STUDIES ON HUMAN PLACENTAL PROTEINS

by

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SUMMARY

Maternal antibodies to placental-specific antigens were not detected in third trimester maternal sera or in concentrated material adsorbed from maternal sera on a placental affinity column using immunofluorescence and gel precipitation techniques. A method was described for the dissociation of antigens of less than 100,000 daltons from soluble antibody-antigen complexes. This method was applied successfully to the dissociation of albumin-anti-albumin complexes formed in antigen excess. Application of this method to pregnancy serum followed by immunisation of rabbits with the dissociated material resulted in anti-SP1 response detectable on AACE. The molecular weight of SP1 is approx. 100,000 daltons and the bulk of SP1 was detectable in the retentate at the end of the dissociation. This argued against a conclusion that SP1 had been dissociated from immune complexes.

Immunisation of rabbits with soluble extract of a placenta which had been adsorbed over anti-human serum column induced AACE-detectable responses to very few serum proteins, PAPP-A, HPL, SP1 and several unidentified antigens found in placental tissue. Placental soluble extract which had in addition been adsorbed over anti-human lung column and anti-human amniotic fluid column induced responses to very few serum proteins, PAPP-A and HPL. Using these latter antisera two other antigens were identified in placental soluble extract (antigens *a* and *b*) which were not detectable in lung soluble extract or in non-pregnant serum. Antigen *b* was also detected in pregnancy serum. By contrast, immunisation with unfractionated placental soluble extract induced extensive anti-serum response, anti-HPL and anti-SP1 but no detectable anti-PAPP-A response.

The extent to which several serum proteins were denatured by a variety of commonly used dissociants of antibody-antigen complexes was assessed by changes in the protein's precipitating behaviour on one-dimensional AACE. AACE was found to be superior for this to double immunodiffusion. The smaller proteins studied were more resistant to irreversible denaturation, but the sample size was too small to seek a general relationship between molecular weight and denaturation. All proteins examined proved resistant to one hour exposure to 1·5 M KI-PBS and to pH 11 buffer.

1·5 M KI-PBS was used in the purification of PAPP-A by antibody affinity chromatography followed by DEAE-cellulose ion exchange and Sepharose 6B gel filtration. Double AACE arcs formed by PAPP-A in term maternal serum were also formed by partially-purified PAPP-A indicating that the antigenic variants had not been separated. The purification product contained PAPP-A which was 427-fold purified in terms of immunological reactivity on AACE. The high degree of purity was also suggested by studies with the radiolabelled purification product. ^{125}I-PAPP-A was shown to have similar molecular weight and electrophoretic mobility to maternal serum PAPP-A. Immune-precipitated ^{125}I-PAPP-A was analysed by 5% and 3% SDS-PAGE and found to contain a major radioactive component of approx. 180,000 daltons and a minor component of between 74,-93,000 daltons. The nature of the minor component has not been determined. It may be tentatively concluded that PAPP-A (mol. wt. 750,000) contains polypeptide subunits of approx. 180,000 daltons and may therefore be composed of up to four such subunits.

A collaborative clinical study in which PAPP-A was assayed in the blood of women during the third trimester of pregnancy by AACE suggested that fetal sex may affect PAPP-A levels with males giving rise

to higher levels than females. No significant difference in the levels of PAPP-A was detected when patients with babies affected with intra-uterine growth retardation were compared with controls. No significant change in the mean concentration of PAPP-A was detected in the group of patients with gestational diabetes, but some very high values were found in this group. In the group of patients with insulin-controlled diabetes the mean concentration of PAPP-A was significantly reduced.

ABBREVIATIONS

AACE	Antibody-antigen crossed electrophoresis
HPL	Human placental lactogen
PAPP-A	Pregnancy-associated plasma protein
PBS	Phosphate-buffered saline solution: 0•16 M NaCl + 5 mM potassium phoshate, pH 7•5
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SP1	Pregnancy specific beta-1-glycoprotein

CHAPTER 1 INTRODUCTION

1. GENERAL INTRODUCTION

This chapter examines the current state of knowledge of the pregnancy-associated proteins, including placental-specific proteins found in sera of pregnant women. As background it is first necessary to describe briefly human placental structure and function. Individual pregnancy-associated proteins are then discussed in turn, together with a review of the areas of current and potential application of pregnancy-associated proteins: obstetric diagnosis, cancer diagnosis and the diagnosis and treatment of conditions of pregnancy with immunological aetiologies. The final discussive part of this chapter considers the use of affinity chromatography in the search for and in the purification of new proteins. The information presented in this chapter is then used to formulate lines of further investigation.

2. HUMAN PLACENTAL DEVELOPMENT, STRUCTURE AND FUNCTION

2.1 Human Placental Development and Structure

The morphological features typical of the human placenta have been discussed in detail in reviews by Boyd and Hamilton (1975), Llewellyn-Jones (1975), Wynn (1975) and Walker and James (1976) which have acted as the sources of information used to construct the following brief account.

Human Placental Development

The human blastocyst implants on the 6th day of gestation. Prior to implantation progesterone and estrogen secreted by the corpus luteum (a body formed in the ovary after ovulation) modify the uterine endometrium to form the decidua which is more suitable for implantation

of the blastocyst and the nourishment of the embryo. Around the time of implantation the trophectoderm of the blastocyst gives rise to two basic forms of trophoblast, an inner cellular cytotrophoblast and an outer syncytiotrophoblast. The syncytial form is derived by differentiation and the breakdown of the cell membranes of the cellular layer. The inner cell mass in the blastocyst which will ultimately form the fetus has sparated from the cells of the trophoblast and has differentiated by the time of implantation.

At the time of implantation the syncytial cells erode the superficial cells of the decidua. During the subsequent few days proliferation of the cytotrophoblast pushes the knobs of the trophoblast deeply into the decidua and by the 10th day of gestation there is much intermingling of trophoblast and maternal tissues. Within the projecting knobs of trophoblast, a third layer of connective tissue, or stroma, the mesoblast is differentiated from the inner surface of the cytotrophoblast. The mesoblast proliferates rapidly and not only forms the core of the projecting knobs of trophoblast but also lines the cavity of the blastocyst which is now termed a chorionic sac. With further proliferation the trophoblastic knobs assume a finger-like shape and are termed chorionic villi. Spaces appear between the closely packed trophoblastic projections so that they are split into separate villi and by coalescence the spaces (lacunae) increase in size. By this time the tips of the villi have reached the decidual capillaries and the spiral arterioles and have surrounded and eroded their walls so that maternal blood sweeps into the lacunae. Maternal blood then fills and expands the lacunae which coalesce further creating large blood-filled spaces segregated by chorionic villi. Once the spiral arterioles have been surrounded fibrin deposits begin to appear between the decidua and

trophoblast. Trophoblast invasion is not uniform - ridges of maternal decidua persist amongst the trophoblast which gives rise to the placental septa seen on the mature placenta. Recent work by Robertson and his colleagues (Robertson et al., 1976) has shown that waves of non-villous trophoblast also penetrate the basal decidua and progress up the spiral arteries in a retrograde direction. Unlike the syncytiotrophoblast of the chorionic villi these interstitial trophoblast cells in the basal decidua are not in direct contact with the intervillous blood.

In the richly nutritive conditions created by maternal blood in the intervillous space syncytial sprouts appear on the primary villi. These develop, subdivide and branch so forming villous trees. Some of the primary villi retain their attachment to the decidua (anchoring villi). By the 18th day of gestation primitive blood vessels have formed in the mesoblastic core of the primary and secondary villous trunks. Around the21st day the intravillous vascular network meets the umbilical vessels to establish the fetal circulation. Towards the end of the 8th week the villi begin to group together at the implantation site. During the third month of pregnancy the conceptus begins to grow to occupy the entire uterine cavity. At this stage the trophoblastic villi which are adjacent to the implantation site develop into placental tissue, whilst the others atrophy. Thus at twelve weeks the placenta is clearly defined. After twelve weeks the number of primary villi does not increase but growth occurs by continuing proliferation of peripheral villi right up to term. Once the establishment of the main placental region is complete, the chorionic membrane, composed of a layer of cytotrophoblast, surrounds the remainder of the fetus.

At all periods of pregnancy a syncytiotrophoblastic

barrier separates the maternal and fetal circulations. However, with increasing placental age the structure of the villi changes. Early (10-18 weeks) villi are large (up to 0•3 mm diameter) and have an outer layer of syncytiotrophoblast and an inner layer of cellular cytotrophoblast, which is initially two cells thick, and a relatively acellular mesoblastic core containing small fetal vessels. By the 24th week cytotrophoblastic cells are few, the stroma is more cellular and compact containing phagocytic cells and the fetal vessels larger and closer to the trophoblastic covering. By the 36th week, the villi are much smaller (0•05-0•1 mm in diameter), the cytoblastic cells have largely disappeared, the syncytium has become attenuated and aggregations of nuclei within it. Fibrin deposits on the surface of the mature villi are commonly seen. Throughout pregnancy the maternal side of the syncytium is in direct contact with the maternal blood in the intervillous space. The fetal side of the syncytium is covered by a basement membrane which abuts onto the cells of the cytotrophoblast, and where these have disappeared, onto the fetal mesoblast and the fetal capillary endothelium (Figure 1.1).

Human Placental Structure at Term

The mature placenta is basically a chorioallantoic structure (also found in all true mammals and a few marsupials) in which the fetal blood supply is received through allantoic, or umbilical, vessels. Mainly on the basis of the degree of invasiveness of its trophoblast cells into the maternal tissues it is further classified as a haemochorial placenta. The degree of trophoblast invasiveness is most extreme in this type of placenta - the uterine capillaries are breached and the trophoblast bathed directly in maternal blood. The placenta at term is disc-shaped,about 15-20 cm in diameter, 2•5-3•0 cm

thick in the middle and tapering towards the edges. It weighs around 650 g (mean figure with considerable variation). It is bounded on its upper (fetal) surface by the amnion beneath which lies the chorion (the chorionic plate). Within the chorionic plate run the fetal vessels. On the lower (maternal) surface the placenta is bound by the decidua (the decidual or basal plate). Between the two surfaces is adense mass of fetal villi bathed by maternal blood in the intervillous space (Figure 1.2). Fetal blood reaches the placenta via the umbilical arteries: from the placenta it reaches the fetus by the umbilical vein. Two umbilical arteries and one umbilical vein are normally contained within the umbilical cord. The fetal placenta is divided into 50-60 lobes or cotyledons. Each fetal cotyledon is a separate vascular system consisting of a single villous tree supplied by one artery and drained by one or more veins. The maternal surface is incompletely divided into about 15-20 cotyledons by septa of various heights and shapes which project from the decidual plate into the intervillous space. These maternal cotyledons correspond to one or more usually several fetal cotyledons. The maternal blood enters and leaves the intervillous space through vessels opening in the decidual plate. During systole the arterial blood spurts into the intervillous space at a pressure of about 80 mm Hg and pushing the villi aside to reach the chorionic plate flows laterally and cascades down bathing the branched villi. It then escapes slowly through the veins which tend to form venous lakes in the decidua (Figure 1.3).

2.2 Human Placental Function

The placenta's functional characteristics include full growth and development within a limited lifespan, anatomic adaptations

to diverse tasks such as transport of gases and metabolites to and from the fetus and elimination of waste products and, of main interest in the context of this investigation, the synthesis of a wide range of hormones, enzymes and other proteins. The placenta can secrete proteins in the absence of a fetus in an anembryonic pregnancy (Bennett et al., 1978). The synthesis of all types of proteins is generally attributed to syncytiotrophoblasts, which have been shown to contain the cell organelles (endoplasmic reticulum) necessary for protein synthesis: cytotrophoblasts, on the other hand, have been shown to contain few such organelles (Pierce and Midgly, 1963; Lister, 1963; Yoshida, 1964). Electron microscope studies (Burgos and Rodrigues, 1966) have pointed to the existence of "thick" and "thin" areas in the syncytiotrophoblast: the former rich in endoplasmic reticulum and microvilli, thought to be specialized in protein synthesis and the latter (known as vasculo-syncytial membranes - Getzowa and Sadowsky, 1950) without microvilli and thought to be specialized in the transport of metabolites and waste products to and from the fetal capillaries which they overlie (Figure 1.1).

Evidence supporting the assumption that specific placental proteins are produced by syncytiotrophoblasts is indirect and comes mainly from immunohistochemical studies (Currie et al., 1966; Horne et al., 1976; Lin and Halbert, 1976). The validity of these studies has however been questioned because of the lack of appropriate controls and because a theoretical analysis suggests that the actual concentrations in the trophoblast cytoplasm could not be detected by the techniques used (Gau and Chard, 1976). The placental proteins appear to be secreted into the intervillous space from which they are carried into the maternal ciculation, rather than through the basement membrane and the

villous stroma and thence into the umbilical cord and the fetal circulation (Diczfalusy, 1974). This has been suggested to be due to the architecture of the chorionic villus described in Section 2.1 of this chapter: the syncytiotrophoblasts are in direct contact with the maternal blood in the intervillous space but are separated from the fetal blood by the basement membrane and the capillary endothelium (Gordon and Chard, 1979).

3. PREGNANCY-ASSOCIATED PROTEINS IN THE MATERNAL CIRCULATION

3.1 Placental-specific Proteins

Currently known placental-specific proteins can be divided into three groups:

(1) The hormones - human chorionic gonadotrophin (HCG), human placental lactogen (HPL) and human chorionic thyrotrophin (HCT).

(2) The enzymes - histaminase, placental alkaline phosphatase and oxytocinase.

(3) Proteins which have been detected immunologically by gel diffusion methods and whose biological functions remain to be elucidated - pregnancy specific $\beta_1$glycoprotein (SP1), pregnancy protein 5 (PP5) and pregnancy-associated plasma proteins A and B (PAPP-A and PAPP-B).

Although development of more sensitive techniques may yet reveal the presence of very low levels of some of the above placental-specific proteins in non-pregnant serum or extracts of adult tissues, for the purpose of this discussion they will be considered specific for pregnancy.

Unlike the steroid proteins produced by the placenta

whose role in the maintenance of pregnancy is well-documented the precise roles of specific protein hormones produced by the placenta are incompletely understood. The two major hormones produced by the placenta, HCG and HPL, have both been purified to high degree and have been demonstrated to have analogues in the pituitary with which they show chemical, biological and immunological similarities.

HCG was the first placental protein hormone to be described. It was discovered as a substance which induced ovarian hyperaemia, oestrus and the formation of corpora lutea in guinea pigs (Aschner, 1912) and mice (Aschheim and Zondek, 1927). Its production by placental tissue grown in culture was first demonstrated by Gey et al. (1938). Immunocytochemical studies point to its synthesis by syncytiotrophoblast cells of placental villi (Midgley and Pierce, 1962; Leznoff and Davis, 1963; Thiede and Choate, 1963). HCG is similar to pituitary luteinizing hormone (LH) in physicochemical and immunological properties but has a higher carbohydrate content and a high content of sialic acid. Its molecular weight is 45,000-50,000 (Bahl et al., 1973; Vaitukaitis, 1977). Like all glycoprotein hormones (luteinizing hormone, follicle-stimulating hormone and thyroid hormone) it is composed of two subunits, an alpha subunit and a beta subunit. With very minor modifications the alpha subunit is common to all the glycoprotein hormones (Pierce et al., 1971) and the beta subunit confers unique specificity to the HCG molecule. Beta-HCG has 147 amino acids: 30 of these residues at its -COOH terminus are not present in the subunits of the other glycoprotein hormones (Carlsen et al., 1973). Information is still accumulating concerning the biological function of HCG and it is not known whether it is essential to normal pregnancy. It is believed to maintain the corpus luteum of the menstrual cycle, permitting its

conversion to the corpus luteum of pregnancy (Johansson and Bosu, 1974) thus allowing the continued production of progesterone necessary for decidual development. The corpus luteum functions maximally during the first four weeks of pregnancy and its functional lifespan may be ten weeks: the placenta gradually takes over progesterone production between the 6th and 10th week of pregnancy. However, a study by Yoshimi and his colleagues has revealed that plasma HCG levels increase throughout the period that plasma levels of progesterone are falling, that is between the 4th and 6th week of pregnancy (Yoshimi et al., 1969). This finding has raised questions about the control of corpus luteum function by HCG. Nevertheless, HCG has been demonstrated to cause an increase in plasma progesterone in non-pregnant women when administered during the luteal phase of the menstrual cycle (Strott et al., 1969). HCG may regulate steroid production in the fetus - dehydroepiandrosterone sulphate (DHAS) by the fetal zone of the adrenal gland and testosterone by the testis (Jaffe et al., 1977; Seron-Ferre et al., 1978). A role for HCG as a thyroid stimulator in pregnancy has also been suggested (Nisula and Ketelslegers, 1974). Pregnancy is known to have a variety of effects on maternal thyroid function some of which may be responsible for the enlargement of the thyroid which occurs frequently (see the review by Turnbridge and Hall, 1975). Although the postulated thyroid-stimulatory role of HCG in pregnancy has yet to be proven, it has been demonstrated that the thyrotrophic activity in pregnancy urine and in the serum of patients with hydatidiform moles is due to HCG (Nisula and Ketelslegers, 1974; Kenimer et al., 1975). HCG has so far been detected in maternal blood and urine: trace amounts have also been detected in amniotic fluid, fetal blood and fetal tissues - ovary, testis, kidney and thymus (Huhtaniemi et al., 1977). In maternal blood and urine it may be detected/

may be detected as early as the tenth day after ovulation, very soon after the implantation of the early developing blastocyst (Mishell et al., 1963). The mean concentration rises rapidly to reach a peak of 60-130 i.u. per ml. (5,000 i.u. = 1 mg. pure HCG: see Vaitukaitisetal, 1972) in the serum between the 60th and 80th day of gestation then declines rapidly before reaching a plateau around the 120th day of pregnancy (Borth, 1971; Varma et al., 1971) and afterwards remains fairly constant until term with a mean concentration in the range 10-25 i.u. per ml. serum. It disappears rapidly from the maternal serum after delivery (Hertz et al., 1959). In placental tissue a mean maximum concentration of 100-500 i.u. per gram placental tissue is reached around the 60th day of gestation. Therafter the mean concentration declines to 10-20 i.u. per gram tissue at term (Diczfalusy, 1953; Hobson, 1971).

