2.2.

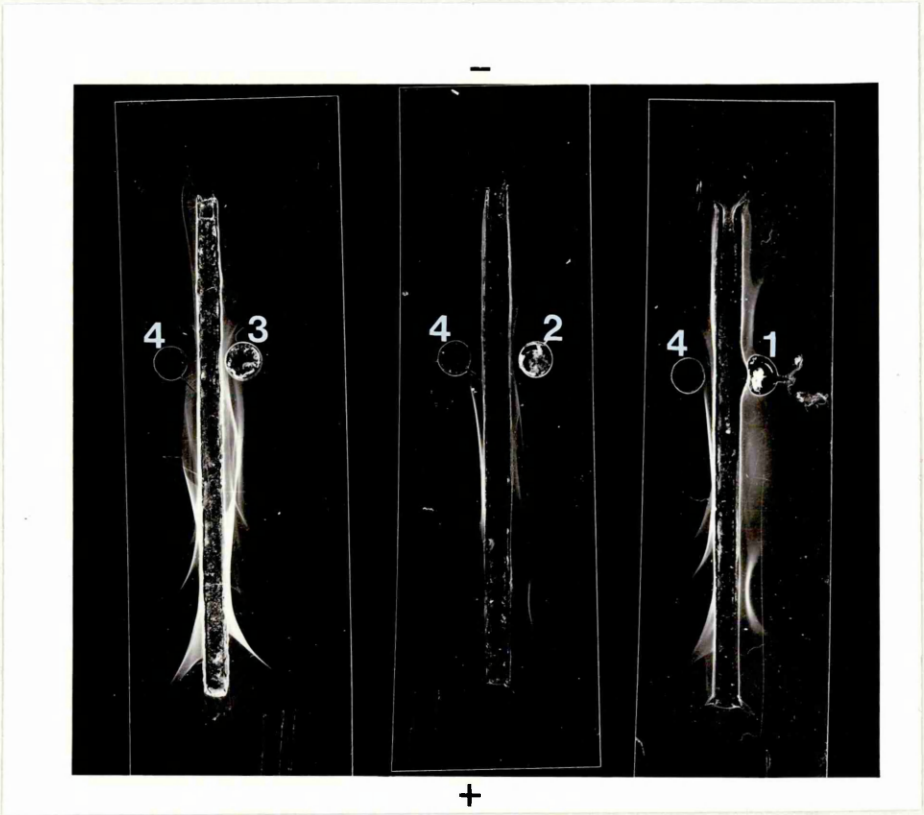

Fig. 3.5 Immunoelectrophoresis of Placental Extract Fractions from Gel Filtration.

For antisera production see p.77, for results see pp.80-81 and for discussion see pp.83-84.

FIGURE 3.6

OUCHTERLONY DOUBLE DIFFUSION USING THE ANTISERUM TO THE THIRD PEAK FROM THE GEL FILTRATION OF WHOLE PLACENTAL EXTRACT

1, 4. 10 µl of pregnant mare serum.

2, 5. 10 µl of stallion serum.

3, 6. 10 µl of placental extract.

Antiserum A 10 µl of rabbit antiserum to the third peak from the gel filtration of whole placental extract.

B 10 µl of rabbit antiserum to the third peak from the gel filtration of whole placental extract adsorbed with 10% stallion serum.

C 10 µl of rabbit antiserum to the third peak from the gel filtration of whole placental extract adsorbed with 15% stallion serum and 10% liver and kidney extract.

Ouchterlony double diffusion was performed using 1% (w/v) agarose in 0.9% (w/v) saline as described in Section 2.2.2.

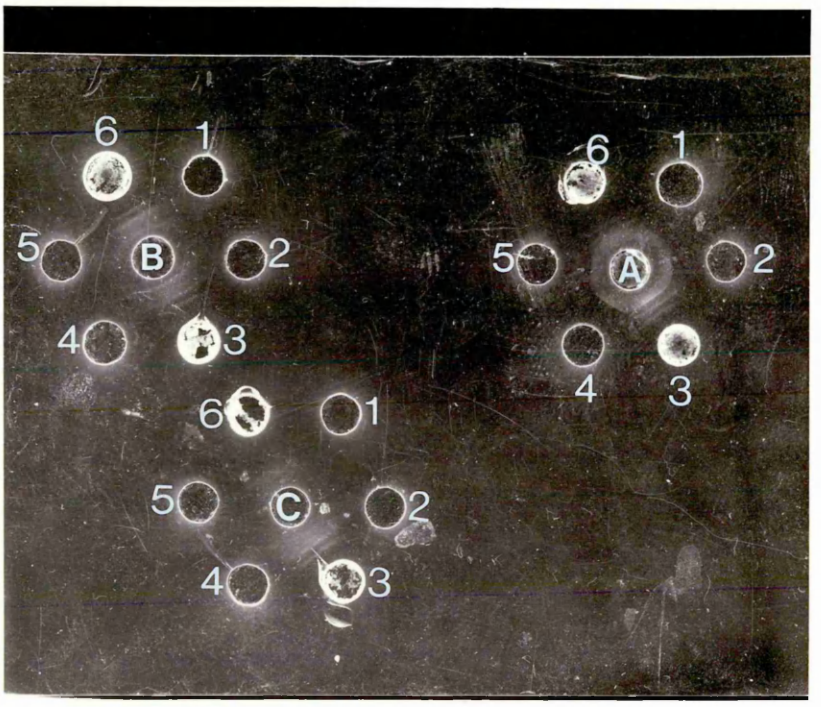

Fig. 3.6 Ouchterlony Double Diffusion using the Antiserum to the Third Peak from the Gel Filtration of Whole Placental Extract.

The results are given on p.80 and discussed on pp.83-84.

With the antisera raised against peak 3 from gel filtration, it was apparent that the red colour, in this peak, was due to haemoglobin. On Ouchterlony double diffusion a reaction of identity was obtained between peak 3 and equine haemoglobin, using the antisera to peak 3. Also, using the antiserum to peak 2, the presence of albumin was demonstrated in peak 2 as a line of identity was obtained between peak 2 and equine albumin on Ouchterlony double diffusion (Fig. 3.4).

On Ouchterlony double diffusion and immunoelectrophoresis, precipitates were obtained between the antisera to the gel filtration fractions 1-3 and the placental extract, pregnant mare and stallion sera (Fig. 3.5). All cross reactivity, between the pregnant mare and stallion sera, was removed by adsorbing the antisera with 10% stallion serum. However on Ouchterlony double diffusion, three precipitin lines were detected between the placental extract and the antiserum to the third fraction (Fig. 3.6). One of these lines gave a reaction of identity with equine haemoglobin as described above and was removed when the antiserum was adsorbed with liver and kidney extract. The other two precipitin lines remained. Neither antigen was demonstrable on immunoelectrophoresis.

A precipitin line was also detected between the placental extract and the antiserum to the first peak which remained after adsorption with 10% stallion serum. However this precipitin line was removed when the antiserum

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FIGURE 3.7

IMMUNOELECTROPHORESIS USING ANTISERA TO THE spt AND ppt FROM THE TREATMENT OF PLACENTAL EXTRACT WITH 50% $(NH_4)_2SO_4$.

1. 10 µl of stallion serum.
2. 10 µl of placental extract.

Antiserum A 200 µl of rabbit anti-spt from the treatment of placental extract with 50% $(NH_4)_2SO_4$.

B 200 µl of rabbit anti-ppt from the treatment of placental extract with 50% $(NH_4)_2SO_4$.

Immunoelectrophoresis was performed at 12v/cm using 1% (w/v) agarose in 20 mM sodium barbitone and 4.3 mM diethyl barbituric acid, pH 8.6, as described in Section 2.2.2.

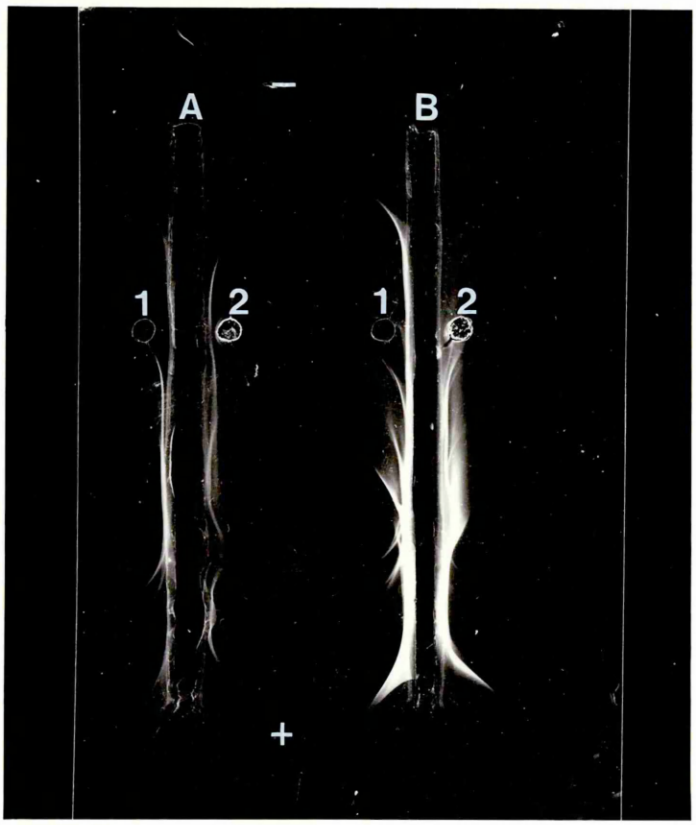

Fig. 3.7 Immunoelectrophoresis using Antisera to the spt. and ppt. from the Treatment of Placental Extract with 50% $(NH_4)_2SO_4$.

The antisera were prepared as described on pp.77-78.

The results are given on p.81 and discussed on p.84.

FIGURE 3.8

DIAGRAMMATIC REPRESENTATION OF OUCHTERLONY DOUBLE DIFFUSION GEL showing the two bands obtained between the antiserum to the 50% $(NH_4)_2SO_4$ supernatant and the placental extract.

1, 4. 10 µl of pregnant mare serum.

2, 5. 10 µl of stallion serum.

3, 6. 10 µl of placental extract.

The antiserum in the centre well was 10 µl of rabbit anti-supernatant from the 50% $(NH_4)_2SO_4$ treatment of placental extract adsorbed with 15% stallion serum and 10% liver and kidney extract.

Ouchterlony double diffusion was performed on 1% (w/v) agarose in 0.9% (w/v) saline as described in Section 2.2.2.

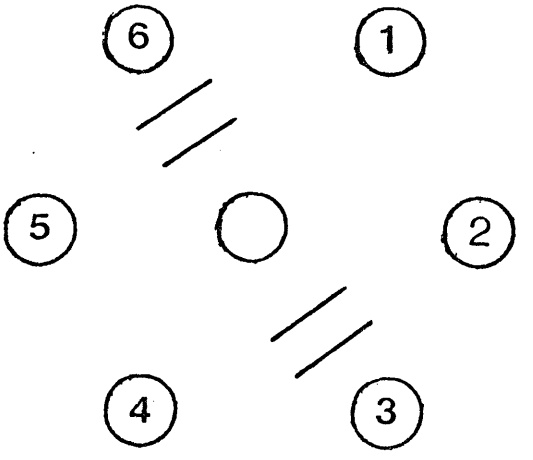

Fig. 3.8 Diagrammatic Representation of Ouchterlony Double Diffusion.

The antiserum was prepared and adsorbed as described on pp.77-78. The results are given on p.81 and discussed on p.84

FIGURE 3.9

IMMUNOELECTROPHORESIS USING ANTISERUM TO THE spt FROM 50% $(NH_4)_2SO_4$ TREATMENT OF WHOLE PLACENTAL EXTRACT.

1. 10 µl of placental extract.
2. 10 µl of stallion serum.

The antiserum in the trough was 200 µl of rabbit antiserum to the supernatant obtained after the addition of 50% $(NH_4)_2SO_4$ to the placental extract adsorbed with 15% stallion serum and 10% liver and kidney extract. Immunoelectrophoresis was performed at 12v/cm in 1% (w/v) agarose in 20 mM sodium barbitone and 4.3 mM diethyl barbituric acid, pH 8.6, as described in Section 2.2.2.

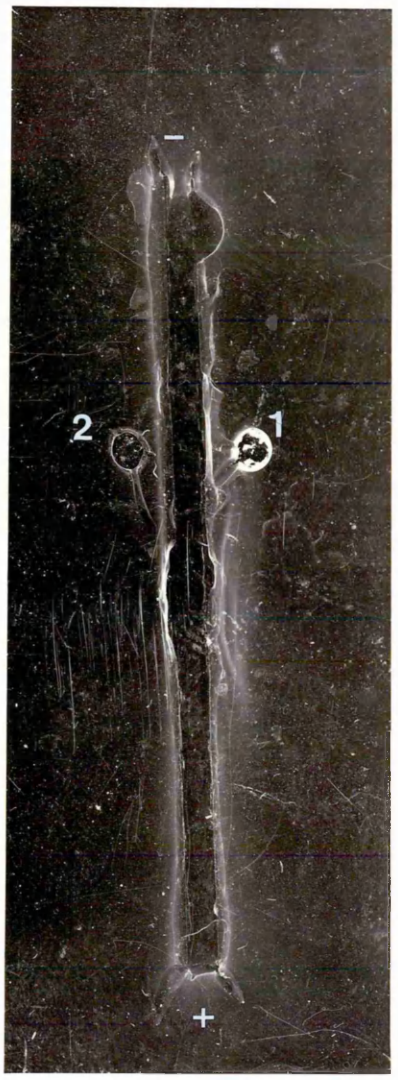

Fig. 3.9 Immunoelectrophoresis using Antiserum to the spt. from 50% $(NH_4)_2SO_4$ Treatment of Whole Placental Extract.

antiserum was adsorbed with liver and kidney extract. No precipitin lines were detected between the antiserum to the second peak adsorbed with 10% stallion serum and the placental extract.

The antisera to the placental extract fractionated by $(NH_4)_2SO_4$ cross reacted on Ouchterlony double diffusion and immunoelectrophoresis. The antisera to the 50% $(NH_4)_2SO_4$ precipitate and supernatant also cross reacted with pregnant mare and stallion sera (Fig. 3.7). The majority of the cross reactivity with the sera was obtained with the antiserum to the 50% $(NH_4)_2SO_4$ supernatant. When the antisera were adsorbed with 10% stallion serum, there was no cross reactivity detectable with pregnant mare and stallion sera. When the adsorbed antisera were tested against the placental extract, three lines were detected between the antiserum to the 50% supernatant on Ouchterlony double diffusion. No lines were detected with the antiserum to the 50% $(NH_4)_2SO_4$ precipitate and the placental extract. When the antiserum to the 50% $(NH_4)_2SO_4$ supernatant was adsorbed with 10% liver and kidney extract, two lines remained detectable with the placental extract on Ouchterlony double diffusion (Fig. 3.8). One of these antigens was also detected using immunoelectrophoresis (Fig. 3.9).

SDS polyacrylamide gel electrophoresis revealed a large number of protein bands in the whole placental extract and the placental extract fractions from gel

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FIGURE 3.10

POLYACRYLAMIDE GEL ELECTROPHORESIS OF WHOLE AND FRACTIONATED PLACENTAL EXTRACTS AND LIVER AND KIDNEY EXTRACTS

Track No. 1. standards (see below).

2. whole placental extract.

3. liver extract.

4. kidney extract.

5. placental extract, gel filtration peak 1.

6. placental extract, gel filtration peak 2.

7. placental extract, gel filtration peak 3.

8. placental extract, 50% $(NH_4)_2SO_4$ ppt.

9. placental extract, 50% $(NH_4)_2SO_4$ spt.

Molecular weight standards (Pharmacia)

	µg/ml	Subunit M.Wt.
1. phosphorylase B	64	94,000
2. bovine serum albumin	83	67,000
3. ovalbumin	147	43,000
4. carbonic anhydrase	83	30,000
5. soybean trypsin inhibitor	80	20,100
6. α - lactalbumin	121	14,400

The polyacrylamide gel electrophoresis was performed in a 15% acrylamide gel under denaturing conditions as described in Section 2.2.2. 10 µl of a 2 mg/ml solution of the extracts was added to the gels. 7.5 µl of the standards solution was added.

FIGURE 3.11

AN IMMUNOBLOT OF THE GEL SHOWN IN FIGURE 3.10

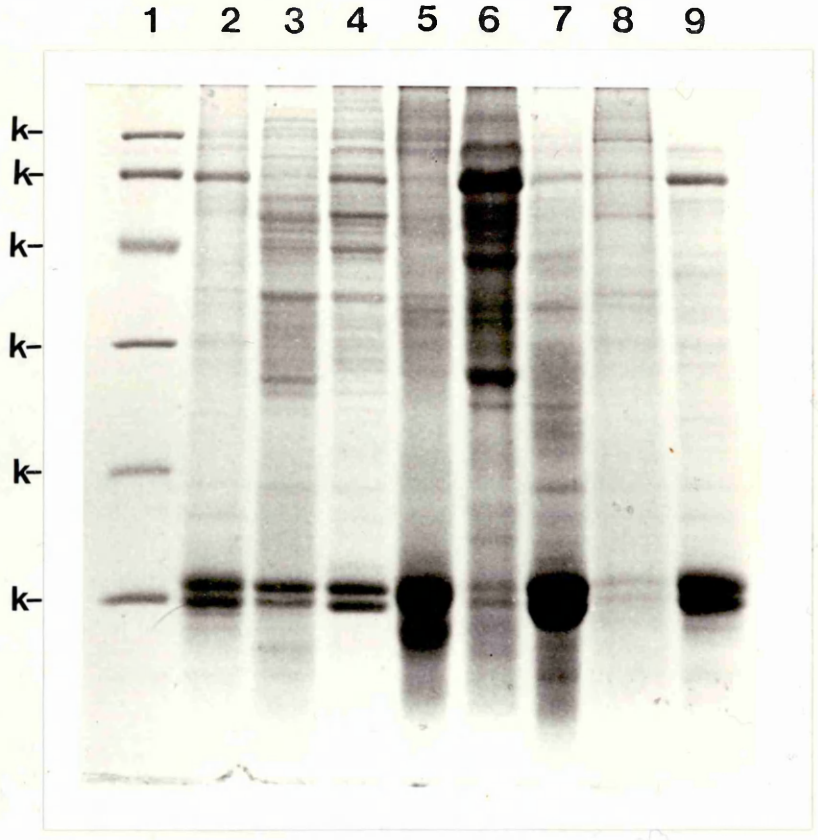
The tracks are identical to those given in Figure 3.10.