HPL, discovered independently by Ito and Higashi (1961) and Josimovich and Maclaren (1962), is also believed to be synthesised by the syncytiotrophoblast cells of the placental villi (Beck and Currie, 1967; Ikonicoff and Cedard, 1973; Lin and Halbert, 1976). HPL is similar to human growth hormone (HGH) in physicochemical and immunological properties. The molecular weight of HPL is 21,000-23,000 daltons and it is a single chain polypeptide with two intra-chain disulphide bonds, composed of 191 amino acids and showing a 96% homology with HGH (Ito and Higashi, 1961; Friesen, 1965; Li et al.,1971; Niall et al., 1971; Josimovich, 1977). Although there is no solid evidence that HPL is essential for pregnancy it is believed to exert important metabolic effects on the mother. The major of these is HGH-like diabetogenic influence on carbohydrate and lipid metabolism which ensures an adequate supply of glucose for the fetus (Grumbach et al., 1973).

As pregnancy progresses the fetus increases its substrate requirement which is thought to lead to an increased functional role for HPL in the second and third trimesters (Kaplan, 1974). HPL has also been demonstrated to have lactogenic properties in the pseudopregnant rabbit and luteotrophic properties in the pseudopregnant rat (Josimovich and MacLaren, 1962): whether or not it has lactogenic and luteotrophic properties in women remains to be established. HPL has so far been detected in maternal blood and urine: trace amounts have also been detected in fetal serum and in amniotic fluid (Kaplan, 1965). In maternal serum it first becomes detectable by radioimmunoassay around the eighth week of gestation. Levels rise steadily to a peak of 6-20 ug. per ml. maternal serum at 32-36 weeks: thereafter concentrations remain steady, or fall slightly, to term (Josimovich and Atwood, 1964; Kaplan and Grumbach, 1965). Postpartum it disappears from maternal serum within 1-2 days (Lin and Halbert, 1976b). The concentration of HPL in placental tissue remains approximately the same throughout gestation (Jasimovich and Atwood, 1964) and rising serum levels reflect the increasing mass of the placenta (Jasimovich et al., 1969). The placenta produces and secretes into the maternal circulation in excess of 1 gram HPL per day during the third trimester of pregnancy.

HCG and HPL have both been attributed with immunosuppressive properties. It has been claimed that they reduce the transformation (proliferation) of lymphocytes that occurs when the lymphocytes are confronted with the T cell mitogen, phytohaemagglutinin (PHA) (Contractor and Davies, 1973). In the case of HCG the effect was thought to be reversible and without cytotoxicity (Adcock et al., 1973; Marz et al., 1973). HCG has also been claimed to inhibit mixed lymphocyte culture (MLC) which involves the stimulation of lymphocytes by foreign

lymphocytes rather than by PHA (Kaye and Jones, 1971; Jenkins and Acres, 1972). HCG and HPL were postulated to exert their effects by blocking the antigen receptor sites of the lymphocytes (Contractor and Davies, 1973). However, the evidence for the immunosupressive role of HCG has proved controversial (Caldwell et al., 1975). The specificity of the inhibition of the lymphocytes (both PHA-induced and in MLC) has been questioned because highly purified preparations of HLC have been demonstrated to be without effect (Carr et al., 1973; Caldwell et al., 1975; Muchmore and Blaese, 1977).

Several investigators have suggested that a third protein hormone, human chorionic thyrotrophin (HCT) is also synthesised and secreted by the placenta (Hennen et al., 1969). HCT shows physico-chemical and immunological similarities to pituitary thyroid stimulating hormone (TSH). The molecular weight of HCT has been determined as 45,000 daltons and it has been shown to be composed of two subunits (Hershman and Starnes, 1971). The physiological role of HCT has not been elucidated clearly. Hennen and his colleagues (Hennen et al., 1969) suggested a role for it in the regulation of thyroid function during pregnancy. However, although has been demonstrated to have thyrotrophic properties (Hershman et al., 1973) its action on the human thyroid is unknown. Thyrotrophic activity in pregnancy urine and in the serum of patients bearing trophoblastic tissue has been demonstrated to be due to HCG, and the thyrotrophic activity of placental tissue in pregnancy postulated to be a function predominately of HCG (Nisula and Ketislegers, 1974; Kenimer et al., 1975). In addition the placental content of HCT is known to be very low and variable (Hershman and Starnes, 1971). Similarly, in normal pregnancy serum it is difficult to detect and several investigators have been unable to do so.

The enzymes presently known to be produced specifically by the fetal placenta are heat-stable alkaline phosphatase and oxytocinase. In addition, a third enzyme, histaminase is thought to be produced specifically by the maternal portion of the placenta.

The first of these enzymes, heat-stable alkaline phosphatase, has been localised on the microvilli of the syncytiotrophoblasts by immunohistochemical studies (Kameya et al., 1973). It has been demonstrated to be an isoenzyme of the heat-labile alkaline phosphatase found in the non-pregnant adult (Fishman et al., 1968a and b; Sussman et al., 1968; Neale et al., 1965). The molecular weight of placental heat-stable alkaline phosphatase has been determined as 128,000 daltons and it was found to be composed of two identical subunits of molecular weight 64,000 daltons each (Luduena and Sussman, 1976). The serum levels of this enzyme have been determined in maternal serum by radioimmunoassay. A 25-fold increase from low levels during the first trimester to 250 ng. per ml. in the 40th week of gestation has been reported (Holmgren et al., 1978).

The second placental enzyme, oxytocinase, has also been localised in the syncytiotrophoblasts by immunohistochemical studies (Small and Watkins, 1971). This enzyme has been demonstrated to be an isoenzyme of the oxytocinase found in the non-pregnant adult (Watkins and Small, 1972; Buul and Van Oudheusden, 1978). The molecular weight of placental oxytocinase has been determined as 300,000 daltons (Yman, 1970). The presence of both the placental and the non-pregnant adult forms of oxytocinase has been demonstrated in maternal serum (Buul and Van Oudheusden, 1978).

Relatively less is at present known about the third placental enzyme, histaminase. Recent immunohistochemical studies have

localised it in the cytoplasm of the decidual cells of the placenta (Weisburger et al., 1978). The molecular weight of histaminase purified from the placenta has been calculated to be 180,000 daltons and it was found to be a dimer composed of identical subunits of molecular weight 90,000 daltons each (Bardsley et al., 1974): similar results were obtained for histaminase purified from pregnancy plasma (Baylin and Margolis, 1975). However, little has been done to compare histaminase purified from the placenta with histaminases found in the non-pregnant adult.

Of the placental proteins which have been detected immunologically by gel diffusion methods, SP1 has been the subject of most studies. It was discovered independently by Tatarinov et al., (1970) and Bohn (1971). Immunohistochemical evidence indicates that its synthesis is initiated by the cytotrophoblast cells of the immature placenta (Tatarinov et al., 1976) and continues in the syncytiotrophoblast of the chorion (Bohn and Ronneberger, 1973; Horne et al., 1976; Lin and Halbert, 1976; Tatarinov et al., 1976). However, a preliminary investigation by Klopper and his colleagues (Klopper et al., 1979) who claimed to have studied representative samples of retroplacental blood has supported the hypothesis that, unlike HPL, the bulk of SP1 does not enter the maternal circulation through the intervillous space. This observation has led them to postulate that a substantial proportion of SP1 may be synthesised by the non-villous interstitial trophoblast in the basal decidua and then makes it way into the maternal ciculation via the channels of uterine drainage not involved in the intervillous space. SP1 has been highly purified by physicochemical methods (Bohn, 1976; Bohn, 1979) and its molecular weight has been estimated at 90,000 daltons in one study (Bohn, 1974) and at 110,000 daltons in another

(Lin et al., 1974a). It is composed of a single peptide chain and contains 28% carbohydrate in addition to iron and sialic acid residues (Bohn, 1972; Lin et al., 1974b). Studies by Bohn (1973) have revealed a degree of chemical and antigenic heterogeneity in the protein. The biological function of SP1 remains unknown but it is known to have an affinity for and may weakly bind steroid hormones especially estriol, 17- estradiol and cortisol (Bohn, 1974). It is also known to exert an inhibitory effect on the PHA stimulation of human lymphocytes and may therefore have immunosuppressive properties (Horne et al., 1976). There appears to be no correlation between maternal levels of SP1 and placental weight (Lin et al., 1976c): this finding supports the hypothesis that SP1 is synthesised by the interstitial trophoblast cells in the basal decidua (Klopper et al., 1979) which, if correct, would suggest a local function for SP1 within the uterine tissue. SP1 has so far been detected in maternal blood and in trace amounts in cord (fetal) blood, amniotic fluid and colostrum (Bohn, 1974; Tatra et al., 1976). In maternal serum levels around 2 mg. per 100 ml. are typical during the 20th week of gestation. Maternal serum levels continue to rise slowly until around the 36th week when a plateau ranging from 5-25 mg. per 100 ml. is retained until term. Postpartum it ceases to be detectable in the maternal circulation within 3-4 weeks and has a half-life of 30-40 hours (Bohn, 1974). Levels in the term placenta average 30 mg. per placenta (Bohn, 1976).

Another placental protein purified to a high degree and characterised by Bohn (1976) and Bohn and Winckler (1977a), PP5, has been demonstrated to be localised in the syncytiotrophoblast and the villous stroma of the term placenta by immunohistochemical methods (Sedlacek et al., 1975). In the early placenta (8 weeks) it is

localised almost exclusively in the syncytiotrophoblast (Jones, 1976). Its molecular weight is 36,600 daltons and it is a glycoprotein containing sialic acid residues. A possible biological function of PP5 may be protease inhibition since studies by Bohn and Winckler (1977a) have revealed that it inhibits proteolytic activity of trypsin and plasmin *in vitro*. In maternal serum PP5 is found in trace amounts: its concentration there has been found to average 0·1 mg. per 100 ml. In term placenta the average PP5 content has been estimated at 1·2 mg. per placenta (Bohn 1976).

The remaining two placental proteins, PAPP-A and PAPP-B, were first identified by Lin and his associates and have been partially purified by physicochemical means by them (Lin *et al.*, 1974a; Lin *et al.*, 1978). However, no physicochemical evidence of purity was provided and the degree of purification (115-fold in the case of PAPP-A and 800-fold in the case of PAPP-B) was indirectly assessed in terms of immunological reactivity. The 115-fold purified PAPP-A preparation contained easily detectable serum proteins. Immunohistochemical studies have localised PAPP-A in the syncytiotrophoblast of the term placenta (Lin and Halbert, 1976). The molecular weight of PAPP-A has been estimated at 750,000 daltons (Lin *et al.*, 1974a; Lin *et al.*, 1974b) but the subunit composition has not been determined. It was found to be a glycoprotein containing sialic acid (Lin and Halbert, 1975). The biological function of PAPP-A has not been determined. A correlation has been found between high concentrations of PAPP-A in maternal serum and large placental or newborn weights and the levels of PAPP-A in maternal serum were found to be higher in primigravid women and in women with high diastolic pressure (Lin *et al.*, 1976a) but the significance of these findings is at present unknown. So far, PAPP-A has not been detected anywhere other than in

maternal serum (Lin et al., 1974d). A steady rate of increase is typical during the second trimester of pregnancy followed by a steep rate of increase during the third trimester. Postpartum the concentration of PAPP-A drops rapidly within 2-3 days and becomes undetectable within 3-4 weeks with a half-life of 3-4 days (Lin et al., 1974c; Lin et al., 1976b).

PAPP-B has so far only been reported and studied by Lin and his colleagues who have shown it to have a molecular weight of 1,000,000 daltons (Lin et al., 1978). The subunit structure of PAPP-B has not been determined. This protein appears to be present in very low concentrations in maternal serum. It was frequently not detected before the 28th week of pregnancy and in over 50% of the samples from the last two months of pregnancy. The mean concentrations show a gradual increase during the second trimester and a more steep increase during the third trimester, reaching a plateau in late gestation. Postpartum PAPP-B disappears from the maternal circulation within one day.

3.2 Other Pregnancy-Associated Proteins

Two proteins found in pregnancy serum have not so far been detected in non-pregnant adult serum: namely pregnancy protein 6 (PP6) and alpha-uterine protein (AVP).

PP6 was first isolated from the placenta and studied by Bohn (1975), who showed it to be localised in the nuclei of the syncytiotrophoblasts. Its molecular weight has been estimated at between 800,000-1,000,000 daltons. It contains 7% carbohydrate and appears to be composed of at least four different subunits of molecular weights ranging from 20,000 to 35,000 daltons which subunits are non-covalently bound. Although its biological function is unknown it is

known to have steroid-binding properties. A protein with identical properties to PP6 has been detected in extracts of other human adult tissues as well as in the erythrocytes but not in non-pregnant serum (Haupt and Bohn, 1975). In pregnant serum PP6 occurs in concentrations of around 1-3 mg. per 100 ml.

AUP was discovered, purified and studied by Sutcliffe and his associates (Sutcliffe et al., 1978; Sutcliffe et al., 1980). It appears to be synthesised by the pregnant uterus but has also been detected in some samples of non-pregnant endometrium. Its molecular weight is 50,000 daltons and it appears to be a dimer composed of two identical subunits. Its biological function is unknown. The demonstration of the presence of AUP in maternal serum requires the use of radioimmunoassay: it is however readily detectable in amniotic fluid.

A fetal-specific protein, alpha-fetoprotein (AFP), found in pregnancy serum is only present in trace amounts in non-pregnant adult serum. AFP is synthesised by the human yolk sac, the fetal liver and gastro-intestinal tract (Gitlin, 1971). The molecular weight of AFP has been calculated as 70,000 daltons; it consists of a single subunit and contains 4% carbohydrate (Nishi, 1970; Ruoslahti and Seppala, 1971). The fiding of an average homology of 45% between the amino acid sequences of fragments of AFP and the corresponding regions in albumin (Ruoslahti and Terry, 1976) and the demonstration of an immunological cross-reaction between unfolded derivatives of AFP and albumin (Ruoslahti and Engvall, 1977) have suggested that AFP may be evolutionarily related to albumin. Both proteins may therefore have similar functions as, for example, carriers in plasma. According to Uriel and his colleagues (Uriel et al., 1972) AFP, like albumin, can bind estrogens. It has been suggested that possession of special functions by AFP, such

as immunosupression, may offer an advantage over albumin to the fetus (Ruoslehti and Engvall, 1977). Although several authors have reported supressive effects of AFP on MLR and PHA-induced transformation of lymphocytes (see review by Lau and Linkins, 1976) recent studies by Labib and Tomasi (1978) have indicated that the supression of lymphocyte activity may be due to contaminating proteins or to certain subfractions of AFP. By means of the technique of radioimmunoassay trace amounts of AFP (less than 10-23 ng. per ml.) have been detected in non-pregnant adult serum (Ruoslahti and Seppala, 1972). In pregnancy serum the concentrations increase to 160-550 ng. per ml. during the third trimester dropping shortly before term to normal (Seppala and Ruoslahti, 1972). In fetal serum maximal concentrations are reached at about the 14th week of intra-uterine development, falling thereafter to low levels in cord serum (Gitlin, 1975).

The remaining pregnancy associated proteins can all be detected in non-pregnant serum but are present in increased concentrations in pregnancy serum. Proteins found in greatly increased concentrations include steroid-binding beta-globulin (SP2), pregnancy zone protein (PZP) and a number of protein hormones.