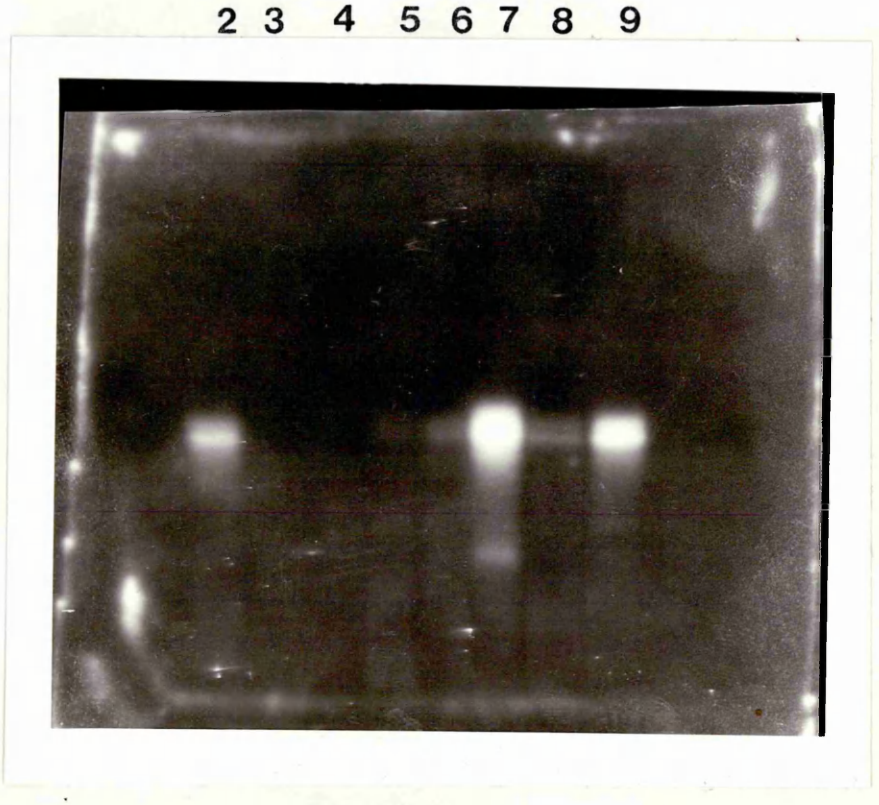
The antiserum was to whole placental extract adsorbed with 15% stallion serum and 10% liver and kidney extract.

Immunoblotting was performed as given in the Appendix.

Fig. 3.10 and 3.11 PAGE and Immunoblot of Whole and Fractionated Placental Extracts and Liver and Kidney Extracts.

For results see pp.81-82 and for discussion see pp.84-85


1 2 3 4 5 6 7 8 9
k–
k–
k–
k–
k–
k–


2 3 4 5 6 7 8 9

gel filtration and $(NH_4)_2SO_4$ precipitation. The patterns of protein bands obtained showed similarities with those of liver and kidney extract. However, owing to the large number of bands present in all samples, direct comparison in order to identify bands unique to the placental extract was not possible (Fig. 3.10).

When an identical gel was subjected to immunoblotting using an antiserum to whole placental extract and adsorbed with 15% stallion serum and 10% liver and kidney extract, two protein bands were specifically identified in the placental extract (Fig. 3.11). No bands were identified in either the liver or kidney extracts. Further, the bands were also demonstrable in the third fraction of the gel filtered placental extract and in the 50% $(NH_4)_2SO_4$ supernatant. The bands were not detectable in the first n but were faintly visible in the second fraction from the gel filtered cental extract. Faint bands were observed in the 50% $(NH_4)_2SO_4$ precipitate. The molecular weights of the two proteins, as calculated from the distances moved by the molecular weight standards were within the range 22,000 - 25,000

3.4 Discussion

The immunological investigation into the proteins of the term equine placenta has successfully identified two proteins which appear to be specific to this tissue. Initially an antiserum to the whole placental extract failed to reveal any proteins unique to the placenta.

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placenta. However from the cross reactivity with pregnant mare and stallion sera, it is probable that the majority of proteins in the crude extract stimulating a response in the rabbit were serum proteins. The tissue proteins present at lower concentrations would require longer to elicit a response.

Consequently the same rabbits were used to raise antisera to the three fractions obtained by gel filtration of the extract. It was assumed that these rabbits would be more responsive to the minor proteins present in the fractions. Using Ouchterlony double diffusion serum albumin was identified in the second peak and haemoglobin in the third. From the molecular weight of the third fraction, 18,000, the haemoglobin was assumed to be in subunit form. The contamination by haemoglobin is due to the fact that delivered rather than surgically removed placentae were used. However during the course of the study there were no placentae from Caeserian sectioned mares available. Excessive washing of the placentae prior to homogenisation did however substantially reduce contamination by haemoglobin subunits.

Adsorbing the antisera to the gel filtration fractions with 10% stallion serum revealed four antigens in the placental extract. Apart from haemoglobin the other three were not of serum origin, one antigen in the first peak and two in the third. Adsorption with a crude liver and kidney extract (10%) removed the

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the antibodies to the antigen from the first peak antiserum and the antibodies to the haemoglobin from the third peak antiserum. The line in the first peak was therefore assumed to be a tissue antigen common to adult tissues such as liver and kidney. The second peak revealed no precipitin lines after adsorption with 10% stallion serum. However the protein content of this fraction was low compared with the other two fractions and it is possible that tissue proteins present were at a too low concentration to stimulate the rabbit immune system. It is possible that if a more extended protocol of injections was used then antibodies would have been produced to some of the minor proteins present in this peak.

The placental extract was fractionated into a precipitate and supernatant using 50% $(NH_4)_2SO_4$. The supernatant contained the haemoglobin. Antisera were raised to the two fractions. Adsorption of the antiserum to the supernatant with 10% stallion serum and 10% liver and kidney extract again revealed two unique lines in the placental extract. Similar treatment of the antiserum to the precipitate revealed no unique placental precipitin lines. It was assumed that the lines identified in the supernatant were identical to those identified in the third peak of gel filtration and this was confirmed by immunoblotting. This method specifically identified the same antigens in the whole placental extract, the third fraction from gel filtration and the 50% $(NH_4)_2SO_4$ supernatant. The SDS PAGE gel of all samples of

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of placental extract revealed numerous staining bands, many of which corresponded to bands in the liver and kidney extracts. However when an antiserum to whole placental extract adsorbed with 15% stallion serum and 10% liver and kidney extract was used in immunoblotting two antigens were identified which were only present in the placental extract. Therefore two equine placental specific antigens have been identified in the term placental extract. These antigens have been designated equine placental antigens 1 and 2 (EPA 1 & 2). Neither antigen was detected in late pregnant mare serum.

In humans pregnancy proteins have been identified using antisera to protein fractions from placental tissue extract. Using the antisera to the placental protein fractions Bohn (1971) identified hPL, SP1, SHBG and PZP in the maternal serum. Further investigations revealed another protein, PP5, specific to pregnancy.

The methods used with equine placenta are similar to those used in humans and have revealed two new proteins specific to the placental tissue. The proteins were not identified in late pregnant mare serum. There has been no previous investigation into the proteins of term equine placenta. However work performed using early equine embryos (Findlay et al, 1979) identified trophoblast specific antigens and it is possible that these antigens may be similar to those identified in this study and are produced throughout pregnancy. However, to confirm this

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this it would be necessary to investigate the placental tissues throughout gestation using the antiserum.

At partuition in the mare the three fetal layers separate leaving the uterine layers intact. Therefore the extract was derived from these fetal layers. However it would be impossible to speculate on the precise source of these antigens since they may be transferred between layers of the placenta. Indeed the antigens may be of fetal origin since contamination of the extract by fetal circulation was possible. Using immunohistochemical methods it would be possible to identify the proteins' location within the placenta.

Only liver and kidney tissue extracts were used to adsorb the antisera and it is possible that the antigens are present in other equine tissues such as those of the uterus or ovary. They may also be present in the endometrial cups which are of fetal origin (Allen, 1980).

The low molecular weights of the antigens is of interest. The molecular weights under denaturing condition from SDS gel electrophoresis (22,000 - 25,000) is in agreement with the approximate weight by gel filtration in non-denaturing conditions and it is probable that the antigens are single polypeptide chains rather than subunits of a larger protein. Since the placentae were obtained after delivery, it is possible that the low molecular weights are due to the degradation of a larger molecule.

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molecule. Apart from placental lactogen (Mol. Wt. 21,000) the EPA 1 & 2 are not comparable in size with the other main human pregnancy proteins which have much higher molecular weights (see Table 1, Chapter 1). However, on the basis of molecular weight, several comparisons with minor human pregnancy proteins are possible, notably placenta specific α_1 and α_2 microglobulins. These proteins have molecular weights 20,000 and 25,000 and are precipitated by 30-50% and 25-60% saturated $(NH_4)_2SO_4$ respectively. Both proteins are produced by the trophoblast in largest amounts in the first trimester, PAMG 1 being secreted predominantly into amniotic fluid. PAMG 2 has been identified by immunofluorescence, on premenstrual endometrium, menstral blood and sperm. The function of these proteins is unknown (Tatarinov, 1982). Another human placental protein with which comparison may be made on the basis of molecular weight is the human chorionic thyrotropin. This protein has a molecular weight of 28,000 and displays similarities with human thyroid stimulating hormone (Ashitaka and Tojo, 1982).

This investigation has successfully identified two antigens specific to the term equine placenta. However further investigations would be necessary before any definite conclusions can be made about these proteins. The methods used in this study to successfully identify unique pregnancy specific equine placental proteins were similar to the methods which failed to identify pregnancy specific proteins in late pregnant mare serum. This

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This therefore provides evidence that the failure in the latter case is not due to the methods used.

CHAPTER 4

TWO DIMENSIONAL ELECTROPHORESIS

4.1 Introduction

On the basis of the immunological evidence described in Chapter 2, there appears to be a major difference in the serum proteins of late pregnant mares and those of other species which have been investigated. Methods, successful in these other species, have been unable to identify pregnancy proteins in late PMS. There are two possible explanations for this. Either equine pregnancy proteins are weak immunogens in the rabbit or pregnancy proteins are absent from late pregnant mare serum. The more appropriate of these two hypotheses could be confirmed if the pregnancy proteins could be identified using a non-immunological technique. In the analysis of protein mixtures the highest resolution possible is obtained with two dimensional electrophoresis (O'Farrell, 1975) and therefore this method was chosen to examine serum from mares throughout pregnancy to identify any proteins which appear but which are not present in non-pregnant mares.

The method optimizes protein separation by combining techniques which depend on independent parameters, isoelectric point and molecular weight. The proteins are separated under dissociating conditions in the first dimension by isoelectric-focusing with 9 M urea/2% Nonidet

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Nonidet P-40 followed by electrophoresis in the second dimension with sodium dodecyl sulphate (SDS). The potential resolution of the technique given by O'Farrell (1975) is the product of the number of proteins resolved by each technique and has been estimated as 7,000 in the case of Escherichia coli components. A protein which constitutes 10^{-4} - 10^{-5}% of the total protein can be detected and quantified by autoradiography.

The process has been adapted to a clinical procedure capable of resolving individual gene products in biological fluids and cell homogenates from different tissues which can be used to detect the appearance of novel proteins associated with pathological conditions in humans (Anderson and Anderson, 1978a and 1978b). This method allows genetic screening and identification of significant increases in the mutation rate due to environmental or other factors to be performed.

The technique has been used to separate the proteins of human plasma and serum (Anderson and Anderson, 1977, Thorsrud et al, 1980) into several hundred gene products. In the study of Thorsrud et al, (1980), the two dimensional gels allowed identification of the pathological immunoglobulin in patients suffering from multiple myelomas, various lymphoproliferative disorders and polyclonal hypergammaglobulinemia.

Although this technique has not been applied to human

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pregnancy serum it is likely that the two dimensional polyacrylamide gel would provide sufficient resolution to identify pregnancy specific proteins in humans and in horses if they are present. To assist in this a high sensitivity silver stain was used on the gels (Morrisey, 1981). This technique has sensitivities of 0.42 ng/mm^2 for BSA, 0.083 ng/mm^2 for ovalbumin and 0.17 ng/mm^2 for cytochrome C. This sensitivity would allow the staining of minor serum components on the gel. With the combination of high resolution and sensitive staining it was hoped to distinguish between the two explanations of the results from the investigation of late pregnant mare serum.

4.2 Materials and Methods

4.2.1 Materials

The equipment for the two dimensional gels was obtained from Dr. D.I. Stott, Department of Immunology, Western Infirmary, Glasgow. The micro-dialyser was obtained from BRL Limited, Cambridge, U.K. Serum was obtained, with the assistance of Dr. D.H. Snow, Equine Research Station, Newmarket, U.K., from one thoroughbred and two pony mares at approximately every fifty days of gestation. Serum samples were also obtained from stallions and non-pregnant mares. All chemicals were of analar grade where possible.

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4.2.2 Sample Preparation

Prior to electrophoresis samples were treated to remove serum albumin, reduced and alkylated. To 0.5 ml of serum, 0.5 ml of saturated $(NH_4)_2SO_4$ was added slowly with constant agitation and allowed to stand at 4^oC for 30 minutes. The precipitate was obtained by centrifugation for 2 minutes at 10,000g, washed with 1 ml of 50% saturated $(NH_4)_2SO_4$ and respun. The pellet was then dissolved in 0.9 ml of 0.25 M Tris/HCl, pH 8.0 containing 8 M deionised urea and 0.01 M EDTA (UTE buffer) and 0.1 ml of UTE buffer containing 0.5 M dithiothreitol. This was incubated for 30 minutes at 37^oC after which time 90 µl of 1 M iodoacetamide in UTE buffer was added and incubated for a further 30 minutes at room temperature. The solution was then dialysed, using a microdialyser, against 10 mM Tris/HCl, pH 8, containing 9 M deionised urea. The supernatant from the $(NH_4)_2SO_4$ precipitation was analysed by SDS PAGE as described in Chapter 2, Section 2.2.2.

4.2.3 Two Dimensional Electrophoresis

The solutions used for the two dimensions, isoelectric focusing and SDS polyacrylamide gel electrophoresis, are given in the Appendix.

The first dimension consisted of isoelectric focusing in tube gels. Tubes were 130 mm x 2.5 mm internal

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internal diameter. Before use tubes were cleaned in chromic acid, washed thoroughly in tap water, rinsed three times with distilled water, dried, siliconised with "Repelcote" and washed as before. One end of the tube was sealed with several layers of parafilm. 10 ml of gel solution was prepared by dissolving 5.4 g of urea in 1.14 ml of acrylamide stock, 2 ml of 10% (w/v) Nonidet P40, 2.2 ml of H_2O, 0.5 ml Ampholine pH 3-10 and 0.1 ml of 0.1 M lysine. Once the urea was dissolved the solution was degassed and 2.5 µl of TEMED and 0.1 ml of solution D (riboflavin) added. Tubes were filled to the 10 cm mark with the above preparation by means of a tube attached to a syringe. The gels were overlayed with overlay Solution A and left to polymerise for 2 hours under a fluorescent lamp.

To prepare a gel containing molecular weight standards 1 ml was removed from the 10 ml of gel solution. 100 µg of BSA, ovalbumin, α chymotrypsinogen and myoglobin were added and the gel poured into the tube and allowed to polymerise under a fluorescent lamp. Once polymerised the gel was extruded and frozen using a solid CO_2/methanol mixture (approx. ratio 1 vol:5 vols) and stored at -70^{o}C until required. A 5 mm length of the gel was removed and applied to the centre of the second dimension gel between the two IEF gels.

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Once polymerised the overlay solution and parafilm were removed and the tube gels placed in the electrophoresis tank. The anode chamber was filled with 1 litre of 0.01 M H_3PO_4. Samples, 40-50 µl, were loaded in 50 µl of sample loading solution. Two blank gels were loaded with 50 µl of sample loading solution. The solution in all tubes was then overlayed with overlay Solution B and the tubes finally filled to the top by overlaying with the catholyte, 0.02 M NaOH, 1 litre of which was added to the cathode chamber. Electrophoresis was performed at 100v for 15 minutes, 200v for 15 minutes, 300v for 30 minutes and 400v overnight. The total number of volt hours per run was 7,200.

The gels were then extruded into 5 ml of equilibration buffer by means of a 10 ml syringe connected to the gel tube by plastic tubing. The gels were equilibrated in this solution for 2 hours, the solution being changed once. After this, gels were either used for the second dimension or frozen in a solid CO_2/ methanol mixture (1 vol:5 vols) and stored at -70°C until required. The blank gels were not equilibrated but cut into 5 mm lengths and placed, along with 0.1 ml degassed 0.01 M KCl in an autoanalyser cup and left to equilibrate for 2 hours at room temperature. The pH of each section was then read using a flat membrane electrode.

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The second dimension was vertical SDS polyacrylamide gel electrophoresis. Gels were cast between two glass plates separated by a 1.6 mm spacer. One glass plate had a notch cut in it to accommodate the first dimensional gels. Plates measured 255 mm x 260 mm and two first dimension gels were applied per run. The resolving gel was prepared by mixing 12.5 ml Solution A, 30 ml of Solution B, 50 ml of Solution E and 7.25 ml of H_2O. This mixture was degassed and 0.25 ml of 10% ammonium persulphate added. This solution was poured between the plates to 200 mm above the bottom spacer, overlayed with H_2O and left to polymerise.