SP2 has been isolated from human placentae and studied by Bohn and Kranz (1973) and Bohn (1974b,c and d). Its molecular weight is 65,000 daltons: it contains 13% carbohydrate and is composed of four identical subunits of 16,000 daltons each held together by non-covalent bonds. Although its biological function is unknown SP2 is known to have steroid binding properties and shows a preferential for testosterone and estrodiol. SP2 is found in trace amounts (less than 1 mg. per 100 ml.) in male and in non-pregnant female sera. It appears to be dependent on the presence of estrogens and is often found in greatly increased

concentrations in the serum of patients treated with estrogens. It may also occur in slightly increased concentrations in the sera of patients suffering from a range of different diseases. During the first half of pregnancy the levels of SP2 rise rapidly to 2-10 mg. per 100 ml. In the last month the range of concentrations found is 2·4-12·6 mg. per 100 ml. After parturition the levels of SP2 return to normal within 5-10 weeks with a half-life of 6-7 days.

PZP has been known under the following names: alpha-2 pregnoglobulin, pregnancy-associated alpha-2 glycoprotein, new serum pregnancy alpha-2 macroglobulin, PAG, pregnancy-associated alpha-2 globulin, SP3, Pal, Xh and Xm (Lin and Halbert, 1975). It appears to be synthesised by peripheral blood leucocytes (Stimson and Blackstock, 1975). Immunohistological studies have indicated that PZP is present in blood vessel cells and parenchymal structures within the placental villous stroma as well as in the trophoblast cytoplasm (Lin and Halbert, 1976). It contains 10-12% carbohydrate and molecular weight is 360,000 daltons: it is made up of two identical subunits of 180,000 each held together by non-covalent bonds (Bohn and Winckler, 1976). The biological function of PZP is incompletely understood. It has been found to be estrogen-induced and to show preferential binding for estriol. A postulated function for it as a steroid-carrier (Bohn and Kranz, 1973) has, however, been rejected (Von Schoultz and Stigbrand, 1973; Stimson, 1973). It has also been found to inhibit the PHA-induced stimulation of lymphocytes (Von Schoultz et al., 1973; Stimson and Blackstock, 1975; Home et al., 1976) and may therefore have immunosupressive properties. The mean levels of PZP are known to increase in multigravidas and one possible interpretation of this finding is that antigenic stimulation has occurred during previous pregnancies. In primigravidas there is an

inverse correlation of PZP concentration with placental weight (Lin et al., 1976a). PZP is found in trace amounts (less than 1 mg. per 100 ml.) in male and in non-pregnant sera, cord sera and amniotic fluid (Beckman et al., 1973; Damber et al., 1976). Like SP2 it appears to be dependent on the presence of estrogens and is often found in greatly increased concentrations in the sera of patients treated with estrogens. A less pronounced increase may also accompany a range of different diseases (Beckman et al., 1973; Bohn and Becker, 1976; Stimson, 1977). PZP appears with increasing frequency during pregnancy between weeks 9 and 25 (Afonso and de Alvarez, 1964). The concentration of PZP increases rapidly at the end of the first trimester, then there is often a decline after reaching a maximum until the third trimester when the concentrations stabilise around 100 mg. per 100 ml. (the range at term is 50-200 mg. per 100 ml.). Postpartum there is a rapid decrease in concentrations which tend to return to normal within 5-10 weeks with a half-life of 6-7 days (Bohn, 1974b).

The protein hormones found in greatly increased concentrations in pregnant serum include adrenocorticotrophic hormone (ACTH), melanocyte stimulating hormone (MSH), LH-releasing hormone, relaxin, oxytocin and vasopressin (see reviews by Diczfalusy and Troen, 1961 and Brody, 1969). Recent evidence indicates that the precursor polypeptide for ACTH and MSH is made in the placenta (Liotta et al., 1980). In vitro biosynthesis of LH-releasing hormone has also been demonstrated (Gibbons et al., 1975). Relaxin, oxytocin and vasopressin have been identified in placental tissue (Zarrow et al., 1955; Caldeyro-Barcia, 1960) and may therefore also be synthesised by the placenta.

In addition the concentrations of several other normal serum proteins increase slightly during pregnancy: for example

ceruloplasm, alpha-1-antitrypsin, transferrin, beta-lipoprotein, lactoferrin and the complement fractions beta-1-A-C (Mendenhall, 1970; Studd, 1971; Stimson, 1972).

4. AREAS OF CURRENT AND POTENTIAL PRACTICAL APPLICATION OF THE STUDY OF PREGNANCY-ASSOCIATED PROTEINS

4.1 Obstetric Diagnosis

Monitoring of placental function may be necessary during pregnancies complicated by any one of the following conditions: uterine bleeding, threatened abortion, hypertension, oedema, proteinuria, preeclampsia, placental insufficiency, post-maturity, diabetes mellitus and erythroblastosis: or for predicting fetal growth retardation, death *in utero* and fetal distress in labour. Several of the biochemical tests presently used to monitor placental function are based upon the measurement of substances synthesised in the feto-placental unit; for example steroid hormones and enzymes. The most common test hitherto used, the estimation of estriol or total estrogens in a 24 hour specimen of urine, is a measure of the function of the placenta and fetus together (Katagiri *et al.*, 1976). Reduced estriol output is associated with an increased incidence of fetal growth retardation, fetal distress in labour and fetal death *in utero* (Beischer *et al.*, 1968). However, about 10% of mothers in whom the fetus is seriously compromised can have urinary values within the normal range (Yousem, 1966). On the other hand, low urinary values may occur without the fetus being metabolically compromised and may be due to a deficiency at a single point in the complex series of steps between the fetal adrenal which initiates synthesis of estriol and the maternal urine used for

analysis (Fliegner et al., 1972; France et al., 1973). Furthermore, the interpretation of the estriol levels can be confused by other factors such as the volume of urine, errors in urine collection and interference from drugs used in the treatment of patients. Wide fluctuations in urinary output make this method unsuitable for use in diabetic mothers (Klopper, 1969). Plasma estriol shows large day-to-day fluctuations and subject-to-subject variability (Klopper et al., 1979). In a controlled study in which plasma estriol levels were used as a marker of fetal well-being in late pregnancy a significant number of false positive results occurred and the conclusion reached was that the test was not valuable in timing delivery of the baby (Duenhoelter et al., 1976). Even assays of plasma estradiol 17-beta, shown to have less diurnal variability than plasma estriol, have not been demonstrated to be superior to that of urinary estrogens (Lindberg et al., 1974).

Placental proteins such as HPL,SP1 and PAPP-A show much smaller day-to-day and subject-to-subject variability than plasma estrogens (Masson et al., 1977b; Klopper et al., 1977). A number of placental proteins have already been used in obstetric diagnosis. The clinical usefulness of these proteins, with the possible exception of HPL, has not been subjected to any critical analysis. The literature abounds with claims and counter-claims as to the usefulness of individual placental proteins: however, most studies lack adequate controls and do not attempt to compare the value of the particular placental protein assay with that of other biochemical, physical or clinical tests available. The uncertainty currently surrounding the physiological role, and the control of production, release and elimination of placental proteins means that nothing definite can be said about the likely clinical significance of individual circulating

placental proteins: it is not known to what extent their concentration reflects placental function. Any variations in the clinical usefulness reported may simply be due to differences in the precision and practicality of the measurement of individual proteins.

The HPL test has been the subject of most studies (see reviews by Loraine and Bell, 1966; Singer et al., 1970; Genazzani et al., 1971; Spellacy et al., 1971; Spona and Janisch, 1971; Karjalainen et al., 1975). Serial determinations are usually required: consistently low values of HPL concentrations in third trimester maternal plasma have been claimed to indicate the risk of fetal distress in labour or neonatal asphxia (Letchworth and Chard, 1972) and have been used to predict the outcome of threatened abortion (Niven et al., 1972; Garoff and Seppala, 1975). In pregnancies comlicated by hypertension low values of HPL have been claimed to indicate the risk of fetal death in utero (Spelacy et al., 1971). However the HPL assay could not be used to predict intrauterine death in the absence of severe hypertensive disease (Spellacy et al., 1975). HPL levels were found to be lower in mild than in severe toxemia (Letchworth and Chard, 1972). Normal or increased values of HPL have been found in pregnancies complicated by rhesus immunisation, maternal diabetes, toxemias and chronic nephritis (Josimovich et al.,1970; Soler, 1974; Lin et al., 1977). Preliminary experiments have recently been carried out aimed at assessing whether the value of the HPL test as a predictor of neonatal morbidity is better than that of some of the other parameters currently used (for example, maternal weight and smoking) and whether the HPL test can be used to predict the optimal time for the delivery of the baby. A prospective study of 1029 women delivered at a London hospital showed that depressed circulating levels of HPL were associated with a higher

risk of neonatal morbidity in late pregnancy than any other antenatal parameter tested, except in severe preeclampsia (Gordon et al., 1978). However, the HPL test was not compared with other biochemical tests (except with the measurement of AFP levels between 16-20 weeks), with ultrasonic measurements of the fetus nor with antenatal fetal heart monitoring in late pregnancy. It is possible that the prediction of fetal risk using ultrasonography will be superior because the fetus is assessed directly whereas HPL is an indirect index (Gordon and Grudzinskas, 1978). A controlled study by Spellacy and his colleagues (Spellacy et al., 1975) indicated that the measurement of HPL was a useful adjunct in decreasing perinatal deaths. However, according to Gordon and Chard (1979) subjects with low levels of HPL do not show a fall: this is consistent with the exponential nature of the rate of increase in circulating concentrations of HPL in late pregnancy.

Information concerning the value of the SP1 test in obstetric diagnosis is slowly accumulating. Low levels of SP1 in the third trimester of pregnancy have been reported to be associated with intrauterine growth retardation and fetal death in utero (Tatra, 1977): furthermore it has been claimed to be superior to HPL in predicting intrauterine growth retardation (Gordon et al., 1977; Towler et al., 1977; Horne et al., 1979). Consistently low levels of SP1 have been reported to be associated with preeclampsia and eclampsia (Afanasjeva et al., 1976; Tatra et al., 1974a). SP1 has also been used to detect pregnancy (Grudzinskas et al., 1977a,b) and identify abnormalities of early pregnancy such anembryonic pregnancy (Bennett et al., 1978), ectopic gestation (Grudinskas et al., 1977a) and predict the outcome of threatened abortions (Schultz-Larsen and Hertz, 1978). In addition there is some evidence which suggests that measurement of SP1 concentrations

in amniotic fluid may be useful in monitoring high-risk pregnancies (Tatra et al., 1976a). In pregnancies complicated with diabetes plasma SP1 concentrations appear to be within normal limits (Lin et al, 1977) even though studies by Tatra and his colleagues (Tatra et al., 1976b) have suggested that the plasma SP1 level in pregnancy may be related to the maternal blood sugar level and carbohydrate metabolism. Normal levels of SP1 have also been reported in pregnancies complicated with toxemias (Lin et al., 1977). A recent suggestion that SP1 may be a protein active locally within the uterine tissue and that the material found in the maternal peripheral circulation may be waste leaking away from the site of action (Klopper et al., 1979; see section 2.1), if correct, would detract from the likely usefulness of SP1 in maternal blood as a means of assessing placental function.

HCG has no established clinical application other than in the early detection of pregnancy (Landesman and Saxena, 1976), although its levels have been found in one study to be above average in pre-eclamptic toxemia and in diabetes mellitus (Loraine and Mathew, 1953): and concentrations below average have been reported by Brody and Carlstrom (1965a) to be associated with pregnancies which threaten to abort and which subsequently end in abortion (see also reviews by Loraine and Bell, 1966 and Saxena, 1971). Only preliminary studies of PAPP-A and PAPP-B levels in normal and abnormal pregnancies have been carried out: they indicate that PAPP-A concentrations may be increased in toxemia but is not affected in pregnancies complicated with diabetes (Lin et al., 1977) while PAPP-B concentrations appear to be decreased in toxemia and in pregnancies complicated by diabetes (Lin et al., 1978b). Measurements of plasma levels of the placental enzymes heat-stable alkaline phosphatase (see reviews by Watney et al., 1970; Curzen

and Varma, 1971; Merret and Hunter, 1973), histaminase (Swamberg, 1950; Beaven et al., 1975) and oxytocinase (Hensleigh and Krantz, 1970; Carter et al.,1974) have been advocated to be of value, but all have proved disappointing in clinical practice (Lind,1976).

Pregnancy-associated proteins of non-placental origin advocated to be of value in assessments of fetal status include PZP and AFP. Determinations of PZP in early pregnancy may be of some predictive value as studies by Beckman and his colleagues (Beckman et al., 1974a) have pointed to a higher than normal incidence of early spontaneous abortion in women in whose serum no PZP could be detected by means of double immunodiffusion. Determinations of AFP in maternal serum or amniotic fluid can reveal neural tube defects and a variety of other prenatal conditions (Brock and Sutcliffe, 1972; Brock 1978a and b; Brock 1979). Persistently low levels of HPL have also been found to be associated with a range of fetal abnormalities (Gau and Cadle, 1974).

4.2 Cancer Diagnosis

Pregnancy-associated proteins are synthesised eutopically in non-pregnant patients with trophoblastic cancer (Bagshawe, 1974) and ectopically in a proportion of other forms of cancer (Gordon et al., 1977). Marker-sided cancer diagnosis is an area which is only beginning to be explored, but it is becoming increasingly clear that specific tumour markers may aid in early tumour detection and localisation as well as the detection of tumour recurrence. Specific placental proteins which have been shown to be associated with cancer occurrence include HCG, HPL, SP1 and placental alkaline phosphatase. At present the only well established use of a placental tumour marker is in the measurement of HCG in the blood of patients with choriocarcinoma and teratomas. HCG

is known to be secreted by choriocarcinoma tumour cells and a proportion of teratoma cells. Measurement of beta-HCG in the blood or urine of women suspected of having choriocarcinoma has made possible the detection of this type of cancer at a treatable stage (Vaitukaitis et al., 1972; Karana and Bagshawe, 1976; Bagshawe 1977; Yuen et al., 1977; Bagshawe, 1978). The measurement of beta-HCG in the blood of patients with teratomas, when combined with the measurement of AFP which is also produced by a proportion of teratoma cells (Abelev, 1974) has been found very useful in the monitoring of the course of these tumours (Perlin, 1976; Lange, 1978; Narayana et al., 1978; Walker, 1978). A wide variety of other malignant tumours, including tumours of the bowel, breast, lung, gonad and melanomas,have also been found to be associated with ectopic HCG production (Sheth et al., 1974; Rosen et al., 1975; Vaitukaitis et al., 1976; Stone et al., 1977; Crowther et al., 1978). The incidence of ectopic HCG production ranged between 9 and 62% among the tumours in the studies cited. However Weintraub and his colleagues (Weintraub et al., 1975) have reported differences in the physical and combining properties of purified ectopic and normal alpha-subunits of glycoprotein hormones such as HCG. They have suggested that these differences may reflect abnormalities of neoplastic protein synthesis or carbohydrate attachment or alternatively the production of as yet unrecognised alpha precursor forms by tumour cells. Unbalanced or isolated ectopic production of the alpha- or beta- subunits of HCG has also been demonstrated in certain ectopic tumours (Tashjian et al., 1973; Vaitukaitis, 1973; Weintraub and Rosen, 1973; Rosen and Weintraub, 1974).

Ectopic production of HPL has been detected in a proportion of patients with malignant tumours of the breast, lung, bowel and

ovarian carcinomas (Rosen et al., 1975; Samaan et al., 1976, Sheth et al., 1977; Crowther et al., 1978). Ectopic production of SP1 has been detected in a proportion of patients with the same types of malignant tumours as have been found to be associated with HPL production as well as in testicular carcinomas and in melanomas (Tatarinov and Sokolov, 1977; Towler et al., 1977; Crowther et al., 1978; Grudzinskas et al., 1978; Searle et al,. 1978). Ectopic production of placental alkaline phosphatase has also been found to be associated with a wide variety of cancers (Fishman, 1973).

4.3 Diagnosis and Treatment of Conditions of Pregnancy with Immunological Aetiologies

In taking stock of the current understanding of the factors involved in the maintenance of the immunological homoeostasis during pregnancy (Section 4.3a) it will become apparent why a straightforward statement cannot yet be made about the role and relative importance of each of the pregnancy- associated proteins whose involvement is implicated. A general statement can, however, be made to the effect that different pregnancy-associated proteins may act to stimulate and/or suppress maternal immune responses during pregnancy and may therefore play a part in abnormal states of pregnancy (discussed in Section 4.3b) which have suspected immunological aetiologies. The elucidation of the precise roles of these pregnancy-associated proteins is a continuing quest which may one day open the way to the diagnosis and treatment of women with immunological complications of pregnancy. Prophylactic immunisation against the offending antigen(s) may then also become possible, either by supplying the antigen prior to pregnancy or by supplying specific blocking factors or non-specific constituents

normally formed during pregnancy.