During polymerisation the stacking gel solution was prepared by mixing 3.8 ml of Solution B, 4 ml of Solution C, 16 ml of Solution E and 4.2 ml of H_2O. The mixture was degassed and 4 ml of Solution D added. The water was removed from the surface of the polymerised resolving gel and the surface rinsed with 2 x 1 ml of stacking gel solution. Stacking gel solution was poured to the base of the notch and two first dimension gels, anodes towards the centre, were added. The stacking gel solution was then poured to the top of the plates and left for 30 minutes to polymerise under a fluorescent lamp.

Once the stacking gel had polymerised, the gel was inserted into the electrophoresis tank, which contained

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anode and cathode chambers, with the spacer removed from the base. Contact at the cathode was via a filter paper wick. Electrophoresis was performed at 20 mA for 16-18 hours until the bromophenol blue dye front reached the end of the gel. The gel was removed from between the glass plates and prepared for silver staining.

4.2.4 Silver Staining

Gels were prepared for silver staining by prefixing in 50% methanol, 10% acetic acid for 30 minutes followed by 5% methanol, 7% acetic acid for 30 minutes. Gels were then fixed for 30 minutes in 10% glutaraldehyde and then soaked overnight in a large volume of deionised H_2O. After rinsing in deionised H_2O the gel was soaked in 5 µg/ml dithiothreitol for 30 minutes. This solution was then poured off and, without rinsing, a 0.1% (w/v) solution of silver nitrate was added and the gel soaked in this for 30 minutes. The gels were handled at all times wearing gloves and both of the above solutions were filtered to remove dust. Staining was performed in a plastic container and, during soaking in the above solutions, the gels were gently agitated.

While the gels were agitated in the silver nitrate solution, the developer was prepared, consisting of

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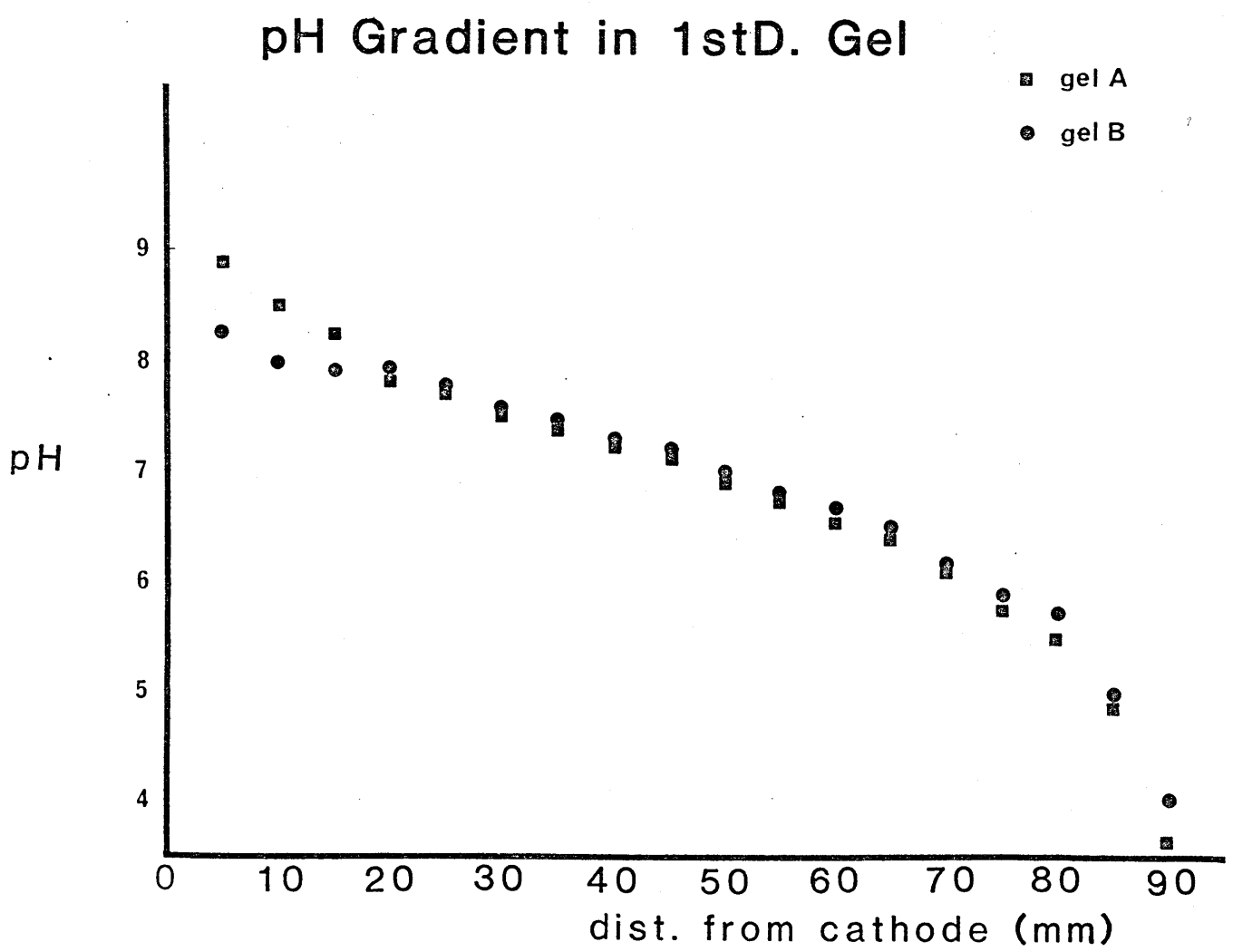
FIGURES 4.1a AND 4.1b

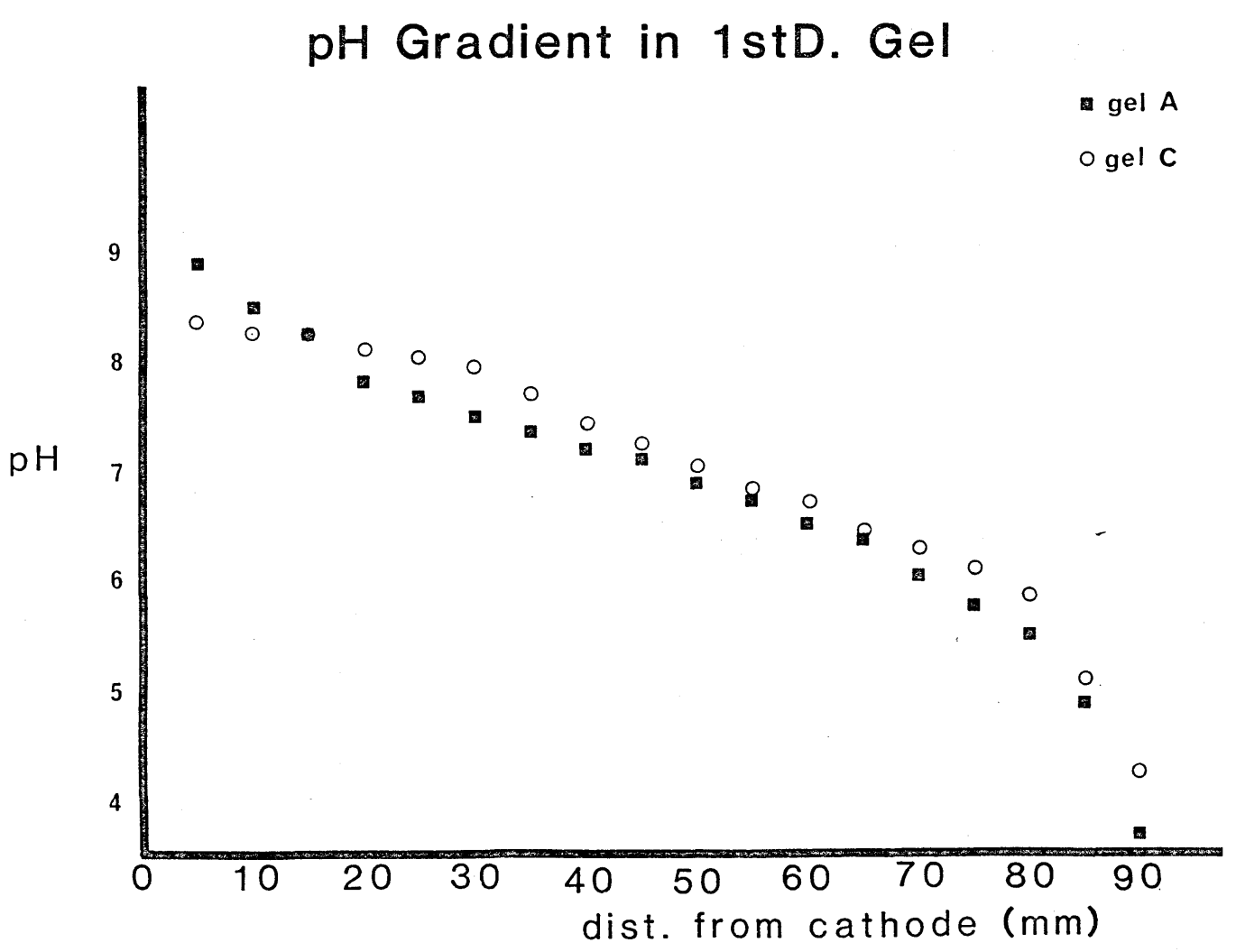
pH GRADIENT IN FIRST DIMENSIONAL ISOELECTRIC FOCUSING GELS

4.1a gels A and B are blank gels taken from the same I.E.F. run.

4.1b gels A and C are blank gels taken from different I.E.F. runs.

The pH gradient was obtained by dividing the blank gel into 5 mm sections and equilibrating each section in 0.01 M KCl for 2 hours at room temperature. The pH was measured using a flat membrane electrode.


pH Gradient in 1stD. Gel
gel A
gel B
pH
9
8
7
6
5
4
0
10
20
30
40
50
60
70
80
90
dist. from cathode (mm)


pH Gradient in 1stD. Gel
gel A
gel C
pH
9
8
7
6
5
4
0
10
20
30
40
50
60
70
80
90
dist. from cathode (mm)

of 3% sodium carbonate containing 50 µl of 37% formaldehyde per 100 ml of developer. Before developing gels were washed once with a small volume of distilled water and twice with small volumes of developer. Developer was then added and the gel continuously agitated until the desired level of staining was attained. The reaction was stopped by adding 5 ml of 2.3 M citric acid per 100 ml developer and the gel agitated for 10 minutes. After this gels were washed in distilled water and photographed.

4.3 Results

The 50% $(NH_4)_2SO_4$ supernatants were tested on SDS polyacrylamide gel electrophoresis and were found to contain principally albumin. Several minor protein bands were visible but these were common to pregnant and non-pregnant mare and stallion sera.

For each isolectric focusing run two blank gels were run to obtain the pH gradient. The gradients were used as a means of comparing the gels in one run with each other and with those from different runs. It was found that all gels conformed to a similar pattern whether they were from the same run (Fig. 4.1a) or from different runs (Fig. 4.1b). A linear gradient was obtained over the range pH 8 to pH 5, there being little variation between gels in this region. However some variation was found between gels in the anode and cathode regions. Despite

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FIGURE 4.2

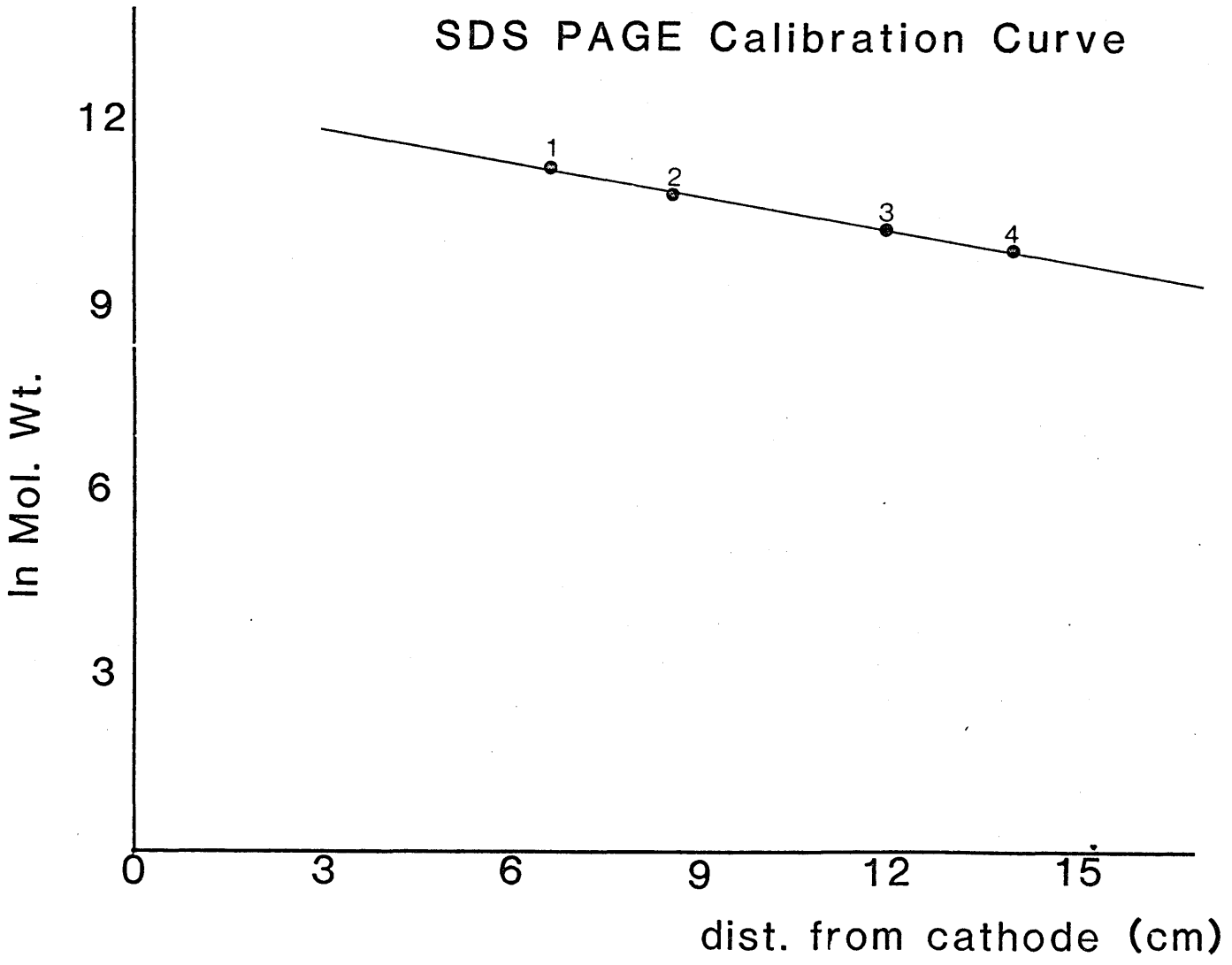
SECOND DIMENSION POLYACRYLAMIDE GEL ELECTROPHORESIS:

MOLECULAR WEIGHT CALIBRATION CURVE

The standards were:-

		mol. wt.
1.	bovine serum albumin	67,000
2.	ovalbumin	43,000
3.	α chymotrypsinogen	25,000
4.	equine myoglobin	18,000

The standards, 100 µg of each, were prepared in a 1 ml first dimensional gel as described in Section 4.2.3. A 5 mm section of this gel was applied per second dimension run and the value given in the graph, distance moved from cathode, represents the mean from four runs in which the dye front moved 180 mm in the resolving gel.


SDS PAGE Calibration Curve
12
9
6
3
ln Mol. Wt.
1
2
3
4
0
3
6
9
12
15
dist. from cathode (cm)

Despite this variation it was concluded that since the greater proportion of the gels was consistent then valid comparisons could be made between gels from the same or different runs. In the second dimension since the current and running time were constant then there would be little variation between gels in the distance moved by proteins and this was found to be the case.

Owing to the thinness of the gels and the agitation during silver staining, many of the gels were damaged by tearing (Figs. 4.5, 4.7 and 4.8). Also on some of the gels intensely staining irregular black spots are present. These are assumed to be due to dust contamination arising during the staining procedure. During silver staining it was found that the standards were overstained by the time that the proteins in the samples showed sufficient staining (Fig. 4.7). In the final gels the standards appeared as a dark line down the centre of the gel. The distances moved by the standards was measured during staining and were used to construct a standard curve (Fig. 4.2).

For the purpose of analysis the proteins in the silver stained gels were referred to by their isoelectric point and approximate molecular weight. The isoelectric point was calculated by the distance moved from the IEF cathode and interpolating this distance on Fig. 4.1. For the molecular weight the distance moved from the top of the gel (SDS cathode) was measured and this was interpolated on

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FIGURE 4.3

A TWO DIMENSIONAL GEL OF PREGNANT MARE SERUM

40 µl of serum from a pony mare in the first 50 days of gestation was applied to the first dimensional gel and isoelectric focusing and polyacrylamide gel electrophoresis performed under denaturing conditions as described in Section 4.2.3.

The gel was silver stained as described in Section 4.2.4.

The scale on the x-axis represents the pH gradient of the first dimension gel as given in Figure 4.1.

The scale on the y-axis represents the molecular weight as calculated from the standard curve (Figure 4.2).

The grided areas on the plate are the groups of spots (A1-A3, B1, and C1-C4) which were unique to early pregnancy (see Table 4.1).

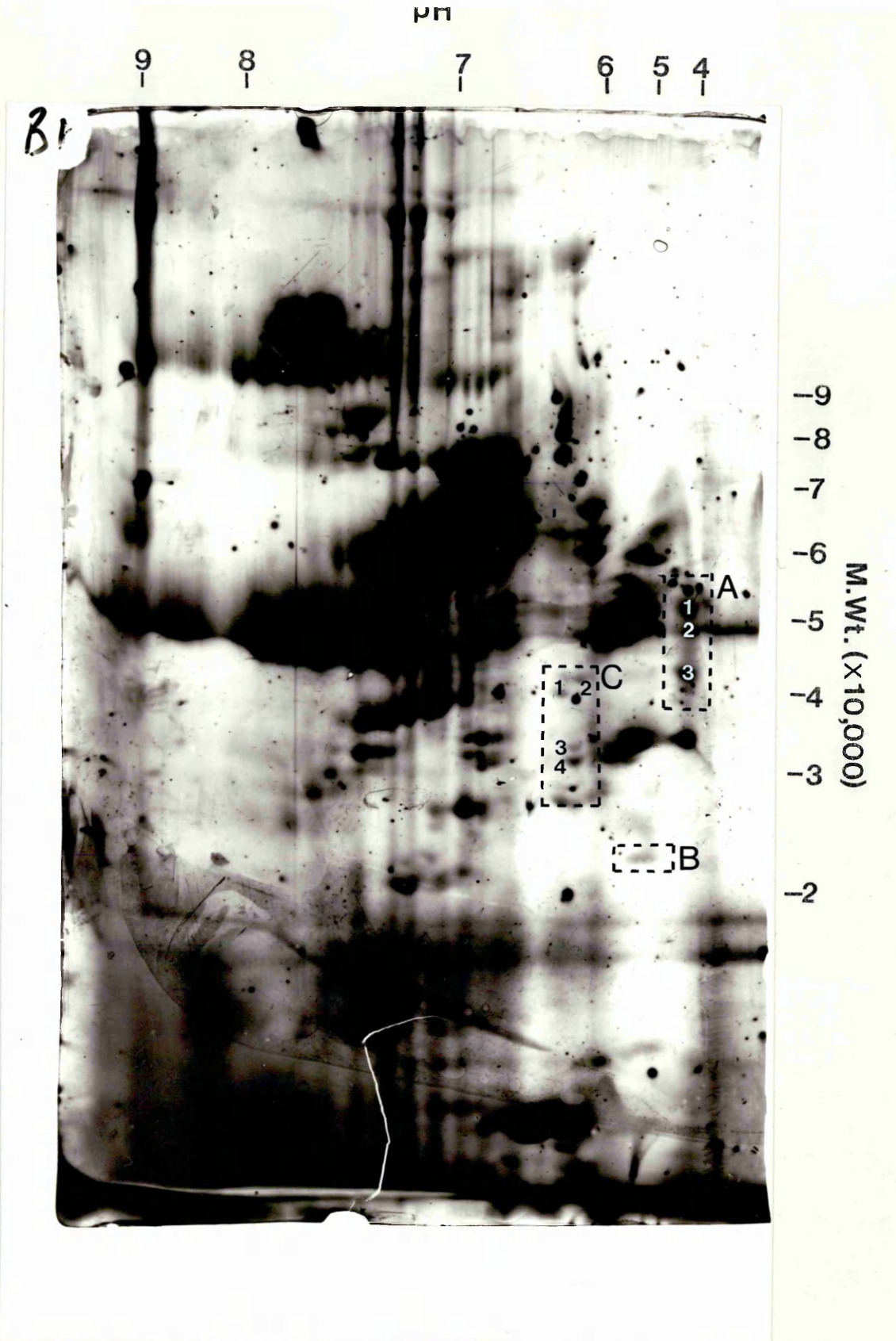


Fig. 4.3 Two Dimensional Gel of P.M.S. in First 50 Days of Gestation.

For results see pp.99-101 and for discussion see pp.102-106.

FIGURE 4.4

TWO DIMENSIONAL GEL OF PREGNANT MARE SERUM

40 µl of serum from a pony mare in the first 50 days of pregnancy applied to the first dimensional gel and isoelectric focusing and polyacrylamide gel electrophoresis performed under denaturing conditions as described in Section 4.2.3.

The gel was silver stained as described in Section 4.2.4.

Note the presence of the spots in the regions A, B and C (see Figure 4.3).

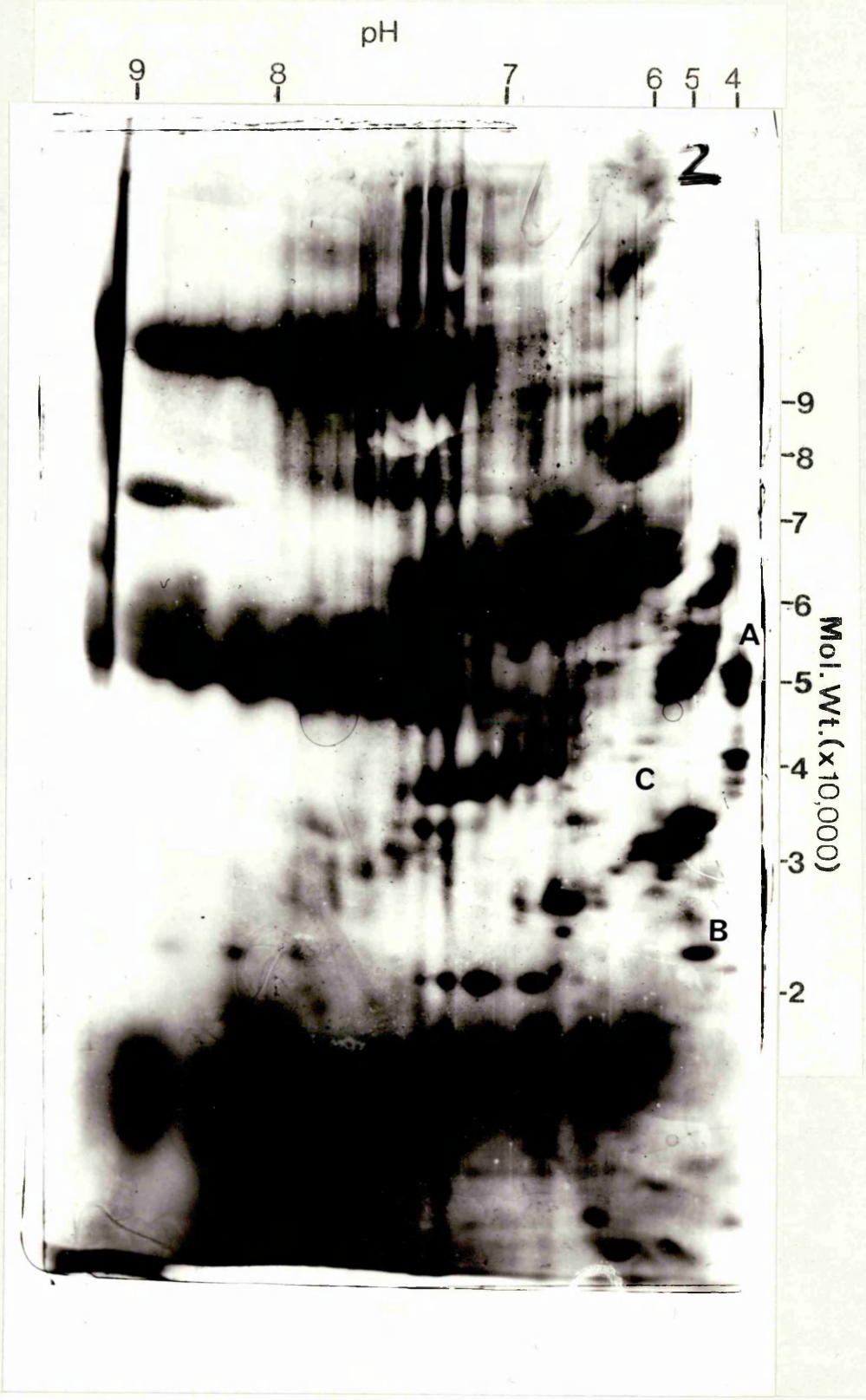


Fig. 4.4 Two Dimensional Gel of P.M.S. in First 50 Days of Gestation.

For results see pp.99-101 and for discussion see pp.102-106.

FIGURE 4.5

TWO DIMENSIONAL GEL OF STALLION SERUM

40 µl of stallion serum subjected to isoelectric focusing and polyacrylamide gel electrophoresis under denaturing conditions as described in Section 4.2.3.

Silver staining was performed as described in Section 4.2.4.

On comparison with 2 D electrophoresis gels of human serum, it is probable that the spots S1, S2 and S3 are albumin, transferrin and IgG light chains respectively.

Note the large number of basic proteins in the grided region and the protein e, f, g and h within this region which were not observed in any sample from mares.

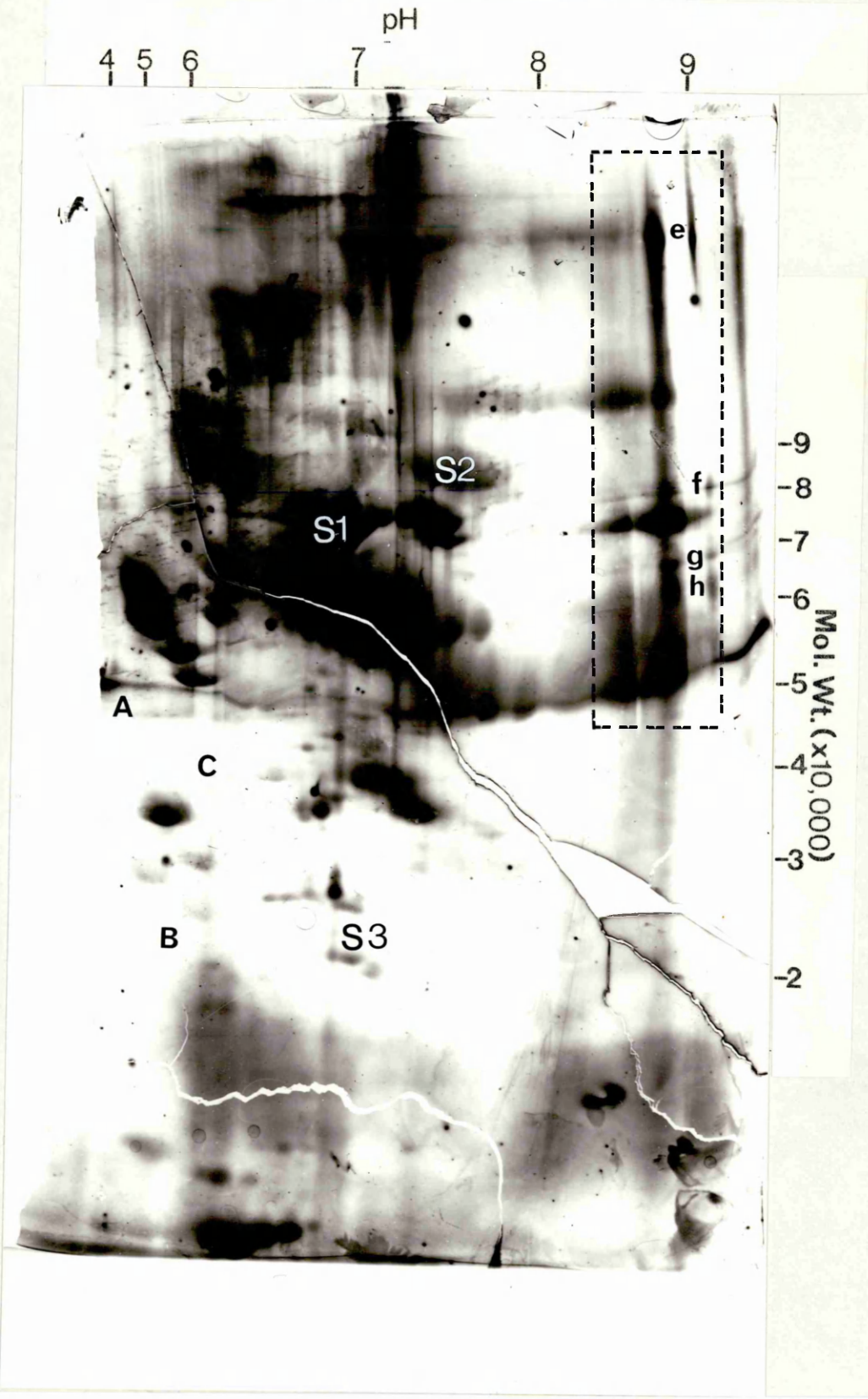


Fig. 4.5 Two Dimensional Gel of Stallion Serum.

The results are given on pp.99-101 and discussed on pp.102-106.

FIGURE 4.6

TWO DIMENSIONAL GEL OF PREGNANT MARE SERUM

40 µl of serum from a pony mare in the last 50 days of gestation was applied. The two dimensional electrophoresis was performed under denaturing conditions as described in Section 4.2.3.

The gel was silver stained as described in Section 4.2.4.

The spots observed in early pregnancy in regions A, B and C are absent from this gel.

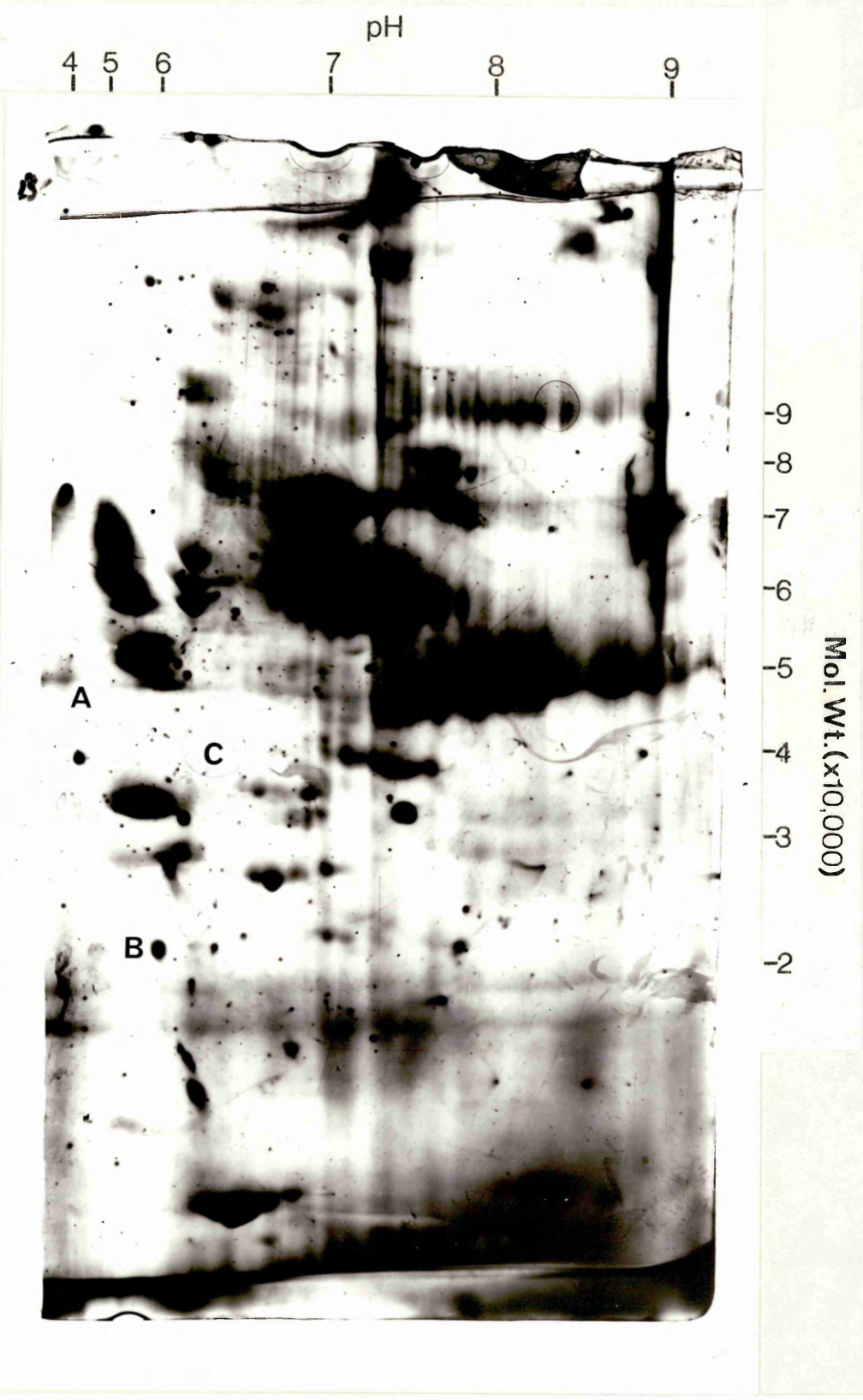
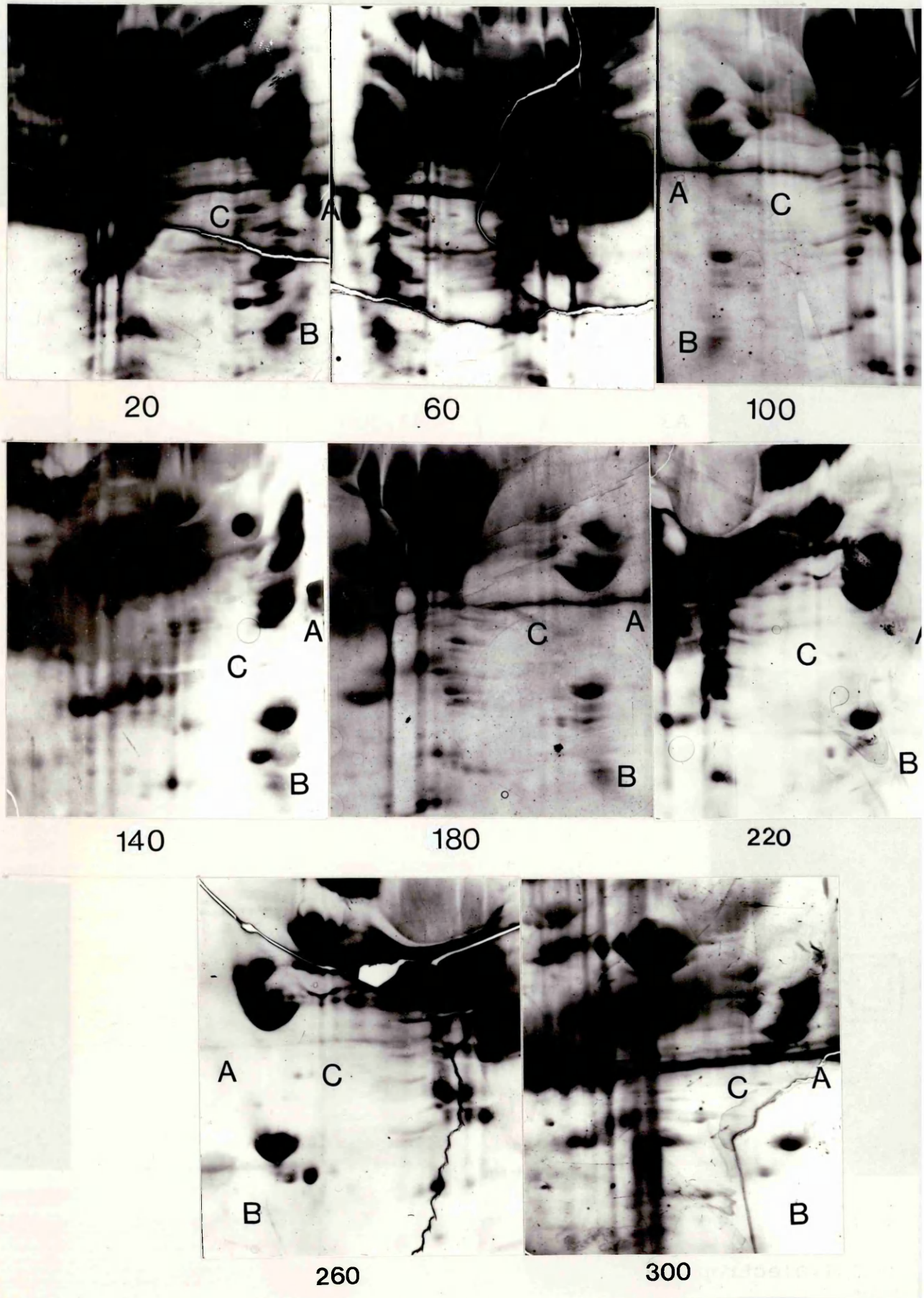


Fig. 4.6 Two Dimensional Gel of P.M.S. in the Last 50 Days of Gestation.