4.3a The Maintenance of the Immunological Homoeostasis During Pregnancy

There is substantial evidence to suggest that maternal humoral and cellular responses to placental and fetal antigens occur in normal pregnancy. Responses to paternal histocompatability (HL-A) allo-antigens have been most extensively studied. Current experiments indicate that developmentally mature and immature human trophoblasts have little or no exposed or masked HL-A determinants on their surface (Faulk and Temple, 1976; Faulk and Johnson, 1977; Faulk et al., 1977). Studies in mice have suggested that loss of histocompatability (H-2) antigens occurs on the trophectoderm at the time of implantation of the blastocyst (Searle et al., 1976). HL-A antigens have, however, been detected on the stromal cells of the placenta (Faulk and Temple, 1976; Faulk et al., 1977). They may also be presented to the mother on fetal cells which frequently enter the maternal circulation (McConnell, 1969; Herzenberg et al., 1979). Cytotoxic antibodies apparently directed against paternal HL-A antigens are frequently found in the sera of multiparous pregnant women (Terasaki et al., 1970) and in one study have been detected in 20% of primiparous pregnancies and in 40% of molar pregnancies, where the mode of antigen presentation may be different (Bagshawe and Lawler, 1975). Complement-fixing antibodies to a major composite protein from placental microsomal fraction, thought to contain HL-A antigens, have been detected in 40% of normal pregnancies (Gaugas and Curzen, 1974). In addition, antibodies to Ia-like allo-antigens expressed on beta-lymphocytes have been identified in sera of multigravid women (Winchester et al., 1975). Cytotoxic effects of maternal and allogeneic lymphocytes on both normal trophoblasts (Currie

and Bagshawe, 1967b) and trypsinised human placental monolayers have been reported (Douthwaite and Urbach, 1971). Other studies of cell mediated immunity in pregnancy using *in vitro* models have revealed a maternal lymphocyte hypersensitivity to unidentified paternal allo-antigens (Rocklin *et al.*, 1973) in multigravida and placental antigens, thought to be either Ia-like alloantigens or placental-specific antigens, in primigravida (Youananukorn and Matangkasombut, 1973; Youananukorn *et al.*, 1974; Stimson *et al.*, 1979). As has already been mentioned, passage of fetal blood elements into the maternal circulation often occurs and antibodies to fetal antigens such as embryonic gut antigens have been detected in the sera of pregnant women (Gold, 1967).

Maternal sensitisation to specific protein products of the placenta has not been unequivocally demonstrated as yet. Antibodies against HCG have been detected in some pregnancy sera (Ruzic *et al.*, 1973). The presence of anti-HPL antibodies in some pregnancy and postpartum sera has been reported (Gusdon *et al.*, 1970; Gusdon *et al.*, 1971; Iannuzzi *et al.*, 1976). However, these findings cannot be accepted as evidence of sensitisation to HCG and HPL since antibodies cross-reacting with HCG as well as antibodies cross-reacting with HPL have been detected in the sera of some non-pregnant individuals (Iannuzzi *et al.*, 1976; Wass *et al.*, 1978). It is not known what provokes the formation of these antibodies. One possibility is exposure to micro-organisms which share antigenic sites with HCG or HPL: supporting this is the finding that some antigens are shared by microorganisms and certain mammalian cells (Kaplan and Suchy, 1964; Chase and Rappaport, 1965) or cancer cells (Minden *et al.*, 1976) and the detection of HCG-producing bacteria in the tissues of patients with malignant neoplasms

(Cohen and Srampp, 1976; Aceredo et al., 1978). Antibodies directed at syncytiotrophoblastic cytoplasm have been reported to occur in some postpartum sera (Hulka et al., 1963), although this finding was not confirmed by other workers (Curzen, 1970). Circulating immune complexes containing an unidentified placental antigen with a molecular weight of 400,000 daltons have been detected in pregnancy sera by Masson et al., (1977).

Tolerance to some pregnancy-associated proteins in the pregnant state, however, may be due to these proteins being also present in small but persistent levels in the non-pregnant adult state. This explanation has been put forward to account for the absence of anti-AFP antibodies in the pregnancy serum (Ruoslahti and Pihko, 1976). In the case of cross-reacting antigens such as HCG and HLH or HPL and HGH exposure to HLH and HGH in the non-pregnant adult state may result in tolerance to HCG and HPL in the pregnant state.

Of the many mechanisms proposed to account for the success of pregnancy in immunological terms, the following are best supported by the available experimental evidence:

(i) The acquisition of special protective properties by the trophoblast cells.

(ii) The production of non-specific immunosupressive factors by the mother and/or fetus.

(iii) The production of specific blocking factors to balance the developing cell-mediated immunity.

The evidence for each of the above mechanisms will now be reviewed in turn:

(i) The acquisition of special protective properties by the trophoblast cells. Since the placenta is the site of contact and

exchange in the materno-fetal relationship a mechanical barrier on its surface would segregate the placental antigens and any any fetal allo-antigens present on trophoblasts from potentially agressive maternal lymphocytes. In the case of HL-A and Ia-like alloantigens, however, protection may result from the partial or complete loss of these antigens from the trophoblasts' surface (Faulk and Johnson, 1977; Faulk et al., 1977). As has already been mentioned, a layer of fibrin or sialomuco-protein is known to exist in the hemochorial placenta. In the mouse this contains sulphate and sialic acid residues which confer upon the surface a relatively large negative charge (Bradbury et al., 1965; Kirby et al., 1964). Transplanted mouse blastocysts have been shown to develop normally in the uterus but not in the kidneys of hyperimmune recipient mice: this effect has been attributed to the immunologically protective role of the sialomucoprotein layer associated with the decidua (Kirby et al., 1966). In man the layer contains sulphates but little sialic acid (Bradbury et al., 1969). This layer may form an electrostatic barrier, preventing the attachment of cytotoxic lymphocytes (Currie and Bagshawe, 1967a) or complement-fixing antibody molecules. There is, however, disagreement as to continuous nature of this layer on the mammalian placenta (Martinek, 1970). The involvement of HCG, which has been postulated to be a structural antigen as well as a secretory product (Smith and Brush, 1973), in barrier formation has been implicated by experiments in which HCG treatment was shown to prolong the survival of transplanted neuraminidase treated human trophoblast in the guinea pig (Borland et al., 1975). It is also possible that the barrier consists of the so-called 'symbodies' - serum sialomucins which interact with conventional antibodies to provide a protective coat around the trophoblast cells (Apffel and Peters, 1969). Although there is

extensive contact between the fetal chorionic membrane - known to be antigenic in the mouse - and the uterine tissues away from the placental site, the membrane is not vascularised by the uterine vessels which could partly account for its lack of susceptibility (Billington, 1975).

(ii) The production of non-specific immunosuppressive factors by the mother and/or fetus. Early studies of maternal immunological activity that the maternal immune system is normal (Medawar and Sparrow, 1956; Billingham and Lampkin, 1957) or only slightly impaired during pregnancy (Lichtenstein, 1942; Breyere, 1961; Lajos et al., 1964). Subsequent studies of maternal lymphocyte reactivity produced conflicting results. Although some workers could find no evidence for either increased or reduced non-specific proliferative responses of maternal lymphocytes stimulated with allogeneic lymphocytes, mitogens or soluble antigens (Carr and Stites, 1972; Harrison, 1972), other workers reported that they are reduced in the second and third trimesters of pregnancy (Kasakura, 1971; Finn et al., 1972; Purtilo et al., 1972; Petrucco et al., 1976) and suggested that the suppression of the immunological reactivity of maternal lymphocytes occurs in late pregnancy. However, there is general agreement that the responses of human lymphocytes stimulated with mytomycin-C treated allogeneic cells, mitogens or soluble antigens can be inhibited by plasma from pregnant women (Ceppellini et al., 1971; Kasakura, 1971; Leventhal, 1971; Buckley, 1972; Hill et al., 1973; Jha et al., 1975; Pence, 1975; Petrucco et al., 1976; Stimson, 1976; Stimson and Blackstock, 1976). The serum concentrations of the immunoglobulins IgG, IgM and IgA were found to decrease in the second half of pregnancy (Maroulis et al., 1971; Tatra et al., 1974b) while IgM-bearing B-lymphocytes were found to increase in the peripheral blood during pregnancy (Loke et al., 1975),

suggesting the suppression of immunoglobulin synthesis during pregnancy. Other studies have demonstrated that the composition of the lymphoid tissues changes during pregnancy (Nelson and Hall, 1964; Strelkauskas et al., 1975).

A large number of substances produced during pregnancy have been reported to have immunosuppressive properties and some or all of them could therefore play a part in bringing about abrogations of maternal thymo-lymphatic activity. They include the products of the placenta and the fetus as well as proteins of maternal origin. Placental products include hormones: HCG, HPL (see Section 3 of this chapter) and steroids (Barnes et al., 1974). It is uncertain whether these placental hormones are inhibitory at serum concentrations, although HPL has been reported to be inhibitory by Petrucco et al., 1976 and HCG has been reported to be inhibitory at those levels found in early pregnancy (8-11 weeks) by Jenkins et al., 1972. Placental hormones could, however, exert their effects during the circulation of maternal lymphocytes past the placenta where they are present in high concentrations (Amoroso and Perry, 1975). Other placental products include SP1 (see Section 3 of this chapter) and three unidentified proteins: a protein precipitating with heparin (Lozovoy et al., 1975), an alpha-2-mobility glycoprotein (Riggio et al., 1971) and a protein of molecular weight less than 150,000 daltons (Hanrahan et al., 1975). The fetal-specific protein, AFP, is also known to have immunosuppressive properties (Lau and Linkins, 1976). Maternally-derived proteins with immunosuppressive properties include serum sialomucins, which are usually elevated in the first and second pregnancy and could represent an acute phase response (Good et al., 1974; Good , 1974), prolactin (Skutsch, 1974; Skutsch, 1975), cortisol (Lewis et al., 1966; Petrucco et al., 1976) and SP2 (see Section 3 of

this chapter). It has been claimed by Beckman and his colleagues (Beckman et al., 1974b) that genetic incompatability may stimulate the production of another immunosuppressive protein, PZP. However, out of four genetic systems studied by these workers only two showed this relationship.

(iii) The production of specific blocking factors to balance the developing cell-mediated immunity. It is evident that other factors are present in pregnancy sera which specifically block maternal lymphocyte reactivity to fetal and trophoblast antigens. Autologous maternal serum was shown to inhibit specific responses of maternal lymphocytes stimulated by fetal lymphocytes (Robert et al., 1973), paternal cells (Rocklin et al., 1976) and placental antigens (Stimson et al., 1979). Presence of autologous maternal serum in tissue culture was found to inhibit trophoblast lysis by sensitized maternal lymphocytes (Taylor et al., 1975).

The possibility that cell-mediated immunity in tumour progression, allotransplantation and pregnancy to antigens which are either weak or are supplied in large or continued doses may be balanced by production of enhancing antibodies or antibody-antigen complexes (blocking factors) has been discussed in a review by Halliday (1972), who argued that in pregnancy the initial requirement for immunosuppression to allow blocking factors to form could be fulfilled by some of the non-specific immunosuppressive factors, notably placental hormones. It has been observed that lymphocyte reactivity to trophoblast antigens is not detectable during the first trimester of pregnancy (Taylor et al., 1975). Antibody-antigen complexes formed in antigenic excess are known to be profoundly tolerogenic at very low concentrations. This observation is reflected in the Effector Cell Blocade theory which postulates a

state of activated B and T-lymphocytes held in blocked state by antibody-antigen complexes in tumour progression (Nossal, 1974; Schrader and Nossal, 1974) and could equally well apply to the pregnancy situation. It has also been postulated that cell-mediated immunity originally developed to destroy malignant cells, then with the advent of placentation humoral immunity was developed to provide specific blocking factors to block cell-mediated immunity (Coggin and Anderson, 1974).

In pregnancy the blocking activity has been found to increase with parity and has been assigned to gel-filtration fractions of maternal sera containing IgG antibodies by a number of workers (Buckley, 1972; Robert et al., 1973; Gatti et al., 1975; Pence et al., 1975; Rocklin et al., 1976). Although the specificity of these antibodies has not been determined recent studies on primiparous women have established that in such women they are not directed at HL-A antigens (including products of HL-A A,B and C loci) but appear instead to be directed at HL-A DR (Ia-like) alloantigens or placental-specific antigens (Stimson et al., 1979)

Studies of placental eluates also point to the presence of enhancing maternal IgG antibodies, although their nature is incompletely understood. Revillard et al. (1976) reported the presence of two antibody populations in human placental eluates. One of these antibody populations was shown to have anti-HL-A specificities and the antibodies exhibited lymphocytotoxicity in the presence of complement. However, evidence has been put forward for complement inactivator production by the human placenta (Strachan, 1976). Antibodies directed at HL-A antigens were also detected in placental eluates by Doughty and Gelsthorpe (1976). The other antibody population detected in placental eluates by Revillard et al. (1976) was shown to have anti-

Ia-like specificities and the antibodies were noncytotoxic. Using immunofluorescence techniques McCormick et al. (1971) and Faulk and Johnson (1977) have detected maternal IgG antibodies on the human trophoblastic basement membrane which, when eluted, were shown to inhibit most mixed lymphocyte reactions. The inhibition was independent of anti-HL-A activity (Faulk et al., 1974) and was postulated to be due to antibodies directed at Ia-like alloantigens (Johnson and Faulk, 1976). However, McCormick et al. (1971) were unable to detect any anti-trophoblastic basement membrane antibodies in pregnancy or in post-partum sera, possibly due to a low titre present. Antibodies capable of inhibiting most mixed lymphocyte reactions and thought to be directed at Ia-like alloantigens were also detected in placental eluates by Jeannet et al. (1977). These findings are of interest as antisera directed at Ia-like antigens have been shown to depress mixed lymphocyte reactions and to impair in vitro antibody-dependent cellular cytotoxicity and immunoglobulin synthesis (Meo et al., 1975; Chess et al., 1976; Davies and Staines, 1976; Hirschberg et al., 1977).

It also appears that receptors for aggregated IgG are present on placental endothelial cells whose function may be to keep immune complexes from entering fetal circulation (Johnson et al., 1976). Finally, mention should be made of the specific immunosuppressive mechanism which results in a degree of selective unresponsiveness between maternal and fetal lymphocytes and which has been postulated to involve specific surface repellent molecules on fetal lymphocytes (Finn, 1975). Such molecules are thought to cross the placenta and coat maternal lymphocytes thus preventing them from attacking fetal cells. This mechanism would explain the generally observed lack of graft-versus-host reactions, despite frequent migration of maternal

lymphocytes across the placenta into the fetal circulation (Kadawaki et al., 1965).

4.3b Abnormal States of Pregnancy with Suspected Immunological Aetiologies

It is well known that both passive and active immunization of pregnant and non-pregnant animals with fetal and placental proteins is capable of inducing abortions and abrogations of reproductive performance (see review by Stevens, 1974). Abortions have been induced by passive immunization of animals possessing cross-reacting antigens with anti-AFP, anti-HCG, anti-SP1 or anti-HPL antisera. Although the possibility that the abortifacient effects were simply a consequence of the damage caused by the deposition of antibody-antigen complexes in the placenta or in fetal tissues cannot be excluded, these findings can equally well be interpreted as indicating that all the antigens involved are essential for pregnancy. It is interesting to note that AFP, HCG, SP1 and HPL are all believed to have immunosuppressive properties. Active immunization of animals possessing cross-reactive antigens with HPL or HCG results in reduction in reproductive function. Immunization of baboons with the beta-subunit of HCG has lasting anti-fertility effects (Stevens, 1979).

Immunological response against the placenta is suspected in pre-eclampsia, which is a disease of primigravidas with many of the hallmarks of immune-complex disease (Kitzmiller and Bernirschke, 1973; Petrucco et al., 1974; Yang et al., 1975; Scott and Beer, 1976). Injection of pregnant rats with heterologous anti-placenta serum can lead to a pre-eclampsia like syndrome characterised by hypertension associated with placental and kidney damage (Lanford et al., 1967; Smith et al., 1967). The nephrotoxicity of anti-placenta serum suggests

the involvement of antigens common to both placenta and kidney. Studies by Koren and his associates (Koren et al., 1970) have indicated that some, though not all, of the renal damage caused by anti-placenta sera is attributable to the deposition of antibody-antigen complexes in glomerular capillary walls. Unlike normal pregnancy, cell-mediated immunity is not reduced in the second and third trimesters of severe pre-eclamptic pregnancy (Petrucco et al., 1976). Furthermore, the levels of maternal serum sialomucins are frequently raised (Good et al., 1974).

Complications of pregnancy due to maternal sensitization to fetal alloantigens are well documented. These include erythroblastosis fetalis, anaemia, leucopenia (Jensen, 1966), thrombocytopenia (Sitarz et al., 1976), haemolytic disease of the newborn (Faulk et al., 1968) and congenital nephrosis (Fudenberg and Fudenberg, 1964). Rocklin and his colleagues (Rocklin et al., 1976, Rocklin et al., 1979) reported that blocking IgG antibodies (thought to be directed at Ia-like alloantigens) present in the sera of pregnant women were absent from the sera of chronic aborters but appeared in their serum during subsequent successful pregnancies. Also, Stimson et al. reported that blocking IgG antibodies (thought to be directed either at Ia-like alloantigens or at placental-specific antigens) present in the sera of primigravida were absent from the sera of a group of abortion-prone women studied. Several other conditions of pregnancy, such as unexplained intrauterine death and intrauterine growth retardation may well have immunological factors in their aetiologies. However, the evidence is in most cases fragmentary or even circumstantial. It has been discussed in a review by Jenkins (1975).