For results see pp.99-101 and for discussion see pp.102-106.



For further information see results pp.99-100 and discussion p.103.

TABLE 4.1 UNIQUE SPOTS ON PMS TWO DIMENSIONAL GEL

Protein	pI	Molecular Weight	Appearance in Pregnancy
A1	4	50,000	First 2 months only
A2	4	47,000	
A3	4	43,000	
B1	5	24,000	First 6 months
C1	6.5	43,000	Concentration higher in early pregnancy
C2	6.2	43,000	
C3	6.2	34,000	
C4	6.2	30,000	

Fig. 4.6.a

Two Dimensional Gels from the Thoroughbred Mare at Various Stages of Pregnancy

The section of the gels shown is the area which contained the proteins in Groups A, B and C, as marked on the photographs. 40 µl of pregnant mare serum obtained at days 20, 60, 100, 140, 180, 220, 260 and 300 post fertilisation were applied per gel. Two dimensional gel electrophoresis and silver staining were performed as described in Section 4.2. This information was used to obtain the value for "Appearance in pregnancy" in Table 4.1.

on Fig. 4.2 to give the molecular weight. Fig. 4.3 has the isoelectric point and molecular weight scales marked out on it.

The gels from the pregnant mares were compared with those from the stallions and non-pregnant mares. Examination revealed that the gels from pregnant mares contained spots which were absent from the non-pregnant and stallion gels. In Figs. 4.3 and 4.4, spots are present which cannot be detected on the stallion gel (Fig. 4.5). In Fig. 4.3, three groups of spots have been marked out A, B and C. Group A contains three spots A1, A2 and A3, Group B contains one spot B1, and Group C contains four spots C1, C2, C3 and C4. The corresponding isoelectric points and molecular weights for these spots are:

A1	pI 4	50,000	C1	pI 6.5	43,000
A2	pI 4	47,000	C2	pI 6.2	43,000
A3	pI 4	43,000	C3	pI 6.2	34,000
B1	pI 5	24,000	C4	pI 6.2	30,000

(Table 4.1)

Figures 4.3 and 4.4 are from pony mares in the first 50 days of gestation. In subsequent gels from these mares, even at 100 days gestation, the spots were undetectable. They were also undetectable in the gels from late pregnancy (Fig. 4.6). The gel from the thoroughbred mare in the first month of pregnancy revealed spots corresponding to A3, B1, C1 and C2. It is possible that two of the other spots, C3 and

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FIGURE 4.7

TWO DIMENSIONAL GEL OF PREGNANT MARE SERUM

50 µl of serum from a thoroughbred mare 20 days after fertilisation was applied. Isoelectric focusing and polyacrylamide gel electrophoresis were performed under denaturing conditions as described in Section 4.2.3.

Silver staining was carried out as described in Section 4.2.4.

Spots corresponding to A_3, B_1, C_1 and C_2 are present.

The spot labelled TS on this gel was observed only in this thoroughbred mare.

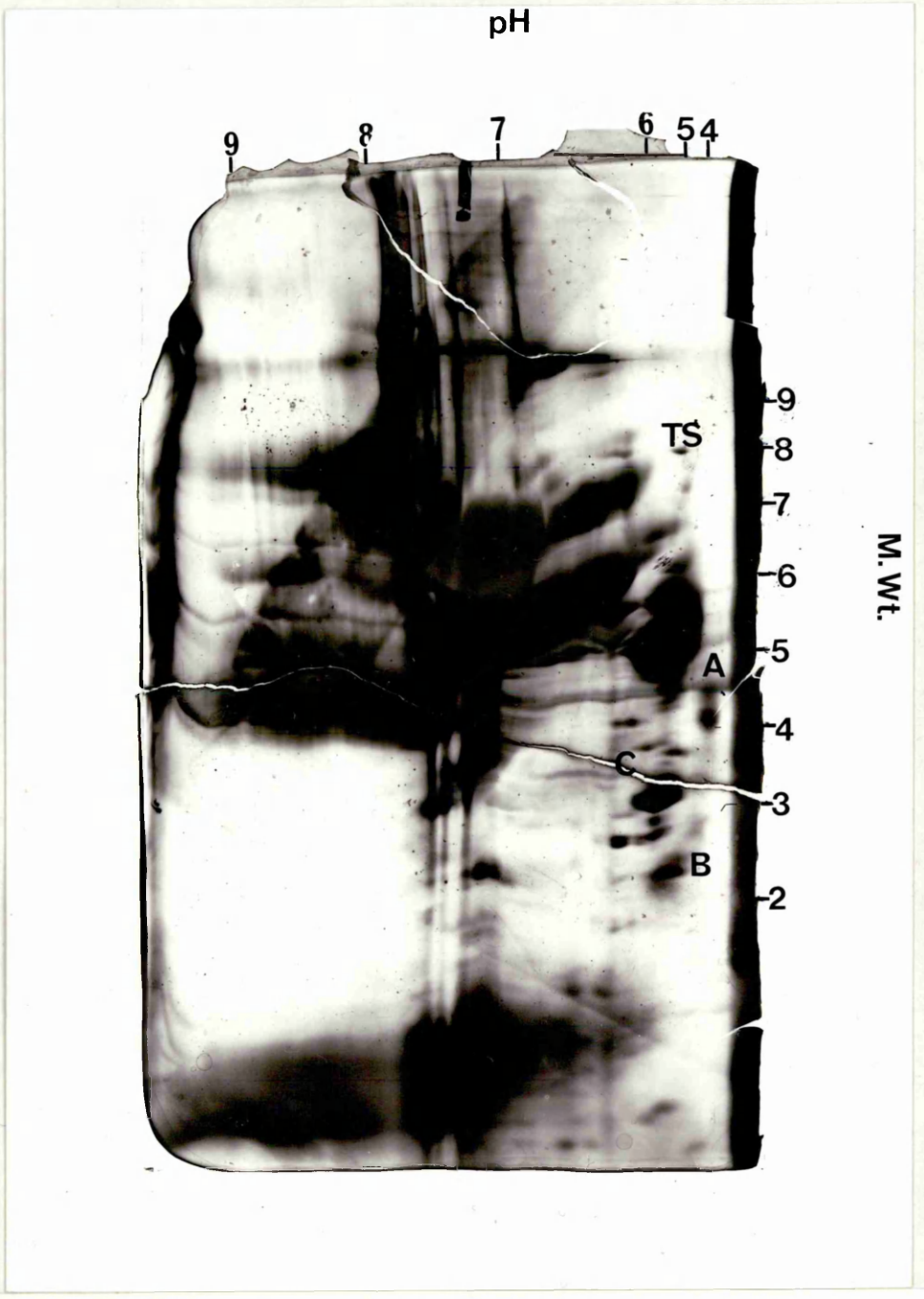

Fig. 4.7 Two Dimensional Gel of P.M.S. in the First 50 Days of Gestation.

The results are given on pp.99-101 and discussed on pp.102-106.

FIGURE 4.8

TWO DIMENSIONAL GEL OF NON-PREGNANT MARE SERUM

40 µl of non-pregnant mare serum was subjected to two dimensional gel electrophoresis under denaturing conditions as described in Section 4.2.3 and the gel silver stained as described in Section 4.2.4.

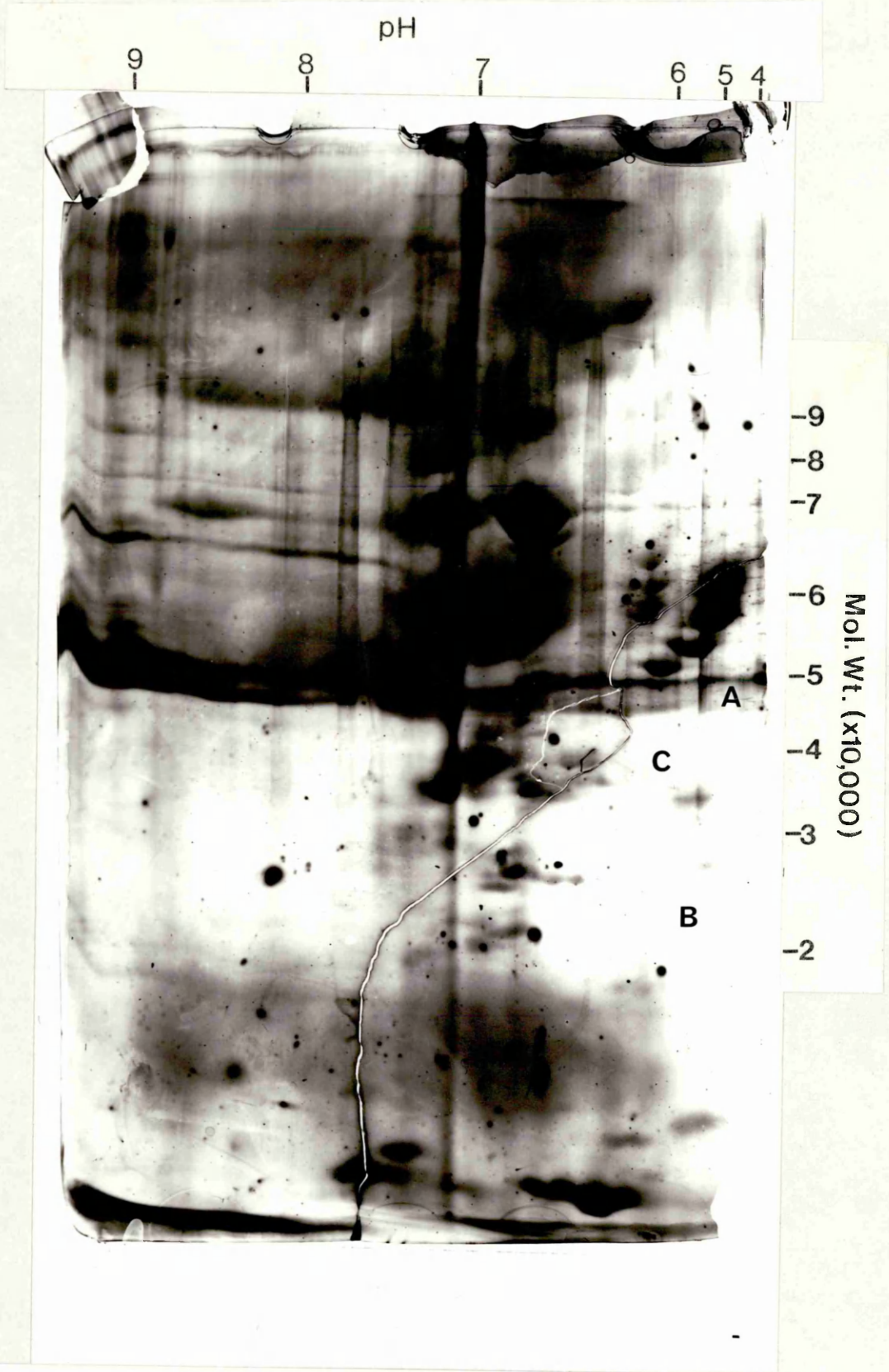

Fig. 4.8 Two Dimensional Gel of Non-Pregnant Mare Serum.

The results are given on pp.99-101 and discussed on pp.102-106.

and C4, are present but are not visible due to overstaining of an adjacent spot (Fig. 4.7). All these spots were present in a similar intensity in the gel of the sample taken from the second month of pregnancy. By the third month the spot in Group A had disappeared and the spots in Group C were only faintly visible. These proteins remained faintly visible until term in the thoroughbred mare but not in the pony mares. The protein in Group B was present until the sixth month of gestation, the spot becoming progressively fainter.

Comparison of the gels from late pregnancy (Fig. 4.6) with those from early pregnancy (Figs. 4.3, 4.4 and 4.7) and those from non-pregnant mares and stallions (Figs. 4.5 and 4.8) indicated that at this stage there were no unique spots.

Several other interesting points can be observed on the gels. On the thoroughbred mare a distinct spot was observed on all gels throughout pregnancy with a pI 5.2 and a molecular weight of 79,000. This spot was not observed in any other of the serum samples from pregnant or non-pregnant pony mares or stallions (Figs.4.3, 4.4, 4.5, 4.6 and 4.8). Also in the gel of stallion serum (Fig. 4.5) a total of 14 proteins can be observed in the basic region of the gel between pH 8.5 and pH 9.0 and in the molecular weight range180,900D to 45,000D. In any sample from a pregnant or non-pregnant mare, the maximum number of proteins recorded in this region was 6. In

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In the non-pregnant mare only 4 of these proteins can be observed (Fig. 4.8). Of particular interest are the four basic proteins, all of pI 9 and molecular weights 180,900, 88,000, 64,000 and 60,000 which were not observed in any samples from mares.

4.4 Discussion

The results show that this high resolution two dimensional system separating proteins on the basis of charge and molecular weight is capable of identifying unique substances in pregnant mare serum. However the unique proteins identified are restricted to the early stages of pregnancy in particular the first two months. The silver stain with its high sensitivity makes visualization of these proteins possible. However in the case of serum it has one major drawback. Serum represents a heterogeneous mixture of proteins which show a wide range of concentrations. There may even be variations in the amount of a particular protein present in the serum between individuals. Therefore it is inevitable that on silver staining proteins in higher concentrations will appear sooner and stain quicker than those at lower concentrations. By the time some of the lower concentration proteins begin to stain the higher concentration proteins will be overstained and may obscure information from the gel. On silver staining it was necessary to obtain a balance which allowed for optimum staining of minor components but did not lead to excessive overstaining of higher concentration

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concentration proteins. The point chosen to stop the staining reaction was when the bands present in the region of pI 5.3 and mol. wt. of 45,000 began to merge due to overstaining. Some of the information was obtained as a result of observing the gels during the staining process. Since the photographs represent arbitrary points in the staining process ("the desired level of staining", Morrisey, 1981) then care must be taken in interpreting the results in view of the fact that the concentrations of proteins may vary considerably between individuals. Therefore results were only taken to be valid if they were observed in several individuals. Also the spots had to be stained in sufficient intensity in the individuals that their presence was not due to a concentration effect. Weakly staining spots observed in single individuals were discarded.

On average the process was capable of resolving 80-100 spots from a 40-50 µl sample of serum. Several of the spots were comparable with those present in humans (Thorsrud et al, 1980). Fig. 4.5 has these proteins marked on it with S_1 most probably being albumin, S_2 transferrin and S_3 IgG light chains. Apart from these proteins there appears to be little similarity between the equine and human gels.

From the gels two types of unique proteins could be identified: (1) those proteins which were pregnancy specific, and (2) those which appear to be specific to the

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the sex or breed of horses. Within the first group eight proteins were identified which were absent from non-pregnant and stallion sera. However these proteins were associated with the early stages of pregnancy. Further subdivision could be made based on the length of time that the proteins were present. Thus, although all proteins in all three groups A, B and C, stained strongly in the first two months of pregnancy, the three proteins in the first group A1, A2 and A3 had disappeared by the third month and the one protein in the second group, B1, had disappeared by the sixth month. The proteins in the third group stained strongly in the first two months of gestation and thereafter stained but only very weakly until term. It is probable that the proteins in Group C are normal serum components raised in concentration in early pregnancy but returning to normal levels after the third month. The failure to detect them in normal equine sera could be due to poor staining in this region of the gel and a lower concentration than in late pregnancy. However the other proteins A1, A2, A3 and B1 are early pregnancy specific since they can only be detected in early pregnancy serum and stain strongly in these gels.

The reduction and alkylation treatment of samples prior to electrophoresis would effectively dissociate subunits held together by disulphide bonds. The DTT would reduce these disulphide bridges and the alkylating reagent, iodoacetamide, would prevent them reforming. The dissociating conditions of the electrophoresis would also

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also reduce non-covalently bonded subunits to single polypeptide chains. Therefore each spot or band on the silver stained gels represents a single polypeptide chain. It cannot be deduced from the gel whether these single polypeptide chains represent individual proteins or subunit chains of a larger protein.

The four polypeptide chains A1, A2, A3 and B1 are the earliest recorded proteins to be detected during pregnancy in the mare being present in the thoroughbred mare by day 20 after fertilisation. Previous reports indicate that the earliest pregnancy proteins recorded in the mare are MPP1 by day 30 (Gidley-Baird et al, 1983) and eCG by day 40 (Allen, 1969). Although the pregnancy specific spots A1, A2, A3 and B1 cannot be identified some speculation is possible. Of particular interest for comparison in the mare are MPP1 and the early pregnancy factor. The low pI of eCG (pI 1.8) means that it would not focus within the gradient of the gels used and was lost. The molecular weight and isoelectric point of MPP1 is unknown but this protein has been detected by day 30 and it is probable that one or several of the spots represents MPP1 or its subunits. The early pregnancy factor has not been recorded in the horse but during pregnancy in sheep it exists in multiple forms notably a 250 K form, a 50 K form and a 20 K form (Clarke et al, 1980). It is possible that two of the spots A1 (M. Wt. 50 K) and B1 (25 K) represent forms of the early pregnancy factor in the mare. In early pregnancy in the sheep the 250 K and 20 K form only are

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are present but the 50 K form can be obtained by removal of a carrier component from the 250 K form (Clarke et al, 1980). The 50 K form then predominates in mid pregnancy whereas in this study protein A1 had disappeared by the third month of gestation.

Therefore although no specific comparisons can be made novel proteins have been detected within 20 days of fertilisation and have disappeared from the serum by the sixth month of gestation. It would be of great interest to fractionate early pregnancy serum from the mare to obtain sufficient protein for characterisation.

The other class of proteins identified in this study relate to the sex and breed of the horse. Thus in the stallion a large number of basic proteins were present, 14 in total. The maximum recorded in the mares was 6 but with longer staining times these proteins may have appeared. However the fact that they readily stain in the stallion indicates that if they are present in the mare then it is at a much lower concentration. Before any definite conclusions can be drawn that these spots represent sex specific proteins, a far larger sample of mares and stallions would have to be examined. The same applies to the single spot recorded exclusively in the thoroughbred mare.

From this investigation it can be concluded that pregnancy proteins are present in the mare in the early

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early stages of pregnancy. These proteins were detected in high levels in early pregnancy and either disappeared with the advancement of pregnancy or, in the case of the thoroughbred mare, decreased to very low levels by term. Four proteins were identified in the former case (Groups A and B) differing in the time of their disappearance from pregnancy serum and four proteins were identified in the latter. This therefore supports the hypothesis that pregnancy proteins, similar to those identified in other species, are absent from late pregnant mare serum.

CHAPTER 5

ALKALINE PHOSPHATASE AND SEX HORMONE BINDING GLOBULIN

5.1 Introduction

During pregnancy in humans the circulating levels of a number of normal serum proteins increase significantly in response to the pregnant state. Some of these can be measured in assays depending on their biological activities and two of these, alkaline phosphatase and sex hormone binding globulin, were investigated in the pregnant mare.

Levels of alkaline phosphatase show a measurable increase in the third trimester of human pregnancy of approximately 100% (Young et al, 1946). This increase in circulating alkaline phosphatase activity is due to the presence of a placental isoenzyme which is distinguishable from other serum isoenzymes by its remarkable thermostability (Fishman et al, 1972). Originally assays for this placental specific enzyme relied upon this heat stability but recently a highly sensitive radioimmunoassay has been used to measure levels throughout pregnancy (Holmgren et al, 1978). Levels are low during the first trimester with a significant increase occurring around mid-pregnancy. Levels reach a peak of mean value 252 ± 70 ng/ml by the fortieth week of pregnancy, a twenty-five fold increase on first trimester values

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During pregnancy the placental isoenzyme is synthesized by the syncytiotrophoblast after the twelfth week and is secreted into the maternal circulation (Stigbrand et al, 1982). The levels of placental alkaline phosphatase coincide with the growth of the placental microvilli with peak levels occurring when microvilli growth ceases (Fishman et al, 1972). Levels have also been correlated with the increased synthesis of oestrogens (Fishman et al, 1968). However the normal range of placental alkaline phosphatase is considered to be too large to be of any clinical significance in identifying pregnancy disorders (Sigbrand et al, 1982). Little is known of the physiological function of this isoenzyme during normal pregnancy (Holmgren et al, 1978).

A previous study reported a 30% increase in alkaline phosphatase activity during pregnancy in the mare (Earle and Cabell, 1952), however only three samples were taken at random. Although the heat stability of the human placental alkaline phosphatase appears to be unique to man and closely related subhuman primates (Doellgast and Benirschke, 1979) evidence suggests that placental alkaline phosphatases are present in most mammalian species (Goldstein and Harris, 1979). Alkaline phosphatase was measured throughout pregnancy in the mare both for total activity and for a heat stable form.

Sex hormone binding globulin is a pregnancy associated protein, synthesized by the maternal liver, the concentration of which rises during pregnancy in humans (Anderson, 1974). Levels rise steeply in the first trimester and gradually thereafter to reach a maximum by term. There is a rapid return to non-pregnancy levels after partuition. The increase in SHBG levels has been estimated from 50-70 nM in the non-pregnant female to 300-400 nM in the pregnant one (Pearlman et al, 1967). The production of SHBG is controlled by the steroids it binds with oestrogens stimulating its production in the liver (Anderson, 1974). It is assumed that the rise during pregnancy is associated with the increase in unconjugated oestradiol seen in pregnancy (Svendson and Sorensen, 1964). However there is no change in circulating levels of SHBG during the menstral cycle despite the steroid hormone changes which take place. This indicates that the changes in pregnancy are probably due to large increases in endogenous steroid production associated with advancing pregnancy and partuition (Pearlman et al, 1967).

SHBG has been identified in the rabbit (Mahoudeau and Corval, 1973) and similar proteins probably exist in most primates (McCormack, 1971, and Anderson, 1974) but no investigation has been reported in the horse. Therefore, using a method which had successfully identified and measured SHBG in humans (Rosner, 1972), the serum of pregnant mares was investigated for a SHBG analogue.

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Also investigated was the possibility of cross reactivity between antisera to human pregnancy proteins and late pregnant mare serum using antisera to human pregnancy proteins. In previous reports cross reactivity between antisera to most human pregnancy proteins and pregnancy sera from other species has been limited to the closely related subhuman primates (Lin and Halbert, 1978). However in the case of pregnancy zone protein a higher degree of inter-species cross reactivity has been recorded. Hence with an antiserum to human PZP von Schoultz et al (1976) were able to demonstrate a PZP analogue in pregnant beagle dogs and Rhesus monkeys. Anti-dog PZP cross reacted strongly with pregnant cow and sheep sera. Further work with an anti-swine PZP revealed this protein in the serum of pregnant horses (Martinsson and Carlstrom, 1977). Thus apart from PZP no previous work had tested the cross reactivity between antisera to human pregnancy proteins and pregnant mare serum. In view of this pregnant mare serum was tested using antisera to human PAPP A, PZP and two recently discovered pregnancy proteins, α_2 and β_1 pregnancy associated macroglobulins.

5.2 Materials and Methods

5.2.1 Materials

Serum samples for both the alkaline phosphatase and sex hormone binding globulin assays were obtained from one thoroughbred mare every week of gestation and from two pony mares at various stages of

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of gestation. Samples were also obtained from six non-pregnant mares, two stallions, a colt and a gelding. Alkaline phosphatase activity was assayed using a BCL Alkaline Phosphatase Kit (No. 415 286 and No. 415 294), Boehringer Mannheim GmbH, Mannheim, West Germany and a LKB reaction rate analyser (LKB, Bromma, Sweden).

Tritiated dihydrotestosterone was obtained from the Steroids Laboratory, Glasgow Royal Infirmary. The radioinert dihydrotestosterone was obtained from Sigma Chemical Company, Fancy Road, Poole, Dorset, England.

Antiserum to human PAPP A was obtained from Dr. R.G. Sutcliffe, Department of Genetics, University of Glasgow, and the antisera to PZP (α_2 PAG), α_2 and β_1 PAM were obtained from Professor W.H. Stimson, Division of Immunology , University of Strathclyde, as was the thirty-sixth week human pregnancy plasma.

5.2.2 Alkaline Phosphatase Activity

Alkaline phosphatase activity in mares was measured throughout pregnancy using a 1 M diethanolamine buffer, pH 9.8, with 0.5 mM $MgCl_2$ at 37°C with 10 mM p-nitrophenol phosphate as substrate. The samples from the six non-pregnant mares were used to establish a normal range. Pregnant mare serum was also

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also assayed for heat stable isoenzymes which involved heating the sample at 56°C for 20 minutes in a water bath prior to measuring the activity. All activities were calculated in IU/l.

5.2.3 Sex Hormone Binding Globulin

The method used for the measurement of sex hormone binding globulin was that of Rosner (1972). Initially the validity of the assay to distinguish between specific globulin binding and non-specific binding to serum albumin was investigated using 15,000 cpm of ^{3}H dihydrotestosterone added to either 0.1 ml of late PMS or a solution of 50 mg/ml equine albumin. After incubation for 15 minutes at room temperature and 15 minutes at 0°C, 0.9 ml of ice cold ammonium sulphate at 10%, 20%, 30%, 40%, 50%, 60%, 70% and 80% saturation were added to the tubes with constant mixing. Within 2 minutes the precipitate was spun down by centrifugation at 0°C. From the supernatant 0.5 ml was removed, mixed with 10 ml of scintillation cocktail and counted. The amount of radioactivity in the supernatant was then compared with the concentration of $(NH_4)_2SO_4$.

All equine samples were tested at the following specific activities of the ^{3}H DHT:- 8,500 cpm/ng, 5,700 cpm/ng and 1,200 cpm/ng. They were prepared by adding radioinert DHT to the radioactive DHT in

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in specific amounts to give the desired specific activity. Human pregnancy serum from the thirty-sixth week was assayed at a specific activity of 1,200 cpm/ng to monitor the success of the assay. A sample of equine seminal plasma was also assayed at this specific activity.

The assay was performed in duplicate for samples and in triplicate for the control. 1,500 cpm of ^{3}H DHT in methanol were added per assay tube. 120 ng of radioinert DHT in methanol were added to the control tubes and the solvent evaporated at 40^{o}C. Serum was diluted in an assay buffer, pH 7.0, which consisted of 0.05 M sodium phosphate and 0.15 M NaCl. For human pregnancy serum and equine seminal plasma the dilution was 1:20 whereas dilutions of 1:5, 1:10, 1:15 and 1:20 were tried for all equine samples. 0.5 ml of dilute serum was used per assay tube. Samples were incubated at room temperature for 15 minutes then transferred to an ice bath for a further 15 minutes. 0.5 ml of ice cold saturated ammonium sulphate was added with constant vortexing to give 50% saturation and the precipitate spun down in a refrigerated centrifuge. From the supernatant 0.5 ml was removed and mixed with 10 ml of scintillation cocktail and counted. The amount of radioactivity added per tube was taken as the mean of the three controls. The results were expressed as follows:-

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$$\% \text{ DHT specifically bound} = \frac{A - B}{A} \times 100\%$$

and also using the formula given by Rosner (1972)

$$\text{TeBG} = \frac{A - B}{C} (D)$$

where TeBG = capacity of serum to bind DHT in µg/100 ml, A = cpm per assay tube (from controls), B = cpm in supernatant of assay tube, C is the specific activity (cpm/ng) and D is a constant allowing for the serum dilution and the change in units (4 in the case of a 1:20 dilution and 3 in the case of a 1:15 dilution).

5.2.4 Antisera to Human Pregnancy Proteins

The antisera obtained to human pregnancy proteins were tested for cross reactivity with pregnant mare serum from mid and late gestation by Ouchterlony double diffusion (Section 2.2.1). Stallion serum and plasma from a thirty-sixth week pregnant woman were used as controls.

5.3 Results Alkaline Phosphatase

A similar pattern was observed in all three pregnant mares throughout gestation although there was some individual

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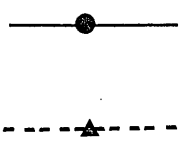
FIGURE 5.1

ALKALINE PHOSPHATASE ACTIVITY THROUGHOUT PREGNANCY IN THE MARE

Serum alkaline phosphatase activity as measured throughout pregnancy in one thoroughbred and one pony mare. Samples were obtained from the thoroughbred mare every month and from the pony mare approximately every 50 days.



thoroughbred mare

pony mare

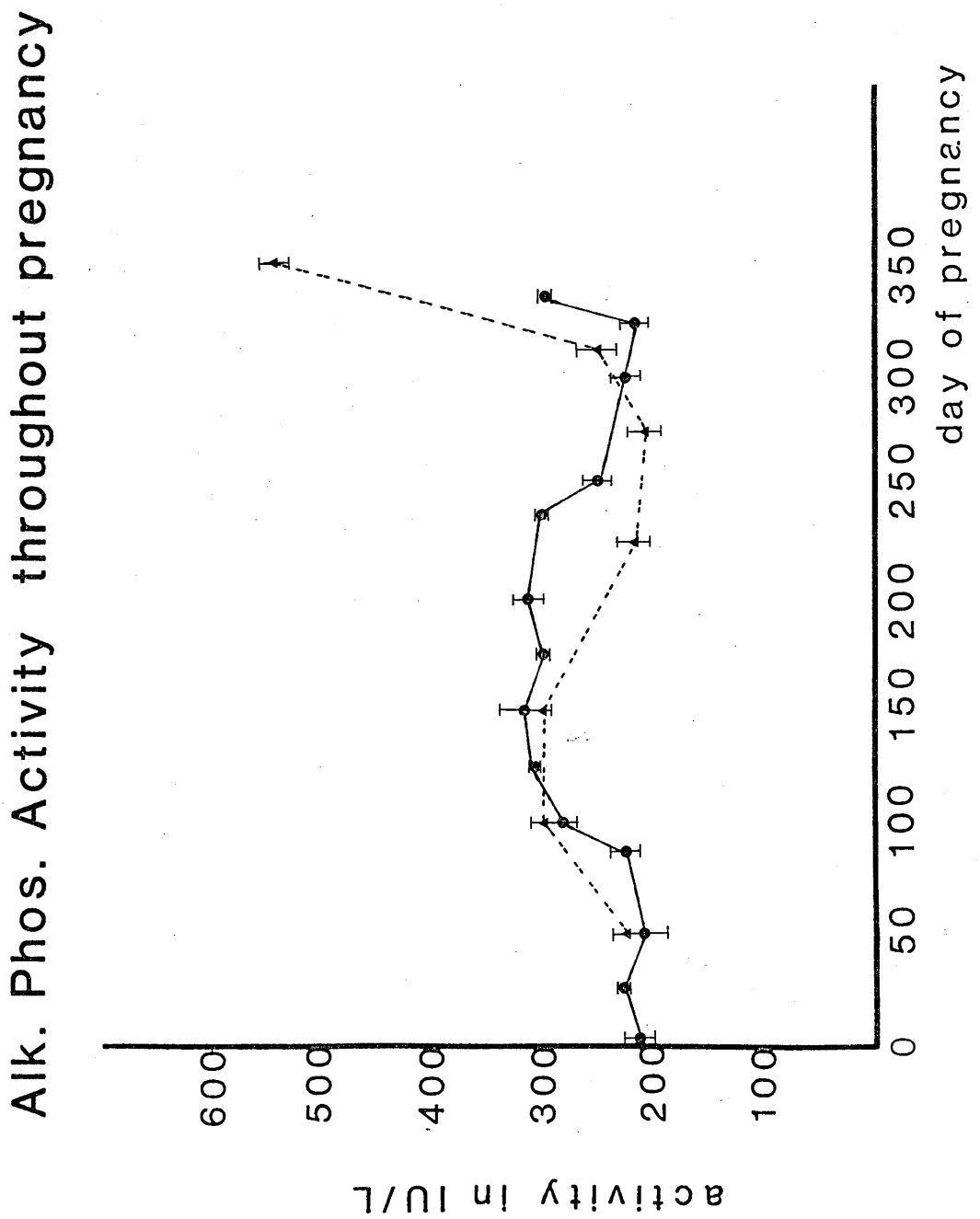


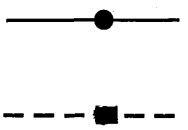
Fig. 5.1

The results are given on pp.114-115 and discussed on pp.116-118.