5. USE OF AFFINITY CHROMATOGRAPHY IN THE SEARCH FOR, AND PURIFICATION OF, NEW PROTEINS

Serological and electrophoretic methods are very useful in the study of new proteins of unknown function: they suffer, however, from the disadvantage that their ability to distinguish relatively rare tissue specific proteins amonst the wide array of proteins found in all tissues is very limited. It is therefore very useful if contaminating proteins can be removed from the crude tissue extract used for immunisation so as to evoke the maximum immune response. Protein fractionation can be achieved by means of conventional physico-chemical methods or, alternatively, immunochemical methods such as antibody affinity chromatography (Parikh and Cuatrecasas, 1977), which consists in principle of the selective adsorption of a specific antigen to a solid support on which the corresponding antibody has been covalently attached. One particularly useful variant of the latter method involves the removal of the contaminating proteins from the crude material by affinity columns of sepharose coupled with the antibodies against the contaminating proteins and has been referred to as negative antibody affinity chromatography (NAAC) (Sutcliffe, 1976). An enriched single fraction of a specific protein for immunisation can be collected after it passes unretarded through the NAAC column. This is not possible with the use of conventional protein separation techniques, where several fractions usually result which have each to be analysed by raising a corresponding antiserum and the average degree of purification of a specific protein is proportional to the total number of protein fractions obtained.

Once an antiserum to the specific protein obtained by the use of the NAAC purification procedure is available, positive affinity

chromatography may be a suitable method for subsequent rapid and efficient purification of the specific protein from crude extracts: it is particularly useful for those proteins which are at a low concentration in the initial extract or where there is a contaminant with similar physico-chemical characteristics to the antigen to be purified.

The efficiency with which an antigen is obtained from positive antibody affinity chromatography is mainly affected by the dissociation constant of the antibody and by the stability of the antigen under the elution conditions. It is suspected that in some cases the covalent linkage of antibodies to a solid matrix may result in an apparent increase in the affinity for antigens so that relatively more severe conditions may be required to dissociate the immobilised antibody-antigen complex compared to the effort required when they are formed in solution (Palmer, 1972). Occasionally other problems may also arise in individual systems such as non-specific adsorption and the co-elution of contaminating proteins, especially when crude preparations of antibodies or antigens are used as the immobilised affinity ligand. Non-specific binding may be due to a number of factors, such as hydrophobic interactions with matrix-bound ligands (Yon, 1972; Shaltiel and Er-el, 1973; O'Carra et al., 1974), ion-exchange binding (Dean and Lowe, 1972; Hixon and Nishikawa, 1973; Parikh et al., 1974) to non-specific or conformational occlusion (O'Carra et al., 1974). Another problem which may arise is elution buffer-induced solubilisation of the matrix bound antibody (Parikh et al., 1974). Also, too low a dissociation constant may effectively result in the irreversible binding of antigen and antibody: this can, however, be countered by preparing the antibody from sera taken from animals soon after the onset of the humoral response to

the antigen in question, since there is a higher proportion of low affinity antibodies early in the immune response (Hunter, 1976). All these problems have to be taken into consideration when contemplating the purification of a protein by antibody affinity chromatography or interpreting the results of such a purification.

Although antibodies are relatively stable in the wide variety of strong reagents which are used to dissociate antibody-antigen complexes in affinity chromatography, such as chaotropic thiocyanate and iodide ions at 1•5M to 3M concentration (Avrameas and Ternynck, 1969; Dandliker et al., 1967; Dandliker et al., 1968; de Saussure and Dandliker, 1969), 5M guanidine-HCl (Dandliker et al., 1968), 8M urea (Slobin and Sela, 1965), glycine-HCl buffer, pH2•8 (Avrameas and Ternynck, 1969), glycine-NaOH buffer, pH11 (Omenn et al., 1970) and 3M Magnesium Chloride (de Souza et al., 1976), many antigens may be more sensitive to at least some of these dissociating agents. The sensitivities of many pregnancy-associated proteins are unknown and each should therefore be carefully tested before proceeding with antibody affinity chromatography: many are large, probably complex proteins - for example, PAPP-A, PAPP-B, PZP, and PP6 - and may therefore be particularly susceptible to denaturation.

NAAC has been applied before to the fractionation of placental extracts (Anderson et al., 1974), as well as to soluble tumour antigens (De Carvalho et al.) amniotic fluid and fetal serum (Sutcliffe, 1976). Although affinity chromatography has been employed in the purification of a great many proteins (Parikh and Cuatrecasas, 1977), including the pregnancy-associated proteins AUP (Sutcliffe et al., 1978), PZP (Folkersen et al., 1978) and AFP (Pihko et al., 1973).

6./

FIGURE 1.1a

Early Stage in Development of Chorionic Villi

(a) The trophoblast projection has occurred with the development of lacunae, and much intermingling with maternal tissue, which for clarity is not shown. No mesoblastic core has yet entered the villus.

(b) The mesoblastic core is now developing within the villus.

Redrawn from Patten (1959).

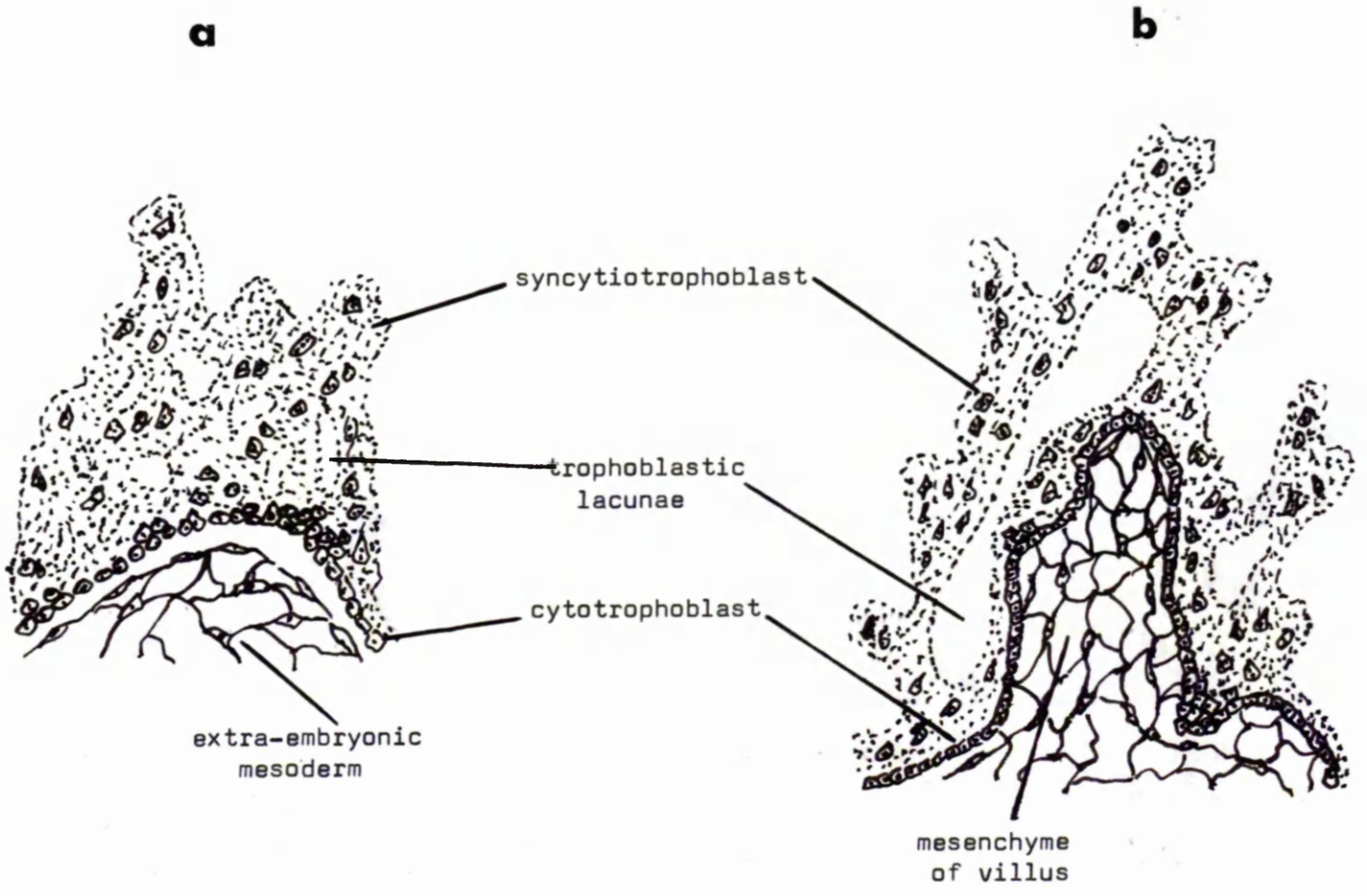

a
b
syncytiotrophoblast
trophoblastic
lacunae
cytotrophoblast
extra-embryonic
mesoderm
mesenchyme
of villus

FIGURE 1.1b

Chorionic Villi at Different Ages

(a) 10 - 18 week embryo.

(b) 24 week embryo.

(c) Term placenta.

Adapted from Patten (1959) and Getzowa and Sadowsky (1950).

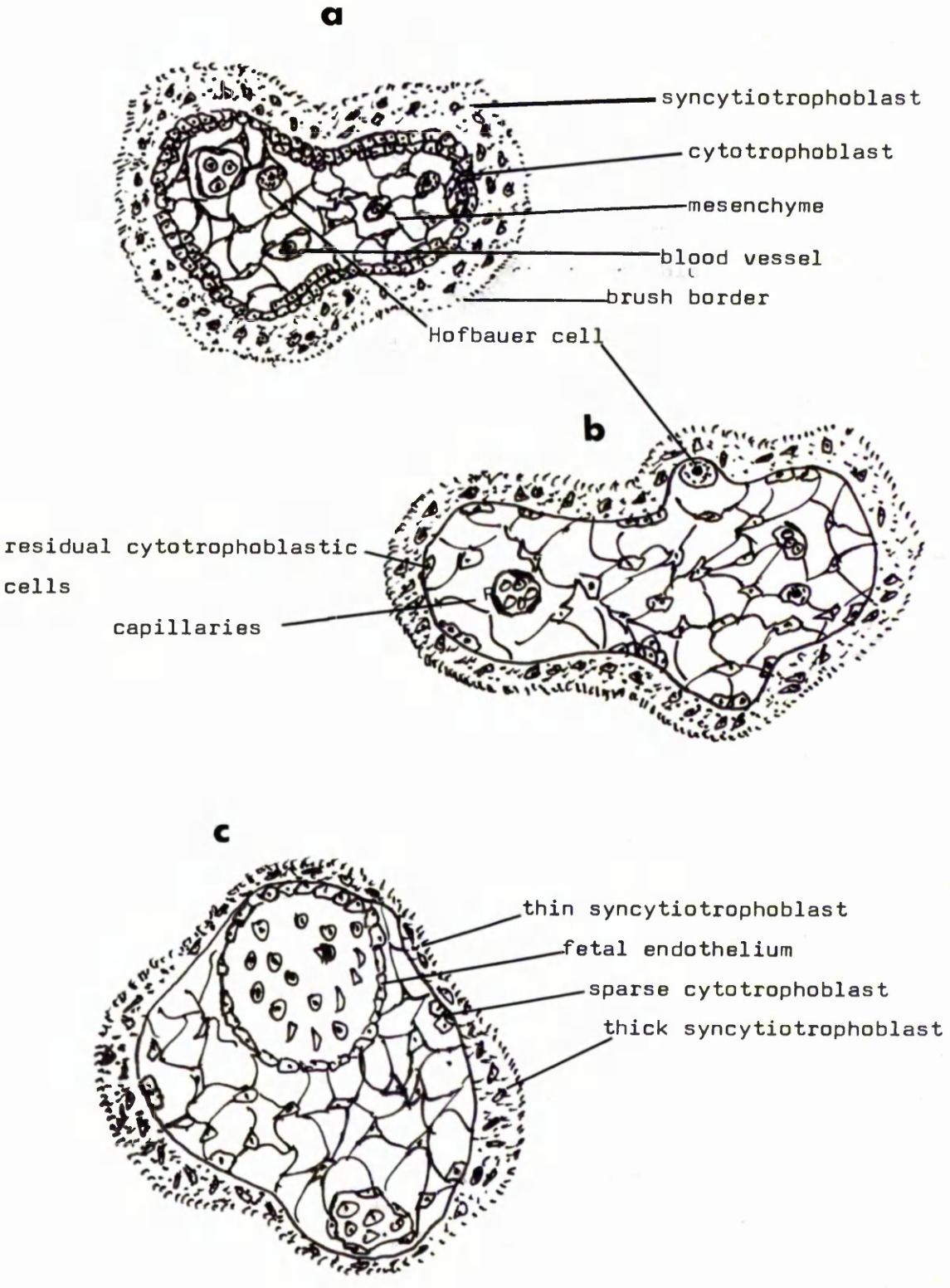

a
syncytiotrophoblast
cytotrophoblast
mesenchyme
blood vessel
brush border
Hofbauer cell
b
residual cytotrophoblastic cells
capillaries
c
thin syncytiotrophoblast
fetal endothelium
sparse cytotrophoblast
thick syncytiotrophoblast

FIGURE 1.2

The Term Placenta in situ and its Membranes

Redrawn from Boyd and Hamilton (1970)