FIGURE 5.2

COMPARISON OF THE DHT BINDING PROPERTIES OF EQUINE SERUM AND ALBUMIN

To 0.1 ml of late pregnant mare serum or a solution of 50 mg/ml equine albumin 15,000 cpm of ^{3}H DHT were added. After 30 minutes incubation, 0.9 ml of ice cold ammonium sulphate at 10% - 80% saturation were added and the cpm remaining in the supernatant obtained.



equine albumin

late pregnant mare serum

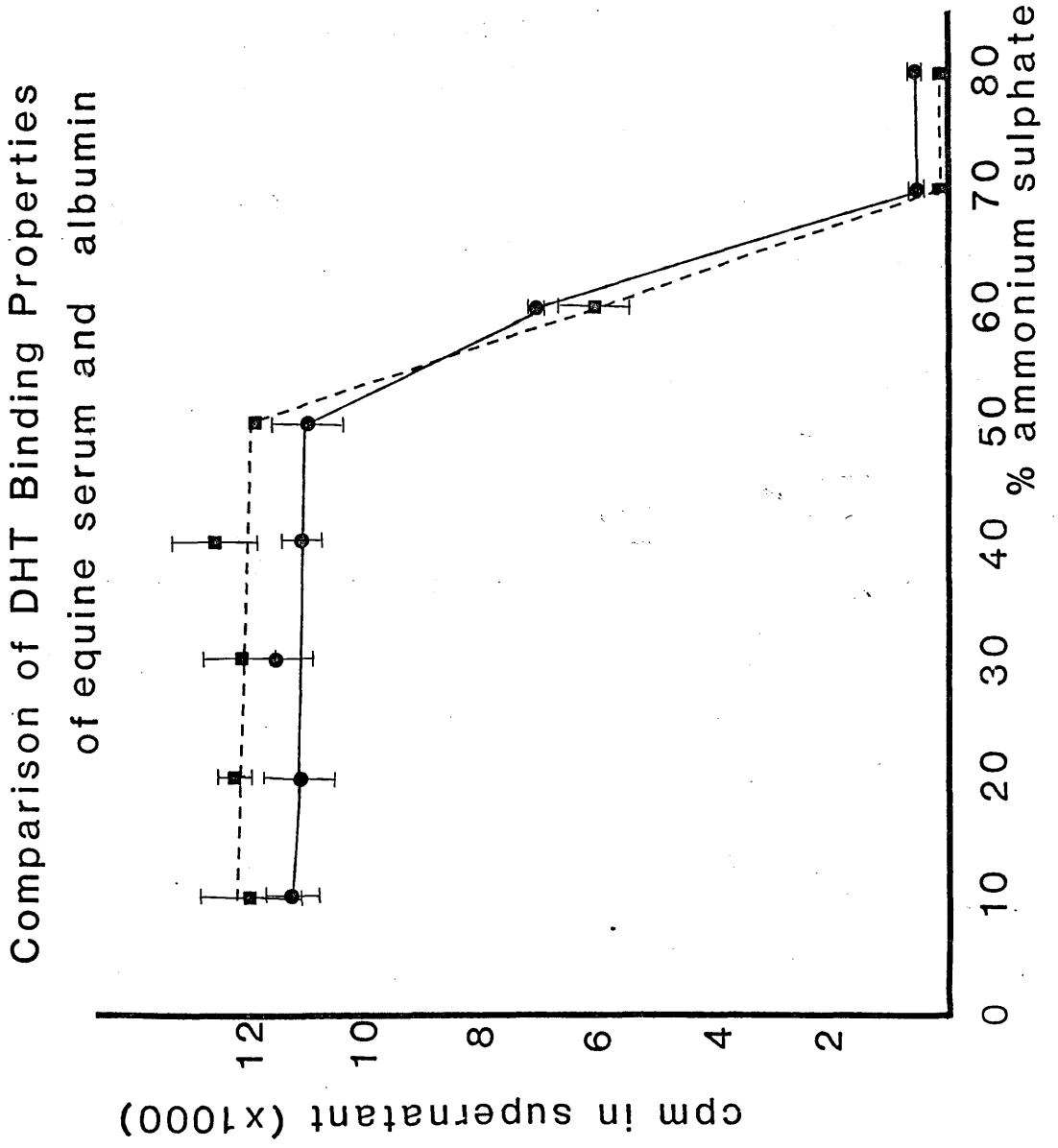


Fig. 5.2

The results are given on pp.115-116 and discussed on pp.118-121.

individual variation. Fig. 5.1 gives the results obtained from the thoroughbred mare and one of the pony mares. About day 100 there was a 25% increase in serum activity, this higher level being sustained until approximately day 230. At this point levels declined to their original value until partuition. Post-partum levels were very high increasing sharply by approximately 100%. All samples except the post-partum ones were within the normal range obtained with six different non-pregnant mares, i.e. 200-500 IU/l. Heating pregnancy serum at 56^{o}C for 20 minutes reduced the alkaline phosphatase activity to undetectable levels in the system used.

Sex Hormone Binding Globulin

The initial investigation into the validity of the assay in horses indicated that the agent responsible for binding the ^{3}H DHT in serum was soluble in 50% $(NH_4)_2SO_4$. The pattern obtained with serum was identical to that obtained with equine albumin (Fig. 5.2), the radioactivity being precipitated in both at 60-70% $(NH_4)_2SO_4$. This result was further verified by the behaviour of the different equine sera in the assay. With all samples of equine sera at each dilution and specific activity the radioactivity remained in the supernatant. The % DHT bound specifically to SHBG in all equine serum samples was 0%. The value obtained for thirty-sixth week human pregnancy serum was 96% and equine seminal plasma

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FIGURE 5.3

ANTISERUM TO HUMAN PZP TESTED AGAINST EQUINE PREGNANCY SERUM

1. 10 µl of third trimester pregnant mare serum.
2. 10 µl of 36th week human pregnancy plasma.
3. 10 µl of second trimester pregnant mare serum.
4. 10 µl stallion serum.
5. 10 µl of third trimester pregnant mare serum.
6. 10 µl of non-pregnant mare serum.

As 10 µl of anti human PZP Ouchterlony double diffusion was performed on 1% (w/v) agarose in 0.9% (w/v) saline as described in Section 2.2.2.

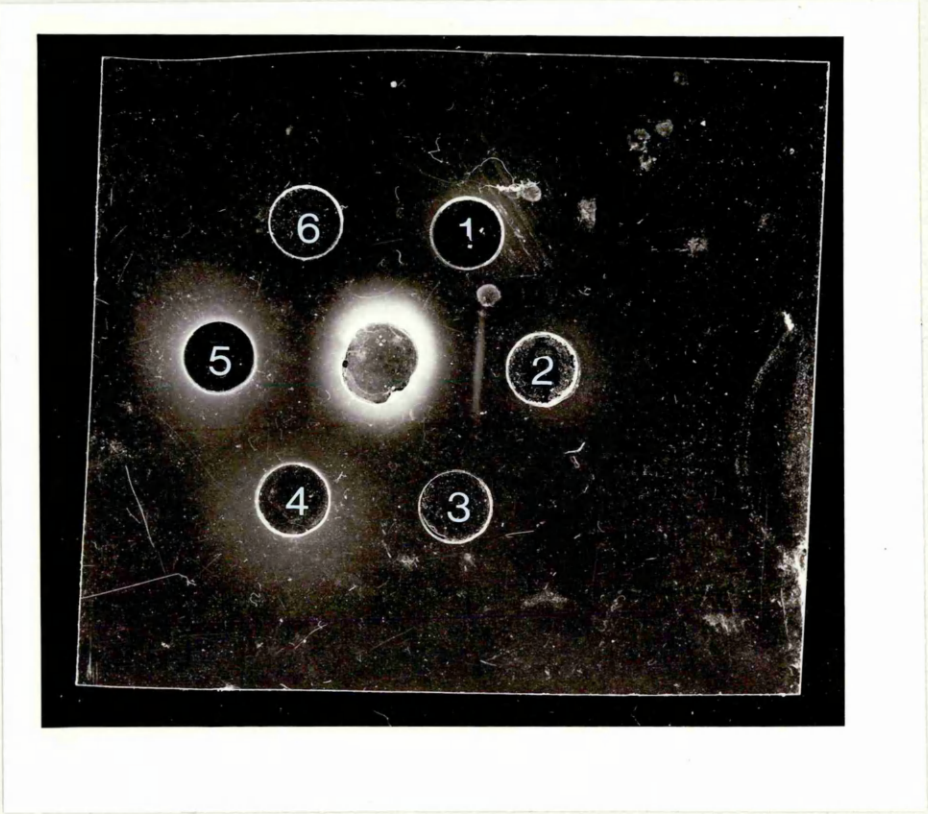

Fig. 5.3 Antiserum to Human PZP Tested Against Equine Pregnancy Serum.

The results are given on p.116 and discussed on pp.121-122.

plasma specifically bound 82% of the DHT. Values of TeBG were 0 µg/100 ml in all equine samples, 4.68 µg/100 ml in human pregnancy serum and 3.28 µg/100 ml in the case of equine seminal plasma.

Antisera to Human Pregnancy Proteins

The antisera to human PZP cross reacted strongly with thirty-sixth week pregnant human plasma. No cross reactivity was observed with either mid or late PMS or stallion serum (Fig. 5.3). A similar result was obtained with antisera to PAPP A and α_2 PAM although the cross reactivity with thirty-sixth week human pregnancy plasma was not as strong. No cross reactivity was observed between the anti β_1 PAM and human pregnancy plasma or equine sera.

5.4 Discussion

The alkaline phosphatase which accounts for the increase in serum activity during pregnancy in humans is a heat stable placental isoenzyme synthesized by the syncytiotrophoblast (Stigband et al, 1982). This isoenzyme significantly increases the total serum activity by the third trimester, the increase being approximately 100% by term (Young et al, 1946). The levels of the placental alkaline phosphatase correlate with the growth of the placenta (Fishman et al, 1968).

In the mare the changes in alkaline phosphatase serum activity in pregnancy did not resemble the human pattern. In particular a decrease in activity was observed during the third trimester when levels returned to normal and it can therefore be concluded that if the changes in serum alkaline phosphatase in mares are caused by an isoenzyme of placental origin it is not secreted into the maternal circulation in a manner which reflects placental growth.

The placental isoenzyme in humans appears to be a late evolutionary event in that it has only been identified in man, chimpanzee and orangutan (Doellgast and Benirschke, 1979), the placental form of alkaline phosphatase in other eutherian mammals such as cat, dog, guinea pig, cow, sheep and pig being similar in its thermolability and chemical inhibitors to the liver form (Goldstein and Harris, 1979). It is possible that the gene locus for placental alkaline phosphatase in Hominidae represents a late evolutionary event, the placental locus in other species being identical to that of the liver (Goldstein and Harris, 1979). No heat stable alkaline phosphatase was identified in pregnant mare serum and the horse could therefore be included in the group of species examined by Goldstein and Harris (1979) which had placental isoenzymes identical to the liver isoenzyme.

In the mare levels of serum alkaline phosphatase were found to be unrelated to placental growth but significant changes did occur. Previous work has reported a 30%

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30% increase in serum alkaline phosphatase activity during pregnancy and a further rise on lactation (Earle and Cabell, 1952), however samples were not taken consistently throughout pregnancy. In this study samples from three pregnant mares throughout gestation revealed a consistent pattern of a 25% increase by day 100 followed by a plateau until day 230 at which point a fall was detected to early pregnancy levels. A substantial increase was recorded at the time of partuition coinciding with the onset of lactation. The pattern of serum activity during pregnancy in the mare resembles that of the conjugated and unconjugated oestrogens in the maternal circulation. The principal oestrogens in the pregnant mare at this time are oestrone, equilin and equilenin. Oestrone levels rise dramatically during the fourth month of pregnancy, this level being maintained for a further four or five months (Cox, 1975). Further investigation would be necessary to confirm this relationship; however it is interesting that a relationship has been noted in pregnant humans between oestrogen synthesis and alkaline phosphatase activity (Fishman et al, 1968). Whether the variation in serum activity was from placental or liver origin is difficult to determine since in the horse these isoenzymes are assumed to be identical in their low heat stability.

The method employed by Rosner (1972) to measure sex hormone binding globulin in humans relies upon the fact that the dihydrotestosterone binds strongly to SHBG but has a low affinity for corticosteroid binding globulins.

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globulins. Also the assay allows the DHT bound specifically to SHBG to be separated from any which may be bound non-specifically to serum albumin. In humans this was readily demonstrated by comparing the ^{3}H DHT binding properties of a solution of albumin and pregnancy serum. Comparison was made of the radioactivity precipitated at a variety of $(NH_4)_2SO_4$ concentrations. All the ^{3}H DHT in the serum was removed from solution at 50% saturated $(NH_4)_2SO_4$, bound to SHBG, whereas in the albumin solution the radioactivity remained in the supernatant until 60-70% $(NH_4)_2SO_4$. In order to validate the assay in equids this procedure was applied to a solution of equine albumin and late pregnant mare serum. However both samples behaved identically in that all the ^{3}H DHT remained in the supernatant until 60-70% $(NH_4)_2SO_4$. Therefore it was concluded that an analogue to human sex hormone binding globulin was absent from pregnant mare serum. That an analogue to SHBG was absent from all equine sera is demonstrated by the assay performed on the selection of equine sera. No variation was found in any of the samples from stallions, pregnant and non-pregnant mares, a gelding and a colt with all the ^{3}H DHT remaining in the supernatant at 50% $(NH_4)_2SO_4$.

During pregnancy in humans levels of SHBG increase markedly. Using this technique Rosner (1972) described an increase in levels from a mean of 1.85 µg/DHT bound/ 100 ml of serum to a mean of 12.4 µg DHT bound/100 ml of serum by the thirty-sixth week of pregnancy. As an assay

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assay control a sample of thirty-sixth week human pregnancy serum was included along with the equine samples. A value of 4.68 µg DHT bound/100 ml serum was recorded for this sample which is low in comparison with the mean given by Rosner (1972) but is nevertheless high when compared with the non-pregnant woman value.

An androgen binding globulin has been identified in the testis of rats and boars which is secreted into the tubular fluid and is transported into the epididymis (Sanborn et al, 1976). It is believed to perform a local role in maintaining high levels of testosterone in the epididymis (Hansson et al, 1974). A similar protein probably exists in the stallion testis which would account for the levels of ^{3}H DHT binding detected in the sample of equine seminal plasma.

In humans the increase in SHBG levels during pregnancy is associated with the increase in unconjugated oestrogens seen in pregnancy (Svendson and Sorensen, 1964) which stimulates the synthesis of this protein in the liver (Anderson, 1974). In mares there is a marked increase in conjugated and unconjugated oestrogens at day 120 (Nett et al, 1975). Assays performed on samples from throughout pregnancy in mares did not detect any appearance of SHBG at this stage. Therefore the results demonstrate that there is no sex hormone binding globulin in the serum of horses. A sex hormone binding globulin exists however in equine seminal plasma. It is possible that a sex steroid

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steroid binding protein exists in equine serum. However a more sophisticated assay such as Sephadex equilibration (Pearlman and Crepy, 1967) or competitive adsorption using a florisil adsorbent (Heyns et al, 1968) would be needed to distinguish it specifically from low affinity steroid binding with albumin.

The cross reactivity of four antisera to human pregnancy proteins and late pregnant mare serum was tested by Ouchterlony double diffusion. However no cross reactivities were found between the antisera to PAPP A, α_2 PAG (PZP) and α_2 and β_1 PAM. The failure to obtain cross reactivity between the anti β_1 PAM and the thirty-sixth week pregnancy plasma was probably due to the fact that this protein shows very variable individual levels during pregnancy (Stimson and Farquharson, 1982). Previous work with an antiserum to hPAPP A indicated that cross reactivity was confined to closely related species (Lin and Halbert, 1978). The antiserum gave reactions of identity with pregnancy sera from chimpanzee and orangutan, partial identity with sera from pregnant Old World monkeys and no reaction with pregnancy sera from New World monkeys. Some cross reactivity might have been expected between the antiserum to α_2 PAG (PZP) and equine serum since this protein has been identified in horses using a swine PZP antiserum (Martinsson and Carlstrom, 1977). However von Schoultz et al (1976) reported little or no cross reactivity between their anti-dog PZP and pregnant mare serum. In view of this the failure to detect cross

cross reactivity between antisera to human pregnancy proteins and pregnant mare serum was not unexpected.

CHAPTER 6

GENERAL DISCUSSION

The major conclusion to be drawn from the preceding chapters is that, within the limits of the systems used pregnancy proteins are undetectable in the circulation during late gestation in mares. However it would appear that the equine placenta does produce pregnancy specific protein and that during early pregnancy serum does contain a number of extra proteins.

In humans the major pregnancy specific proteins, SP1 hPL, PAPP A and B, reach a maximum concentration in the last month of pregnancy and are readily demonstrable by immunochemical methods (Lin et al, 1974a). Indeed since 1970 the identification of the numerous novel proteins associated with pregnancy in humans, subhuman primates and laboratory rodents has relied solely upon antisera to pregnancy serum or placental extracts. From the selection of successful methods, three were chosen to investigate whether similar proteins existed in pregnant mares. However, despite the fact that all three methods had produced highly poly-specific antisera to pregnant mare serum, all cross reactivity was removed by adsorbing with stallion serum. Two hypotheses were suggested to explain this. The first suggested that pregnancy proteins were present in late pregnant mare serum but were poor immunogens in the rabbit. Alternatively pregnancy

pregnancy proteins were indeed absent from the maternal circulation in late gestation. In order to differentiate between these two possibilities high resolution two dimensional polyacrylamide gel electrophoresis was performed on PMS, the gels being stained with a highly sensitive silver stain. Although pregnancy serum in humans, subhuman primates and laboratory rodents has not been investigated by this technique it was assumed that the pregnancy proteins would be at sufficiently high concentrations to be identified by this system. Indeed electrophoresis has been used to identify a pregnancy specific protein in mink (Larsen et al, 1970), the protein being readily visible on cellulose acetate gels. The dissociating conditions of the two dimensional system reduces proteins to single polypeptide chains. However only very faint spots were identified in the late pregnant mare gels and this in only one thoroughbred mare, and it was concluded that pregnancy proteins were virtually absent from the maternal circulation in late gestation.

A possible explanation for this lack of pregnancy specific proteins in the mare is based on the comparative placental morphology of mammals. Reasonable evidence exists that in humans both PAPP A and PP5 have local roles within the placental area (Bischof, 1981, Siiteri et al, 1982). The function of the other proteins is unknown but it has been suggested that the placental pregnancy proteins are merely by-products of a more fundamental process within the placenta, their presence in

in the mother being unimportant (Chard, 1982b).

In Chapter 1 the outline of a recent theory on the factors which control the levels of placental proteins in the mother was given. The essential feature of this hypothesis is that an equilibrium exists between the site of synthesis of these proteins, the syncytiotrophoblast, and the rate of blood flow across this site. Any change in uteroplacental blood flow would effectively change the concentration of the secreted proteins within this area and either increase or decrease the rate of synthesis accordingly. This could explain the changes in levels of pregnancy proteins associated with complicated pregnancies which affect the uteroplacental blood flow.

The levels of placental pregnancy proteins in the foetus are 10-1000 fold less than in the maternal circulation despite the fact that equal secretion rates into the maternal and fetal circulations would result in higher levels in the foetus (Chard, 1982b). This difference could be explained by assuming that secretion into the fetal circulation would be prevented by the basement membrane and fetal endothelium which would form a barrier between the circulation and the syncytiotrophoblast (Chard, 1982b). It therefore follows that disruption of the fetal barrier during pregnancy should result in an increase in levels of pregnancy proteins in the fetal circulation. This has been shown to be the case in pregnancies complicated by placental insufficiency where

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where levels of hPL were found to be raised in cord blood (Geiger et al, 1971).

The hypothesis may explain two other aspects of placental protein production. Firstly non-placental areas of the trophoblast, such as the chorion, produce only small amounts of placental proteins and this could be due to the fact that these areas are not in direct contact with the maternal blood. The other is the observation that some adult non-trophoblast tumours produce placental proteins such as hCG, hPL and SP1 (Vaitukaitis, 1977, Rosen et al, 1975, and Grudzinskas et al, 1980). It is possible that many normal adult cell types synthesize these proteins but because they are not in direct contact with the blood flow only small amounts are produced. However in the case of a tumour associated with the invasion of blood vessels contact with the cells and blood flow would be more intimate. This cell/blood contact may be important in stimulating the production of placental proteins in cells containing the appropriate synthetic machinery (Chard, 1982b).

Therefore, according to the hypothesis, the appearance of placental pregnancy proteins in the maternal circulation in humans is due to direct contact at the placental/maternal interface. Comparison of human and equine placental morphology may explain why no pregnancy specific proteins were detected in late PMS.

The classification of placental morphology has proven problematical since it does not coincide with any taxonomic pattern. A useful classification, based on the number of layers of tissue between the maternal and fetal circulation is that of Grosser (1909). Although this classification was based on the misconception that the efficiency of the placenta is inversely proportional to the number of layers between maternal and fetal circulation it still provides a useful means of discerning placental morphology. Thus the placentae were classified epitheliochorial (three maternal and three fetal layers), syndesmochorial (two maternal and three fetal) endotheliochorial (one maternal and three fetal), haemochorial (no maternal layers and three fetal) and haemendothelial (no maternal and one fetal).

In humans the placenta is haemochorial with the trophoblast actively phagocytosing adjacent maternal tissues, maternal blood corpuscles and the secretions of the uterine glands. The trophoblast differentiates into an irregular cytotrophoblast surrounded at the point of invasion by an extensive outer syncytium, the syncytiotrophoblast (Renfree, 1982). The maternal blood flows across the syncytiotrophoblast and, according to the hypothesis, stimulates the production of the placental proteins. It is interesting to note that in early pregnancy in the mare a similar invasion of the uterus by fetal cells in the formation of the endometrial cups leads to the appearance in the maternal circulation of a pregnancy specific

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specific protein, eCG.

In the mare the trophoblast tissue is separated by three layers of maternal tissue and there is no direct contact with the maternal circulation. Thus in the epitheliochorial placenta there is no intimate contact at the placental/maternal interface and hence no stimulation of synthesis and secretion of placental pregnancy proteins.

An example of this may be found in the comparison of levels of alkaline phosphatase in humans and mares. Although the evidence indicates that a placental alkaline phosphatase exists in both species the pattern is entirely different during gestation. In humans the levels rise in a similar pattern to placental growth. In the mare levels do not show any such similarity. This difference could be explained using the hypothesis since in humans the maternal blood would remove the alkaline phosphatase, performing a local role, and stimulate its synthesis. In the mare although the alkaline phosphatase may perform a similar local role the lack of direct contact between the placenta and maternal circulation would prevent its secretion into the maternal circulation.

If it is assumed that the number of layers between fetal and maternal circulation affects the secretion of proteins into the mother then the reciprocal case, the flow of material from mother to foetus, might also be affected. This can be seen when considering the

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the placental transfer of IgG (see Tizard, 1982). There is a direct relationship between the amount of IgG transferred from mother to foetus and the number of layers in the placenta. In humans the transfer of IgG is high. It is even higher in species possessing haemoendothelial placentae with only one intervening layer between maternal and fetal circulations. In dogs and cats which have endotheliochorial placentae only 5-10% of maternal IgG is transferred. In the ruminants (syndesmochorial) and the horse with 5 and 6 layers respectively the transfer of IgG from mother to foetus is non-existent, the offspring of these species relying on protection from colostral transfer of IgG.

Thus the hypothesis proposed states that pregnancy proteins in the maternal circulation are a result of intimacy of contact between the maternal circulation and trophoblast tissue. In humans the proteins are present in the maternal circulation because the maternal blood flows across their site of synthesis. In the mare the three layers of uterine tissue between the maternal circulation and the trophoblast exclude the secretion of placental proteins.

In other species in which pregnancy proteins similar to the human ones have been identified the placental morphology is either haemochorial or haemendothelial. Thus analogues to the human placental pregnancy specific proteins have been identified in subhuman primates (Lin

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(Lin and Halbert, 1978) and rodents (Lin et al, 1974c, Tatarinov, 1982) all of which possess trophoblast tissue in direct contact with maternal blood. There are no reports of similar pregnancy specific proteins in late pregnancy in species like dogs, cats, horses or pigs and only placental lactogens have been identified in ruminants. However, this could be due to the situation in other species not being investigated, or at least not reported if negative results have been found.

Therefore a bias may exist in that species investigated for pregnancy specific proteins in late pregnancy have been restricted to those species possessing similar placental morphology to humans. If other species with either epitheliochorial or syndesmochorial placentae were investigated in a similar manner for pregnancy specific proteins in late gestation, then this may provide evidence to prove or disprove the hypothesis. Examination of pregnancy proteins, if present, in the dog and cat may provide further evidence since their placental morphology is intermediate between horse and human.

This hypothesis relating direct blood flow across the placenta with the appearance of proteins peculiar to pregnancy in the maternal circulation does not exclude the existence of such proteins in the mare. However it would be necessary for these proteins to be localised within the placenta. Here they would perform roles necessary for the maintenance of fetal and placental

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placental development. Two equine placental antigens were identified using antisera to placental extracts. These two antigens were not detected in serum or extracts of liver and kidney and were designated equine placental antigens 1 and 2. On the basis of their molecular weights comparisons were made with the human placental proteins and several possible analogues were identified. Their detection means that pregnancy proteins exist in the mare but they are restricted to the placenta. Perhaps if a more extensive study was performed on the equine placental extract using longer injection protocols then more equine placental proteins could be identified. Studies in humans have revealed at least twenty soluble and eleven solubilized, apparently membrane bound proteins (Bohn, 1979 and Bohn et al, 1982).

Further investigations could be performed on the nature of EPA 1 and 2 using the antiserum obtained. Only liver and kidney extracts were used to adsorb the antisera and it would be of interest to investigate whether the antigens were present in other equine tissues such as uterus or ovary. This could be done using either homogenates of these tissues or by immunohistochemical techniques such as immunofluorescence. This technique could also be used to identify in which layer(s) of the equine placenta the antigens were localized. Their occurrence in the fetal circulation and amniotic fluid could also be tested for.

Using embryo homogenates gathered from various stages of gestation the behaviour of the proteins could be investigated giving information on their time of appearance and peak placental production. Another consideration would be whether the antigens were present in the endometrial cups which are of fetal origin.

The antiserum could be used to monitor purification of the antigens. Various physical separation techniques such as gel filtration, chromatofocusing and ion exchange chromatography could be used and the presence of the antigens in the various fractions detected using the antiserum. Once purified the physico-chemical properties of the two antigens could be determined more accurately. Once all this information had been assimilated then more viable comparisons could be made with human placental proteins.

Although the two dimensional gel electrophoresis failed to resolve any proteins unique to late pregnant mare serum, it identified several in early pregnancy serum. Of particular interest were three proteins present only in the first two months of pregnancy and one present for the first six months only. All four spots stained strongly within 20 days of fertilisation, the earliest recorded detection of pregnancy proteins in the mare. The proteins they represent may therefore be involved in the maternal recognition of pregnancy. They may represent a previously reported early pregnancy

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pregnancy specific protein , MPP1, or the early pregnancy factor which has so far not been reported in the mare.

The fact that these proteins stained strongly on the gels would indicate that they are present in the serum at reasonable concentrations. An immunological investigation similar to that used on late PMS applied to early pregnant mare serum would therefore reveal more information about them. An antiserum to early pregnancy serum adsorbed with stallion serum would perhaps be sufficient to provide a specific antiserum. This antiserum could then detect the proteins throughout a purification procedure and the number of proteins involved could be determined since the spots on the two dimensional gels represent polypeptide chains which may be individual proteins or subunit parts of a larger protein. The physico-chemical properties of the pure proteins could be more accurately determined in both dissociating and non-dissociating conditions. Using the antiserum the levels of the proteins throughout their appearance in the serum could be investigated as could their origin. From this information more valid comparisons could be made between these proteins and proteins known to be present at this stage of gestation.

The proposal that a lack of direct contact between maternal blood and placenta results in the absence of novel proteins in the serum adequately explains the failure to identify pregnancy specific protein in late pregnant mare serum. However it does not explain why

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why on adsorbance of the antisera to pregnant mare serum with stallion serum no pregnancy associated proteins, synthesized at for example the maternal liver, were identified. The major protein in this group is PZP which has been demonstrated in pregnant mare serum and is known to be a potent immunogen (Martinsson and Carlstrom, 1977) and may have a possible role as a "feeder protein" within the placenta, providing amino acids by its degradation (von Schoultz and Stigbrand, 1982). However it has been found that levels of PZP actually decrease during pregnancy in some monkey species (Lin et al, 1976a) and in the pig (Martinsson and Carlstrom, 1977). Since PZP was not demonstrated in late pregnant mare serum then the horse may be included within this group. In view of the feeder protein hypothesis and the fact that new born pigs and horses are highly mature when compared with humans then much higher levels might have been expected in these species.

Another pregnancy associated protein in humans, sex hormone binding globulin, was investigated in the mare throughout pregnancy. However no analogue of this protein was identified in equine serum. As the assay relied upon a relatively non-specific precipitation procedure it is possible that a sex steroid binding protein does exist in equine serum.

Therefore in the mare there appears to be very little change in the serum proteins in the later stages of

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of pregnancy. There is no detectable increase in levels of normal serum proteins and no appearance of proteins unique to pregnancy. A hypothesis has been proposed which may explain the lack of pregnancy specific proteins in the serum of mares in late gestation. However there may be other reasons which are worthwhile considering. In early pregnancy there occurs an event which is unique to horses, the appearance of the endometrial cups and their secretion of eCG which coincides with the appearance of a "crop" of secondary luteal structures (Allen, 1980). However, recent evidence indicates that these secondary luteal structures are a result of maternal pituitary hormones such as LH and FSH and a possible fetal factor and it may be that eCG represents "an irrelevant by-product of the endometrial cups which themselves have a more important and unrealised function" (Allen, 1984). It is interesting to note the similarity between this and the human placental pregnancy specific proteins. A further observation is the crop of secondary luteal structures also appear unnecessary for the maintenance of pregnancy since normal pregnancies have been observed in their absence (Allen, 1982, Allen, 1984). Therefore during the course of equine evolution this sytem has arisen which appears to have little to do with the maintenance of pregnancy and any advantage to the mare gained from it remains unclear.

It is interesting to speculate on the possibility that the production of eCG by the endometrial cups in the mare and production of pregnancy specific proteins in humans

humans represent parallels in that both are by-products of more fundamental reproductive processes. Perhaps if such a process occurred once in early pregnancy in the mare this would considerably reduce the possibility of it occurring again in late pregnancy. A reason for this may be in the immunological aspects of pregnancy maintenance. The endometrial cups are rejected by the maternal immune system and it may be that if in later pregnancy numerous novel antigens appeared in the maternal circulation this could lead to a similar rejection of the foetus by the mother. This would therefore suggest an evolutionary mechanism to explain the absence of pregnancy specific proteins in late pregnant mare serum.

Prior to this investigation it had been stated that pregnancy specific proteins similar to human ones probably occur widely in mammals indicating that they perform some essential role (Klopper, 1980). Now however this would appear not to be the case and the species difference in pregnancy associated proteins is another of the features of pregnancy which show the large variety of reproductive strategies adopted by mammals. Examples of this can be found when comparing the factors affecting luteal maintenance in early pregnancy in primates and ruminants. In primates this process is performed by a chorionic gonadotrophin of trophoblast origin transported in the maternal circulation. In ruminants the process is carried out within the uterus (Heap et al, 1979).

It has been assumed that in all species progesterone is essential for the maintenance of pregnancy. However an exception to this rule has been found in the African elephant which is capable of maintaining its pregnancy in the absence of progesterone (Smith et al, 1969). Therefore the absence of pregnancy specific proteins in late pregnancy in the mare may be another unique feature in the reproductive strategy of the horse. During the course of evolution in eutherian mammals although the essential features of pregnancy such as fertilisation, the maternal recognition, growth and development of the foetus and partuition remain constant various unique features may develop within a certain genus. The lack of pregnancy specific proteins in the mare may be just such a unique feature. It is only by examination on a similar basis of a wide diversity of mammalian species that the hypotheses proposed in this thesis can be proven or disproven.

APPENDIX

S.D.S. Polyacrylamide Gel Electrophoresis

Stock Solutions

1. Acrylamide 30.0 g

   NN[1] methylenebisacrylamide 0.8 g

   Dissolve and make up to 100 ml with dist. H_2O.

   Mix for 30 minutes with 2.5 g of Amberlite Monobed Resin MB1.

   Filter and store at $4^{o}C$ in a light proof bottle.

2. 1 M Tris/HCl buffer pH 8.8.

   2 M Tris 50 ml

   1 N HCL 16.2 ml (approx.)

   dist. H_2O to 100 ml

3. 0.5 M Tris/HCl buffer pH 6.8

   1 M Tris 50 ml

   1 N HCl 45 ml (approx.)

   dist. H_2O to 100 ml

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4. Tris/glycine electrophoresis buffer pH 8.3 (10 x conc.)

glycine	144 g
Tris	30 g
dist. H_2O to 1 litre	

Check pH after dilution

Adjust with tris or glycine only

5. Ammonium persulphate — 0.8% w/v

make up fresh

6. S.D.S. — 20% w/v

7. Bromophenol blue — 0.1% w/v

8. Solubilizing buffer

	Amount	Final concn. in sample
0.5 M Tris/HCl pH 6.8	25 ml	0.0625 M
SDS	20 ml	2%
β mercaptoethanol	10 ml	5%
glycerol	20 ml	10%
bromophenol blue	2 ml	0.001%
dist. H_2O	23 ml	

Gel /

Gel Preparation

1. Resolving gel

Acrylamide	36.7 ml
1 M Tris/HCl	37.6 ml
20% SDS	0.5 ml
TEMED	50 µl
Ammonium persulphate	10 ml
H_2O to 100 ml	

Mix thoroughly.

2. Stacking gel

Acrylamide	16.7 ml
0.5 M Tris/HCl	25 ml
20% SDS	0.5 ml
TEMED	50 µl
Ammonium persulphate	10 ml
H_2O	47.8 ml

Mix thoroughly.

Immunoblotting

Solutions

1. Transfer buffer

Tris	9.1 g
SDS	0.6 g
glycine	43.25 g

Made up to 3000 ml with distilled H_2O, no need to pH.

2. Blocking buffer

20 mM Tris. HCl

0.15 M NaCl

0.5% Tween 20

0.5 mg/ml Sodium Azide

The SDS gel is placed on top of a sheet of filter paper previously soaked in transfer buffer. Over this was placed a sheet of Nitrocellulose, large enough to cover the gel and presoaked in transfer buffer. A further piece of filter paper was placed on the Nitrocellulose and this assembly was sandwiched between two presoaked "Scotchbrite" pads. This was then placed in a plastic holder and placed in a Bio-rad Transblot cell with the gel at the negative and the nitrocellulose at the positive. A current of 400 mA was then applied for 3 hours. After this

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this the nitrocellulose was removed, placed in blocking buffer overnight and then incubated for 1½ hours in blocking buffer with 5% horse serum and 1:100 dilution of the antiserum. It was then washed five times over a thirty minute period with blocking buffer and incubated for a further 1½ hours with approximately three million counts of ^{125}I-protein A. The nitrocellulose was then washed with blocking buffer until the background was low and exposed to X-ray film.

Two Dimensional Electrophoresis

Solutions for isoelectric focusing

1. Acrylamide stock

Acrylamide	33.3 g
Bis-acrylamide	1.9 g

Dissolve and make up to 100 ml with dist. H_2O.
Mix with 2.5 g of Amberlite Monobed Resin MB1 for 30 minutes then filter.

2. 10% w/v Nonidet P40 in H_2O

3. Overlay Solution A

8 M dionised urea
1 mM Lysine

Store frozen.

4. Overlay Solution B

Urea	4.8 g
Ampholine pH 3.5-10	0.25 ml
0.1 M Lysine	0.1 ml
H_2O	6.1 ml

Store frozen in 200 µl aliquots.

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5. Sample Loading Buffer

9 M deionised urea

0.01 M Tris /HCl pH 8.0

0.001 M PMSF

0.7 µg/ml pepstatin

0.001 M $Na_2 MO_4$

0.001 M Lysine

Store frozen.

Solutions for second dimension SDS PAGE

A. 36.6 g Tris

48 ml of 1N HCl

0.23 ml OF TEMED

H_2O to 100 ml

Adjust to pH 8.8

B. 33.3 g of Acrylamide

0.9 g of Bis-acrylamide

H_2O to 100 ml

Mix with 2.5 g of Amberlite Monobed Resin MB1 for 30 minutes then filter.

C. 5.98 g of Tris

0.46 ml of TEMED

+ 1N HCl to pH 6.8

H_2O to 100 ml

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D. 10 mg Riboflavin

H_2O to 250 ml

E. 90 ml of 9 M deionised urea

2 ml of 10% SDS

H_2O to 100 ml

Prepare fresh.

Equilibration Buffer

54 g urea

10 g glycerol

1.0 ml 0.1 M Lysine

20 ml 5 x Laemmli Sample Buffer

31 ml H_2O

5 x Laemmli Sample Buffer

7.56 g Tris

20 g SDS

0.01 g Bromophenol blue

Adjust to pH 6.8

H_2O to 200 ml

10 x Reservoir Buffer

150 g Tris

720 g Glycine

50 g SDS

H_2O to 5 litres

Adjust to pH 8.3-8.5 on dilution.

N.B. Use tris or glycine to adjust pH

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