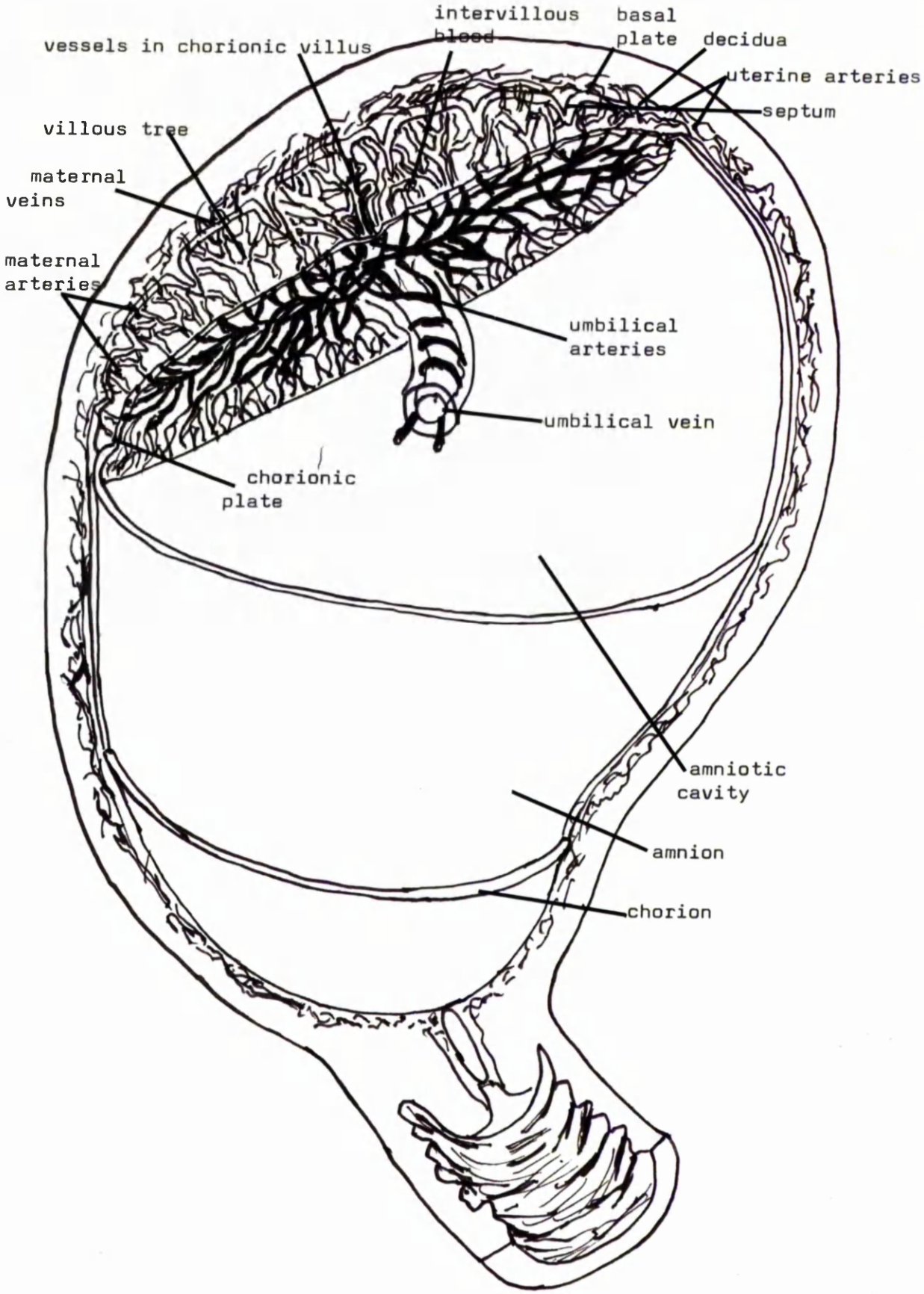
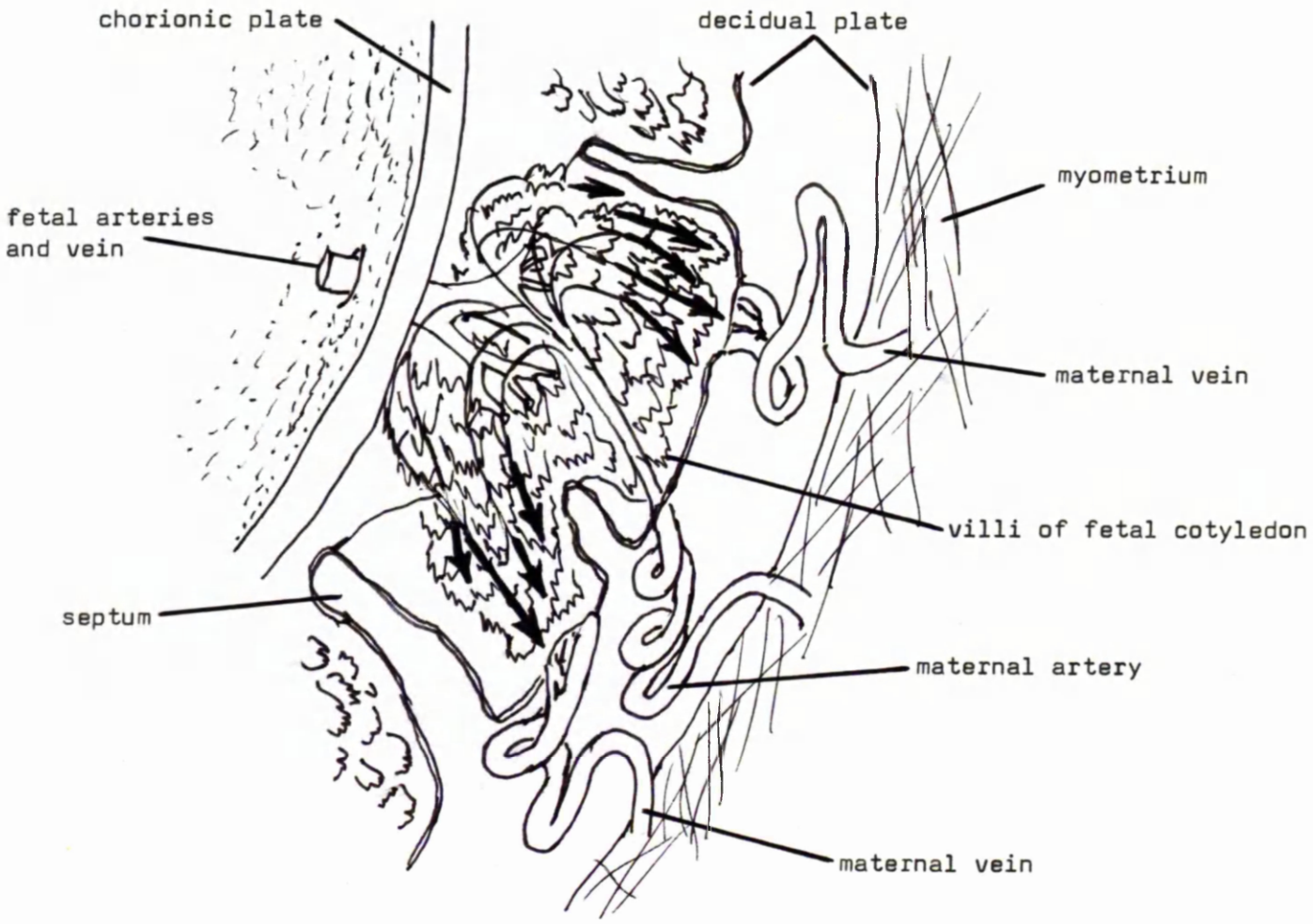

intervillous blood
basal plate
decidua
vessels in chorionic villus
uterine arteries
septum
villous tree
maternal veins
maternal arteries
umbilical arteries
umbilical vein
chorionic plate
amniotic cavity
amnion
chorion

FIGURE 1.3

The Intervillous Space and the Placental Circulation

Redrawn from Llewellyn-Jones (1975)


chorionic plate
decidual plate
myometrium
fetal arteries
and vein
maternal vein
villi of fetal cotyledon
septum
maternal artery
maternal vein

6. LINES OF FURTHER INVESTIGATION

The information presented in the preceding review of the literature has allowed me to formulate objectives for further experimental study which could initially be approached using immunochemical techniques. The first objective was to investigate the existence of naturally occuring maternal antibodies to placental-specific antigens. This investigation concentrated on three approaches - immunofluorescence, antibody affinity chromatography and dissociation of antibody-antigen complexes. Evidence from these approaches is presented in Chapter 2. The second objective was to obtain further information on the specific protein products of the placenta and it gave rise to the experimental work described in Chapters 3 - 7 of this thesis. In Chapter 3 the use of negative antibody affinity chromatography in the search for specific placental proteins in placental soluble extract is presented. The purification and further study of one of the placental-specific proteins, PAPP-A, detected with the use of negative antibody affinity chromatography is described in Chapters 4,5,6 and 7. First, the sensitivity of PAPP-A and other pregnancy-associated and normal serum proteins to the dissociants of antibody-antigen complexes was examined: the results are presented in Chapter 4. These results were used to design a scheme for the purification of PAPP-A by antibody affinity chromatography. The purification and subsequent iodination of PAPP-A is described in Chapter 5. Preliminary studies of the subunit structure of PAPP-A form the subject of Chapter 6. Finally, studies on the concentration of PAPP-A in the blood during normal and complicated pregnancies are described in Chapter 7.

CHAPTER 2 THE SEARCH FOR NATURALLY-OCCURRING MATERNAL ANTIBODIES TO PLACENTAL-SPECIFIC PROTEINS

1. INTRODUCTION

The existence of naturally-occurring maternal antibodies to placental antigens has been investigated using three experimental approaches:

(1) Immunofluorescence studies on placental sections.

(2) Adsorption of maternal serum on a placental protein affinity column.

and (3) Dissociation and separation of the antigen-antibody complexes present in maternal serum.

2. IMMUNOFLUORESCENCE STUDIES ON PLACENTAL SECTIONS

2.1 Introduction

This section deals with the first of the experimental approaches listed above: immunofluorescence studies on sections of placentas obtained from two normal and one agammaglobulinaemic women at delivery. The studies described here were aimed at detecting antibody specificities in maternal sera directed against placental-specific antigens and isoantigens rather than alloantigens. For this reason no attempt was made to test each maternal serum on sections of the placenta obtained from the same pregnancy nor to determine any maternal and fetal allotypes in such matched pairs. Instead, sections of two randomly chosen placentas were used. The immunofluorescence method employed maternal serum in the first layer and anti-human immunoglobulin fluorescent anti-serum in the second layer. A preliminary step involving the use of antibodies to human immunoglobulin was introduced in order to block any endogenous immunoglobulins present in the placental sections. The effect

of this blocking antiserum was compared with that of elution with buffers at different pH in order to determine whether the endogenous immunoglobulins were immunologically bound on the placenta. Sections of a placenta obtained from an agammaglobulinaemic patient and believed to contain little or no endogenous maternal immunoglobulin were used as a control in the study.

The maternal sera investigated in this study came from a range of pregnant subjects which included primi- and multi-parous women with and without a history of previous pathological abortions. The effect of absorption of these sera with placental soluble extract was investigated by substituting absorbed maternal sera for unabsorbed maternal sera in the experimental procedure outlined above. This was in order to determine whether soluble placental antigens were involved in the anti-placental activity (if any) of maternal sera. Sera from a male donor and from an agammaglobulinaemic pregnant woman were used as negative controls and a serum known to give a strong positive immuno-fluorescent reaction - rabbit antiserum to human placental chromatin - was used as a positive control.

2.2 Materials and Methods

Tissues

Three placentas were obtained at the term delivery of normal infants: one of the placentas came from an agammaglobulinaemic patient. Placental tissues were first rinsed with cold PBS. Blocks of tissue were excised from from the midportion of placentas and snap-frozen in an acetone-solid CO_2 mixture. Sections from each block were cut at a thickness of 8 micrometres with a cryostat, then air-dried on slides and stored under polyethylene glycol at -20^{o} C until use. A fourth

placenta obtained at the term delivery of a normal infant was rinsed with cold PBS, homogenised and the soluble extract was harvested.

Sera and Antisera

Serum was extracted from venous blood taken from eleven pregnant women in the third trimester of pregnancy: ten normal women which included two primipara and two multipara with no record of previous spontaneous or pathological abortions, two primipara and four multipara with records of previous spontaneous or pathological abortions (denoted "maternal sera i - x " in Table 2.1) and one agammaglobulinaemic pregnant woman (the donor of the agammaglobulinaemic placenta used in this present study). A twelth sample of serum was obtained from a male donor. Serum samples were stored at -20^{0} C until use. Aliquots of all maternal sera were absorbed with an equal volume of the placental soluble extract prior to use.

Rabbit anti-human placental chromatin antiserum was kindly provided by Dr. Ailsa Campbell (Department of Biochemistry, The University of Glasgow). It had been raised to chromatin extracted from fresh placental tissue using the method of Okita and Zordi (1974) and Zordi (1975). Sheep anti-human serum, sheep fluorescein isothiocyanate (FITC)-antibodies to human Ig and sheep FITC-antibodies to rabbit Ig were purchased from Wellcome.

Immunofluorescence Techniques

The techniques employed here were based on those originally described by Coons and Kaplan (1950). A preliminary step involving the use of sheep anti-whole human serum antiserum ("blocking antiserum") was introduced prior to treatment of the placental sections with the maternal serum to be tested which was followed by treatment with fluorescent antibodies to human Ig.

Procedure

Slides containing fixed placental sections were immersed in a Coplin jar for one minute prior to use. The slides were then dried except for the area around and covering the placental section. All treatments were carried out in a moist chamber at room temperature. A single drop of the treatment solution was applied per section. Unless otherwise stated the duration of each treatment was 45 minutes. Sections were washed after each treatment in three changes of PBS, pH 7·5 for 30 minutes. Unless otherwise stated each of the 32 tests listed in Table 2.1 below was performed on three placental sections - one section from each of the two placentas obtained from normal women, and one section from the agammaglobulinaemic woman's placenta.

Table 2.1: Outline of Experimental Procedure

TEST	TREATMENT (in order shown)		
	1. Sheep anti-human serum	2. Other	3. Test serum ($\frac{1}{2}$ dilution)
1	NO	NO	NO
2	NO	NO	NO
3	YES	NO	NO
4	NO	0·2M glycine-HCl pH 2·8 for 10 min	NO
5	NO	0·2 M glycine-HCl pH 2·8 for 30 min	NO
6	NO	0·2 M glycine-HCl pH 4·5 for 10 min	NO
7	NO	0·2 M glycine-HCl pH 4·5 for 30 min	NO
8 - 17	YES	NO	One of maternal sera i - x per test
18	YES	NO	Agammaglobulinaemic maternal serum
19	YES	NO	Male serum

20 - 29/

/Table 2.1 ctd.

TEST	TREATMENT (in order shown)		
	1. Sheep anti-human serum	2. Other	3. Test serum ($\frac{1}{2}$ dilution)
*20 - 29	YES	NO	One of placental soluble extract-absorbed maternal sera i - x per test
*30	YES	NO	Placental soluble extract-absorbed agammaglobulinaemic maternal serum
31	NO	NO	Rabbit anti-human placental chromatin

* tests 20 - 30 were not performed on sections from the agammaglobulinaemic woman's placenta

As final treatment, the sections in tests 2 - 30 were exposed to sheep FITC-antibodies to human Ig (one-tenth dilution) for 45 minutes and the sections in test 31 were exposed to sheep FITC-antibodies to rabbit Ig (one-tenth dilution) for 45 minutes. The sections in test 1 were not treated with any FITC-antibodies. The sections were then mounted in 90% glycerol buffered at pH 8·5 and examined with a Zeiss fluorescence microscope.

2.3 RESULTS

The untreated placental sections in test 1 (Table 2.1) showed no autofluorescence. Treatment with fluorescent antibodies to human Ig (test 2) did not give rise to any significant fluorescence of the agammaglobulinaemic woman's placental section, but in the two normal placental sections weak and irregular fluorescence. This fluorescence was associated with the trophoblast basement membrane, the fetal blood vessels, the villous stroma and other structures thought to be deposits

of fibrinoid material, but was not associated with the trophoblastic cytoplasm. Pre-treatment of the agammaglobulinaemic woman's placental section with sheep anti-human serum antiserum (test 3), pre-elution at pH2·8 or pre-elution at pH 4·5 (tests 4 - 7) resulted in the sections displaying no significant fluorescence. Pre-treatment of the two normal placental sections with sheep anti-human serum antiserum (test 3) or pre-elution at pH 2·8 for 10 minutes or for 30 minutes (tests 4 and 5) resulted in sections displaying no significant fluorescence. However, limited granular fluorescent staining of the trophoblast basement membrane was observed in sections pre-eluted at pH 4·5 for 10 minutes or for 30 minutes (tests 6 and 7).

Treatment of the placental sections with each of the ten maternal sera (tests 8 - 17), with serum from the agammaglobulinaemic pregnant woman (test 18), with male serum (test 19) or with placental soluble extract-absorbed maternal sera (tests 20 - 30) did not give rise to any significant fluorescence of the placental sections. Only a very dull level of fluorescence was observed on all placental sections in tests 8 - 30 which bore no comparison to the strongly positive reaction produced by rabbit anti-placental chromatin antiserum (test 31) in which very intense staining of the trophoblastic nuclei was observed in addition to some staining of the cytoplasm.

2.4 DISCUSSION

Immunofluorescence techniques involving the use of specific antibodies from animals immunised with purified placental proteins and fluorescein markers have been used to provide information on the localisation of these proteins in the placenta and have localised several

placental-specific proteins in the trophoblastic cytoplasm (Beck et al., 1969; Fox and Kharkongor, 1970; Loke and Borland, 1970; Bohn and Rouneberger, 1973; Bohn, 1976; Lin and Halbert, 1976; Tatarinov et al., 1976; see also Chapter 1, Section 3.1). They have also been used previously in studies aimed at demonstrating the presence of naturally occurring antibodies to placental antigens. Immunofluorescence studies by McCormick and his colleagues (McCormick et al., 1971) and by Faulk and Johnson (1977) of the distribution of immunoglobulin in the human placenta have demonstrated the presence of limited amounts of maternal IgG and traces of IgM on the trophoblastic basement membrane and deposits of fibrinoid material but not in the trophoblastic cytoplasm. In placentas which were not perfused considerable amounts of IgG were also detected by these workers in the villous stroma and around blood vessels: prolonged washing of the placental tissue resulted in diminished staining of these areas. The stromal IgG probably represents maternal immunoglobulin in transit: it is known that maternal IgG is selectively transferred across the placenta (Miller, 1966). Unlike the stromal IgG maternal IgG associated with the trophoblastic basement membrane could not be removed by washing but it could be eluted from the placental tissue with a low pH buffer (pH 2·3) suggesting immunological nature of its deposition there (McCormick et al., 1971; Faulk et al., 1974). IgG thus eluted has been shown to inhibit most mixed lymphocyte reactions and the inhibition was shown to be independent of anti-HL-A activity (Faulk et al., 1974). Although the precise specificity of this IgG has not been established it is thought to be directed at Ia-like alloantigens expressed on human B-lymphocytes (Johnson and Faulk, 1976). McCormick and his colleagues (McCormick et al., 1971) could not, however, detect these antibodies in pre-labour or in post-partum maternal sera: such sera caused no inhibition

of labelled eluate activity on placental sections. IgG antibodies directed at Ia-like alloantigens were also detected in placental eluates by Revillard et al. (1976) and Jeannet et al. (1977). Other immunofluorescence studies aimed at the detection of maternal antibodies to antigens expressed on placental tissue produced conflicting results. Hulka and his associates (Hulka et al., 1961; Hulka et al., 1963) claimed to detect anti-placental antibodies directed at trophoblastic cytoplasm in post-partum, but not in pre-labour maternal serum. However, other workers were unable to confirm Hulka's findings (Curzen, 1970).

The placental tissue used in the present study had been subjected to only very limited washing with PBS and the fluorescence resulting the treatment of this tissue with fluorescent antibodies to human IgG although much weaker than that reported by McCormick (McCormick et al., 1971) formed a pattern similar to that reported by him for unperfused placentas. In both cases fluorescence was found to be associated with placental structures other than trophoblastic cytoplasm. In the present study the maternal origin of the immunoglobulins involved in the staining reactions is suggested by the finding of the lack of comparable staining of the placental sections obtained from the agammaglobulinaemic pregnant woman. Furthermore, the fluorescence-diminishing effect of the blocking antiserum was found to be mimicked by treatment with the pH 2·8 buffer while treatment with a higher pH buffer appeared only to diminish the staining of Ig in the villous stroma and had less effect on the staining of Ig in the trophoblastic basal membrane. The present findings are in agreement with the postulated immunological nature of the binding of this immunoglobulin on the trophoblastic basal membrane.

Treatment of the placental sections with sera from

primiparous and multiparous pregnant women and with the negative control sera - male serum and the agammaglobulinaemic pregnant woman's serum - did not give rise to any significant fluorescence of the normal and agammaglobulinaemic woman's placental sections. Treatment of the placental sections with the positive control - rabbit anti-placental chromatin antiserum - on the other hand produced a strongly positive reaction: very intense staining of the trophoblastic nuclei was observed in addition to some staining of the cytoplasm,probably due to contamination of the original material used for immunisation. Thus, no evidence for the presence of antibodies to placental-specific proteins in maternal pre-labour sera was produced by the present study.

3. ADSORPTION OF MATERNAL SERUM ON A PLACENTAL PROTEIN AFFINITY COLUMN

3.1 Introduction

This section describes attempts to isolate and concentrate maternal serum antibodies directed against placental-specific proteins or placental isoantigens using a placental protein affinity column. Serum samples were obtained from a group of pregnant women and a control group of non-pregnant women. Within each group serum samples donated by women who had similar obstetric histories of previous successful or unsuccessful pregnancies were pooled together. Each serum pool was then adsorbed on a placental protein-affinity column and the adsorbed material eluted from the column with 2 M KI. The eluates were initially assayed for total protein and Ig content and the percentage content of IgG and IgM determined. The eluates were then tested for anti-placental activity by:

(1) Immunofluorescence using placental sections according

to the method described in Section 2 of this chapter

and (2) Double immunodiffusion against soluble placental antigens and, as control, soluble lung antigens.

3.2 Materials and Methods

Tissues

Two placentas were obtained at the term delivery of normal sections of normal infants and used to prepare 8 micrometre for fluorescence studies. The remaining tissue was homogenised and the soluble extract harvested. Adult lung tissue was also homogenised and the soluble extract harvested.

Sera and Antisera

Serum was extracted from venous blood taken from pregnant women in the third trimester of pregnancy, and from non-pregnant women. The serum samples were then pooled according to the scheme set out in Table 2.2, and stored at -20^{o} C until use.

Table 2.2: Serum Pools

Serum pool	Obstetric status of donors	Obstetric history of donors: live births	miscarriages	Number of donors	Volume (ml.)
1	NP(=non-pregnant)	NO	NO	3	23
2	NP	YES	NO	3	16
3	NP	YES	YES	2	8
4	P(=pregnant)	NO	NO	12	46
5	P	YES	NO	16	41
6	P	YES	YES	10	36
7	P	NO	YES	8	36

Standard human serum was purchased from Behringwerke. Sheep fluorescent FITC-antibodies to human Ig, sheep fluorescent FITC-anti-

bodies to rabbit Ig, rabbit anti-human IgG (F_c) and rabbit anti-human IgM (F_c) antisera were purchased from Wellcome. Rabbit anti-human placental chromatin antiserum was the antiserum used in the experiments described in Section 2 of this chapter.

Preparation and Use of Placental Protein Affinity Column

Activation and substitution of Sepharose 4B were performed according to the method of Porath et al. (1967). 500 mg. of protein from the placental soluble extract was coupled to Sepharose 4B and the placental protein-Sepharose packed into a 3 cm. diameter Wright scientific column. 2 M KI-PBS, pH 7·0 was passed through the column for 1 hour at a rate of 160 ml. per hr. following which the column was regenerated in PBS.

Each of the seven serum pools listed in Table 2.2 was fractionated over the placental protein affinity column. All fractionations were carried out at 4°C. In each fractionation the serum pool was passed through the column at a rate of 80 ml. per hr. and washed through with PBS until the eluate from the column gave a zero reading at 280 nm. The column was then desorbed with 2 M KI-PBS, pH 7·0 for 1 hour at a rate of 160 ml. per hr. and regenerated in PBS. The desorbed material was concentrated by ultrafiltration on an Amicon XM-100 A membrane (exclusion limit 100,000 daltons), separated from the KI by dialysis against 1:10 dilution of PBS, lyophilised and redissolved in 0·4 ml. of distilled water. The protein content of the desorbed material was subsequently determined using the folin phenol reagent (Lowry et al., 1951). The IgG and IgM contents were determined by radial immunodiffusion.

Immunochemical Methods

Radial immunodiffusion was performed according to Mancini

et al. (1965). 2 microlitre aliquots of Behringwerke human serum at 1/5, 1/10, 1/20, 1/40, 1/80 and 1/160 dilutions and 2 microlitre aliquots of concentrated desorbed material from each of the seven fractionations over the placental protein affinity column were applied to two gels. One of these gels contained 1·8% rabbit anti-human IgG (F_c) antiserum and the other 1·8% rabbit anti-human IgM (F_c) antiserum. The diameters of the precipitin rings formed after 48 hours were measured and used to construct standard reference curves of antigen concentration versus immunoprecipitate area.

Immunofluorescence studies were performed according to the scheme outlined in Section 2.2 of this chapter. A total of thirteen tests was carried out. Each test was performed on two placental sections, one from each of the two normal placentas (see under "Tissues"). Tests 1 - 3 correspond to the tests 1 - 3 in Table 2.1. Tests 4 - 10 were carried out according to the procedure set out for tests 8 - 17 in Table 2.1: however, in place of maternal sera (treatment 3), concentrated desorbed material from one of the seven fractionations over the placental protein affinity column was used per test. Tests 11 and 12 were carried out according to the procedure set out for tests 18 and 19 respectively in Table 2.1 using agammaglobulinaemic maternal serum and male serum. Test 13 corresponds to test 31 in Table 2.1.

Double immunodiffusion was performed according to Ouchterlony (1968). Duplicate tests were set up: one with well to well distances of 6 mm. and a development time of 24 hours and one with well to well distances of 8 mm. and a development time of 48 hours. 2 microlitre aliquots of concentrated desorbed material from each of the seven fractionations over the placental protein affinity column and 2 microlitre aliquots of rabbit anti-human placental chromatin antiserum were

tested against 2 microlitre aliquots of lung and placental soluble extracts.

3.3 Results

The yields of total protein and IgG and IgM from the fractionation of the seven serum pools (Table 2.2) over the placental protein affinity column are shown in Table 2.3.

The untreated placental sections showed no autofluorescence (test 1). Placental sections treated with fluorescent antibodies to human IgG (test 2) showed weak and irregular fluorescence in structures other than the trophoblasic cytoplasm. Pre-treatment with anti-human serum antiserum (test 3) resulted in sections showing virtually no fluorescence. Treatment with the concentrated desorbed material from each of the seven fractionations over the placental protein affinity column resulted in all the sections exhibiting only very dull fluorescence and a similar effect was produced by treatment with serum obtained from the agammaglobulinaemic woman and with male serum (tests 4 - 12). Treatment with anti-placental chromatin antiserum (test 13) produced an intense staining of the trophoblastic nuclei and moderate staining of the trophoblastic cytoplasm.

No immunoprecipitin lines could be detected in double immunodiffusion experiments in the area of the gel between those wells containing concentrated desorbed material from each of the seven fractionations over the placental protein affinity column and wells containing placental soluble extract or adult lung soluble extract. Faint immunoprecipitin lines were detected in the area of the gel between wells containing anti-human placental chromatin antiserum and wells containing placental soluble extract or adult lung soluble extract.

TABLE 2.3

Yields of total protein, IgG and IgM from fractionations of serum pools over the placental protein affinity column

Serum pool[a]	Obstetric status of donors (P=pregnant NP=non-pregnant)	Folin protein applied to column, mg.	Folin protein recovered in material desorbed with 2 M KI		Ig content of desorbed material, %[c]			
			(mg.)	(%)[b]	IgG	Group mean ± s.d.	IgM	Group mean ± s.d.
1	NP	1580	7•9	0•5	8•1	13•8 ± 5•4	1•1	3•5 ± 2•8
2	NP	1133	3•4	0•3	18•8		2•7	
3	NP	546	7•1	1•3	14•4		6•6	
4	P	2800	5•6	0•2	22•7	13•6 ± 6•5	5•0	5•1 ± 1•5
5	P	3900	3•9	0•1	8•1		3•6	
6	P	1900	1•9	0•1	13•4		7•2	
7	P	2533	7•6	0•3	10•0		4•7	

a for details see Table 2.2

b calculated as: $\frac{\text{Folin protein in material desorbed with 2 M KI (mg.)}}{\text{Folin protein applied to placental column (mg.)}}$

c calculated as $\frac{\text{Ig in material desorbed with 2 M KI (mg.)}}{\text{Folin protein in material desorbed with 2 M KI (mg.)}}$

3.4 Discussion

Inspection of the results in Table 2.3 reveals that in all fractionations over the placental protein affinity column protein desorbed from the column with 2 M KI represented a small percentage (between 0•1 and 1•3%) of the protein in the serum pool applied to the column. When the mean values for the IgG and IgM content of the material desorbed with 2 M KI after fractionation of the seum pools from the non-pregnant group (13•8 $\pm$ 5•4% IgG and 3•5 $\pm$ 2•8% IgM) and the pregnant group (13•6 $\pm$ 6•5% IgG and 5•1 $\pm$ 1•5% IgM) are compared they are seen to be very similar. Furthermore these mean values are similar to normal serum values such as those for Behringwerke human serum which contains 15•9% IgG and 1•2% IgM. These observations could suggest a low level of non-specific adsorption of serum proteins to the column in all fractionations over the column.

Even although the volume of the concentrated 2 M KI eluates (0•4 ml.) represented an average 100-fold volume concentration of sera pools within the pregnant group, as with the non-pregnant group, no evidence of positive immunofluorescent reactions with the placental sections could be obtained. The immunoglobulins in the concentrated 2 M KI eluates also failed to form immunoprecipitates with soluble proteins in the placental and adult lung extracts. These findings contrasted with the positive reactions obtained using rabbit anti-human placental chromatin antiserum in immunofluorescence tests and in double immuno-diffusion.

The results reported here suggest that it may not be possible to use a placental protein affinity column to isolate and concentrate antibodies to placental specific proteins and placental isoantigens from third trimester maternal sera. However, these

conclusions are tentative as the methods of assaying the antibody presence were restricted to those which do not depend on the availability of highly purified placental proteins and to those which may not be able to detect very low levels of antibodies. Antibodies to two placental-specific proteins, HPL and HCG, have been detected in the sera of pregnant women as well as some non-pregnant individuals by means of highly sensitive techniques such as radioimmunoassay and haemagglutination (see Chapter 1, Section 4.3a). The use of such techniques was possible because both proteins are available in highly purified form. The presence of anti-HPL and anti-HCG antibodies could not be detected using gel precipitation techniques indicating that the concentration of these antibodies was extremely small. If antibodies to other placental proteins are also present in maternal serum at a very low concentration there might well be a state of antigen excess and a better approach would be to look for soluble antibody-antigen complexes in maternal serum. This approach is described in the following section.

4. DISSOCIATION AND SEPARATION OF THE ANTIBODY-ANTIGEN COMPLEXES PRESENT IN MATERNAL SERUM

4.1 Introduction

This section describes an attempt to seek soluble antibody-antigen complexes in the sera of pregnant women. Multiparous pregnant women were chosen as the donors of the sera, since in such women stimulation by placental- and fetal-specific antigens may already have occurred during previous pregnancies. The procedure used involved dissociation of antibody-antigen complexes with 2 M KI, followed by the separation of antigens by elution under dissociating conditions through

a filter membrane with an exclusion limit of 100,000 daltons. This approach naturally restricted the search to antigens of molecular weight below 100,000 daltons. This procedure was first tested by applying it to the separation of serum albumin from soluble sheep anti-human albumin - human albumin complexes prepared under conditions of antigenic excess *in vitro*. Antiserum to protein dissociated on XM100 A membrane in 2 M KI from pooled multiparous sera was raised in rabbits and analysed on two-dimensional antibody-antigen crossed electrophoresis.

4.2 Materials and Methods

Tissues

The placenta, placental amnion, placental chorion and decidua were obtained at the time of term delivery of normal infants. These tissues and the following adult tissues - endometrium, myometrium, uterus, ovary, heart, liver, spleen, thyroid, lung kidney, adrenal and pancreas - were homogenised and the soluble extract in PBS harvested.

Sera and Antisera

Samples of venous blood were obtained from fifteen pregnant multiparous women during the third trimester of pregnancy. The serum extracted from each blood sample was pooled. The volume of this serum pool was 120 ml. A sample of venous blood was obtained from an adult male donor and the serum extracted.

Sheep anti-human serum antiserum and rabbit anti-PAPP-A antiserum were prepared in our laboratory (for details of the anti-PAPP-A antiserum preparation see Chapter 4, Section 2). Rabbit antisera against human SP1, HPL and human albumin were purchased from Behringwerke A.G., W. Germany

Immunochemical Methods/

/Immunochemical Methods

One- and two-dimensional AACE was carried out according to Laurell (1966; 1972) in 1% agarose gel. Double immunodiffusion was performed according to Ouchterlony (1968) and allowed to proceed for 24 hours.

Preparation of Soluble Anti-human Serum - Human Serum Complexes

(1) The quantitative precipitation reaction between sheep anti-human serum antiserum and human serum.

To twelve 200 microlitre aliquots of anti-human serum antiserum in test tubes were added aliquots of human male serum beginning with 5 microlitres in the first tube, 10 microlitres in the second and thereafter increasing by 20 microlitres each time to 210 microlitres in the final tube. The volume in each tube was made up to 1 ml. with PBS. After 30 minutes incubation at room temperature and 30 minutes at 4^{o} C the solutions were centrifuged at 3,000 r.p.m. for 10 minutes, the precipitates resuspended in PBS and centrifuged again. The precipitates were then dissolved in 1 M NaOH and analysed for protein content by the method of Lowry et al., 1951.

(2) The preparation of soluble complexes between anti-human serum and human serum albumin.

To 10 ml. of anti-human serum antiserum were added 17 ml. of human male serum (1:1·7 volume ratio of antiserum:antigen solution) with stirring and the solution left to stand for 30 minutes at room temperature and for 30 minutes at 4^{o} C. The solution was then centrifuged at 3,000 r.p.m. for 10 minutes in order to remove any precipitate formed.

Dissociation and Separation of Antigens from Antibody- Antigen Complexes

The dissociation and separation of antigens and antibodies was attempted from:

(1) soluble complexes between anti-human serum and human serum albumin

and (2) soluble antibody-antigen complexes in pooled multiparous sera.

In both cases the gamma fraction was initially precipitated from solution with 33% saturated $(NH_4)_2SO_4$, pH 8·0 - 8·6, the precipitate removed by centrifugation at 9,000 r.p.m. for ten minutes and redissolved in a buffered solution. Precipitation with 33% saturated $(NH_4)_2SO_4$ was repeated twice. Finally the precipitate was dissolved in 50 ml. PBS and washed with PBS in a thin channel ultrafugation cell (TCF10 model) on an Amicon filter XM 100A (exclusion limit 100,000 daltons). The washing was continued until the filtrate gave a zero reading at 280 nm. The filtrate was concentrated to 10 ml. on an Amicon filter PM 10 (exclusion limit 10,000 daltons). The retentate was concentrated to 25 ml., made up to 2 M KI with 25 ml. 4 M KI, pH 7·0, washed with 2 M KI, pH 7·0, for 12 hours and finally concentrated to 10 ml. Throughout the washing with PBS and 2 M KI the volume inside the ultrafiltration cell was maintained at 50 ml. and the gas pressure at 10 p.s.i. Thematerial eluted through the XM 100A membrane in 2 M KI was concentrated on an Amicon filter PM 10 to 10 ml., then dialysed against diluted PBS and the protein content determined by the method of Lowry et al. (1951).

The gamma fraction was precipitated from 10 ml. human male serum with 33% saturated $(NH_4)_2SO_4$, the precipitation was repeated twice and the final precipitate was redissolved in 10 ml. PBS. The precipitate was then analysed for the presence of albumin on double immunodiffusion.

The material obtained at different stages of the dissociation of anti-human serum albumin - human serum albumin complexes was tested for the presence of albumin on double immunodiffusion. Aliquots of the

material obtained at the different stages of the dissociation of the antibody-antigen complexes from pooled multiparous sera were stored at -20 °C. They were analysed for the presence of SP1 on one-dimensional AACE at a later stage.

An aliquot of pooled multiparous sera was made up to 2 M KI with an equal volume of 4 M KI, pH 7•0, and left standing at 4 °C for 12 hours. It was then dialysed against several changes of PBS and tested for the presence of human serum albumin on double immunodiffusion. The same procedure was repeated at a later stage in order to test for the presence of SP1 on double immunodiffusion.

Preparation of Antiserum to the Protein Dissociated on XM 100A Membrane in 2 M KI from Pooled Multiparous Sera.

Two aliquots of the material dissociated on an XM 100A membrane in 2 M KI from pooled multiparous sera containing 8•6 mg. of protein each were lyophilised, resuspended in 1 ml. FCA and used to immunise two rabbits. 4•3 mg. of protein was used to boost each rabbit four weeks later. Antisera were obtained from the two rabbits on the seventh day following boosting. Absorption of 10 ml. aliquots of antisera was carried out by adding increasing amounts of adult male serum until 5 ml. had been added and the formation of immunoprecipitate was no longer readily detectable. Antisera were then analysed on two-dimensional AACE and on double immunodiffusion using pooled multiparous sera, adult male serum and placental soluble extract as sources of antigens.

4.3 Results

Quantitative Precipitation Reaction between Sheep Anti-human Serum Antiserum and Human Serum

It was found that as increasing amounts of human serum were

added to 200 microlitre aliquots of anti-human serum antiserum the quantity of the precipitate formed increased. The amount of precipitate formed was greatest when 50 microlitres of human serum were added. The addition of larger volumes of human serum resulted in decreased precipitation until a point was reached on the addition of 210 microlitres of human serum where no precipitate could be detected with the methods used (Figure 1).

The Dissociation and Separation of Antigens from Antibody-Antigen Complexes

(1) Soluble complexes between anti-human serum and human serum albumin.

Albumin could not be detected by means of double immunodiffusion in the 33% $(NH_4)_2SO_4$-precipitated gamma-fraction of human male serum readjusted to the original serum volume. Albumin was not detected by double immunodiffusion in the filtrate collected when the 33% $(NH_4)_2SO_4$-precipitated gamma-fraction of the 1:1•7 anti-human serum:human serum solution was washed on a XM 100A membrane in PBS but it was detectable in the filtrate collected when 2 M KI was used in place of PBS (Figure 2).

(2) Soluble antibody-antigen complexes in pooled multiparous sera.

When the material obtained at different stages of the dissociation of antibody-antigen complexes from pooled multiparous sera was analysed for the presence of SP1 on one-dimensional AACE the following results were obtained:

(1) SP1 was readily detectable in the 33% $(NH_4)_2SO_4$-precipitated gamma-fraction of pooled multiparous sera

(2) No SP1 could be detected in the filtrate collected when the gamma-fraction of multiparous sera was washed on a XM 100A

membrane with PBS.

(3) The presence of SP1 in the filtrate collected when the gamma-fraction of multiparous sera was washed on a XM 100A membrane with 2 M KI could not be determined though it was suggested by the formation of a faint precipitin line immediately above the antigen well.

(4) SP1 was readily detectable in the material retained on the XM 100A membrane at the end of the dissociation procedure (Figure 3).

The material dissociated on the XM 100A membrane in 2 M KI from the gamma-fraction of pooled multiparous sera was found to contain 30 mg. of protein.

Precipitin lines were formed on double immunodiffusion by SP1 in pooled multiparous sera treated with 2 M KI for 12 hours. These lines showed cross reactivity with those formed by human serum albumin and SP1 in untreated pooled multiparous sera.

Antisera to Protein Dissociated on a XM 100A Membrane from the Gamma Fraction of Pooled Multiparous Sera

Unadsorbed rabbit antisera to protein dissociated on a XM 100A membrane in 2 M KI from the gamma fraction of pooled multiparous sera formed three precipitin lines with human male serum on two-dimensional AACE. When these antisera were absorbed with human male serum to the point where precipitin formation was no longer readily detectable and analysed on AACE, one or two small precipitin arcs were still detectable when concentrated human male serum was used as the source of antigens. The antisera were therefore judged to be incompletely absorbed. One or possibly two small precipitin arcs and an additional large arc was detected when concentrated pooled multiparous sera was used as the source of antigens. A single large arc was detected when soluble placental extract was used as the source of antigens. The antigens in

pooled multiparous sera and soluble placental extractwhich gave rise to the large precipitin arcs were shown to cross-react (Figure 4). Both rabbit antisera showed reactions of identity with commercial anti-SP1 antiserum with respect to:

(i) the large precipitin arc formed on AACE (Figure 5)

and (ii) the single precipitin line formed on double immunodiffusion when pooled multiparous sera was used as the source of antigens

Using the incompletely adsorbed rabbit antisera to the protein dissociated on a XM 100A membrane in 2 M KI from multiparous sera or commercial anti-SP1 antiserum, SP1 was also detected on one-dimensional AACE in extracts of placental chorion and amnion. It was not detected in extracts of decidua, endometrium, myometrium, uterus, ovary and in extracts of the following adult tissues tested: heart, liver, spleen, thyroid, lung, kidney, adrenal and pancreas.

4.4 Discussion

Since the main precipitating activity of anti-human serum antiserum is due to anti-human serum albumin antibodies it is to be expected that the quantitative precipitation reaction between anti-human serum antiserum and human serum is a reflection in the large measure of precipitate formation between anti-human serum albumin antibodies and human serum albumin. From the findings that precipitate formation ceases at equal volume ratios of anti-human serum antiserum and human serum (Figure 1) it may be assumed that soluble complexes between anti-human serum and serum albumin were formed at 1:1·7 volume ratio of anti-human serum antiserum to human serum (see Section 4.1, "Materials and Methods").

The results reported herein show that human serum albumin

(molecular weight: 66,000 daltons), which is not precipitated from human serum with 33%-saturated $(NH_4)_2SO_4$, can be dissociated from 33%-saturated $(NH_4)_2SO_4$-precipitated soluble complexes between anti-human serum and serum albumin by washing on an Amicon XM 100A membrane with a commonly used dissociating agent such as 2M KI, but not with PBS. The same procedure was therefore applied to 33%-saturated $(NH_2)_4SO_4$-precipitated gamma fraction of pooled multiparous sera in order to test for the presence of naturally occuring soluble antibody-antigen complexes.

Immunization of rabbits with protein dissociated on an XM 100A membrane in 2M KI from the gamma fraction of pooled multiparous sera produced a strong response to a placental antigen in addition to limited anti-human serum response. The placental antigen was subsequently identified as placental-specific protein, SP1 (see Chapter 1, Section 3.1). Several considerations argue against any conclusion that SP1 has been dissociated from soluble anti-SP1-SP1 complexes in maternal sera. A follow-up study showed that SP1 is a protein which precipitates with 33%-saturated $(NH_2)_4SO_4$ and , since it is unlikely that it was all coupled to antibodies in maternal sera its presence in the solution during dissociation with 2M KI would have depended very critically on the efficiency of the initial washing step. However, Sp1 is too big to be readily washed through an XM100A membrane; this is suggested by its molecular weight - 110,000 daltons according to Lin and Halbert et al. (1974) or 90,000 $\pm$ 5,000 daltons according to Bohn (1974) - and was demonstrated by the failure to wash it through the XM 100A membrane in PBS. Little, if any SP1 could be detected on AACE in the filtrate collected when the gamma fraction of multiparous sera was washed on an XM 100A membrane with 2M KI and

most SP1 was still present in the retentate at the end of the dissociation experiment. Some tunneling of SP1 molecules through the XM 100A membrane would not be surprising and 2 M KI may have aided the tunneling process by inducing reversible or irreversible conformational changes in SP1 molecules. It is unlikely that gross irreversible changes occured since cross-reactivity was observed on double immunodiffusion between SP1 in untreated multiparous sera and in sera exposed to 2 M KI for twelve hours.

Although a simple interpretation of the failure to find other anti-placental specificities in rabbit antisera is that no other placental-specific proteins or placental isoantigens of molecular weight between 10,000-100,000 daltons are involved in immune complex formation during pregnancy in multiparous women, a number of alternative explanations are equally viable. Some placental proteins may have been irreversibly denatured by exposure to 2 M KI for twelve hours (eg. PAPP-A; see Chapter 5, Section 2). Strongly bound antigens might not have been dissociated by 2 M KI. Some antigens may have been present in amounts insufficient to induce immune response in rabbits. Unresponsiveness in rabbits may also have been due to genetic factors. Weak responses would not have been detected with the methods used (AACE). Similarly small amounts of antigen might not have been detected on AACE. Furthermore, had any of the antigens bee monovalent they would not have been detected on AACE.

Few studies of immune complexes in maternal sera have been published. One such study by Masson and his colleagues (Masson et al., 1977) suggested the involvement of a placental antigen of 400,000 daltons in immune complex formation. Although the intriguing

possibility that SP1 may be involved in immune complex formation was raised by the experimental findings described herein, the weight of the evidence produced was insufficient to determine whether or not it is involved. This possibility will need to be investigated more thoroughly. The present study produced no evidence of the involvement of any other placental-specific proteins in the molecular range 10,000 - 100,000 daltons in immune complex formation during pregnancy in multiparous women.

FIGURE 2.1

Quantitative Precipitation Reaction Between Sheep Anti-Human Serum Antiserum and Human Serum

Increasing volumes of human serum were added to 200 microlitre aliquots of anti-human serum antiserum and the quantity of precipitate formed (mg.) was measured.

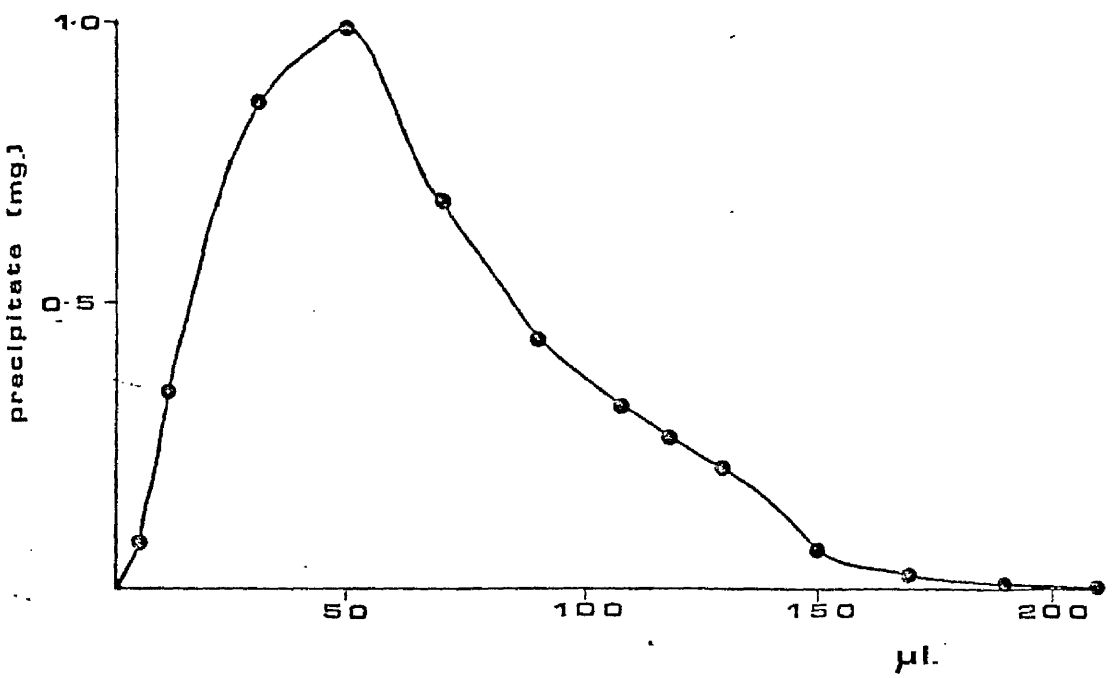

1·0
0·5
precipitate (mg.)
50
100
150
200
µl.

FIGURE 2.2

Double Immunodiffusion Test for the Presence of Human Serum Albumin

The antiserum is anti-human serum albumin

The antigen wells were filled with 2 microlitre aliquots of:

(1) 1/20 dilution human serum

(2) 33% $(NH_4)_2SO_4$-precipitated gamma-fraction of human serum adjusted to the the original serum volume

(3) Material eluted through a XM 100A membrane in PBS from the gamma-fraction of the 1:1•7 anti-human serum: human serum solution (adjusted to the original human serum volume)

(4) Material eluted through the XM 100A membrane in 2 M KI from the gamma-fraction of the 1:1•7 anti-human serum: human serum solution (adjusted to the original human serum volume).

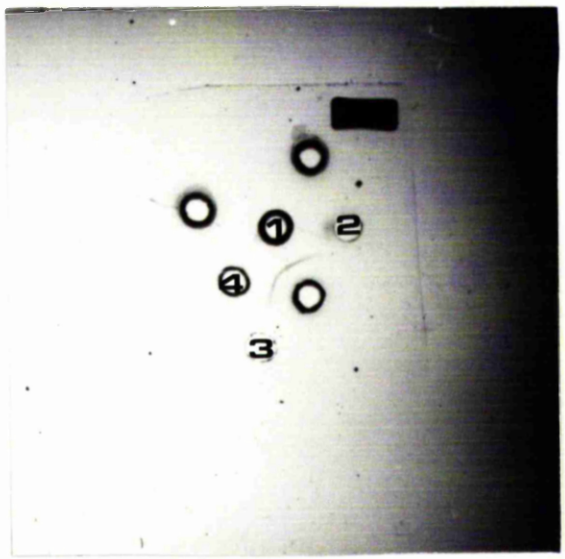

1
2
3
4

FIGURE 2.3

One-Dimensional AACE Patterns Formed by SP1 in 10 microlitre Aliquots of:

(1) 33% $(NH_4)_2SO_4$-precipitated gamma-fraction of pooled multiparous sera (50 ml.)

(2) Material eluted through the XM 100A membrane in PBS from the gamma-fraction of pooled multiparous sera (10 ml.)

(3) Material eluted through the XM 100A membrane in 2 M KI from the gamma-fraction of pooled multiparous sera (10 ml.)

(4) Material retained on the XM 100A membrane at the end of the dissociation procedure (50 ml.).

The antiserum is incompletely adsorbed rabbit antiserum to protein dissociated on the XM 100A membrane in 2 M KI from multiparous sera. The antiserum strength is 20%.

For demonstration of its cross-reactivity with commercial anti-SP1 antiserum and reaction with pooled maternal sera see Figure 2.5.

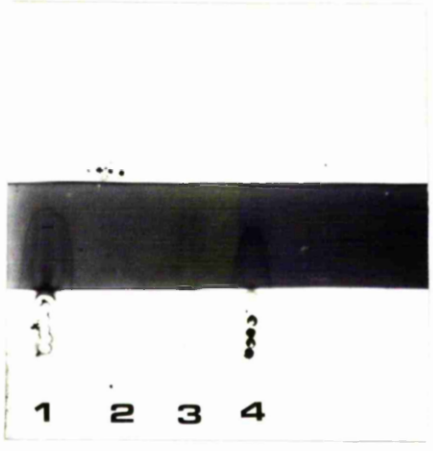

1
2
3
4

FIGURE 2.4

Two-Dimensional AACE Plate Showing Cross-Reactivity between Antigens in Soluble Placental Extract and Pooled Multiparous Sera.

The antigens are:

(1) 6 microlitre aliquot of soluble placental extract containing 600 microgram protein

(3) 4 microlitre aliquot of concentrated pooled multiparous sera containing 1,360 microgram protein

(2) Addition of (1) and (3).

Note that in (2) the main precipitin arc is larger than in (1) or (3).

(4) 4 microlitre aliquot of concentrated male serum containing 1,360 microgram protein.

The antiserum is incompletely adsorbed rabbit antiserum to protein dissociated on a XM 100A membrane in 2 M KI from multiparous sera. The antiserum strength is 10%.

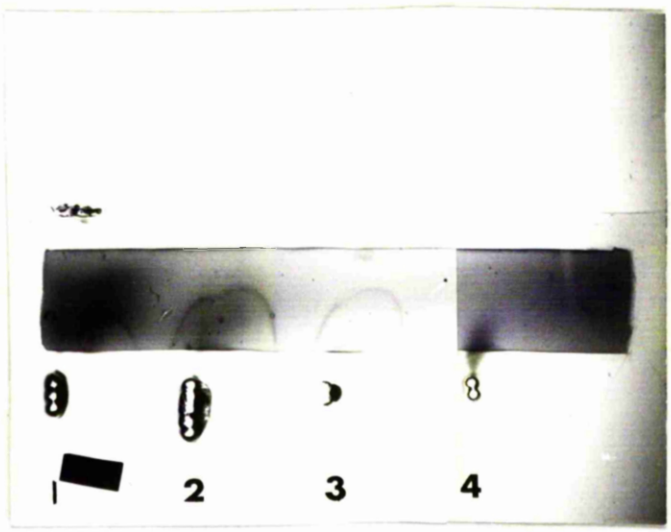

1
2
3
4

FIGURE 2.5

Two-Dimensional AACE Plate Showing Cross-Reactivity Between Commercial Anti-SP1 Antiserum and Rabbit Antiserum to Protein Dissociated on a XM 100A Membrane in 2 M KI from Multiparous Sera.

Antigen (1) is a 10 microlitre aliquot of pooled multiparous sera containing 680 microgram protein.

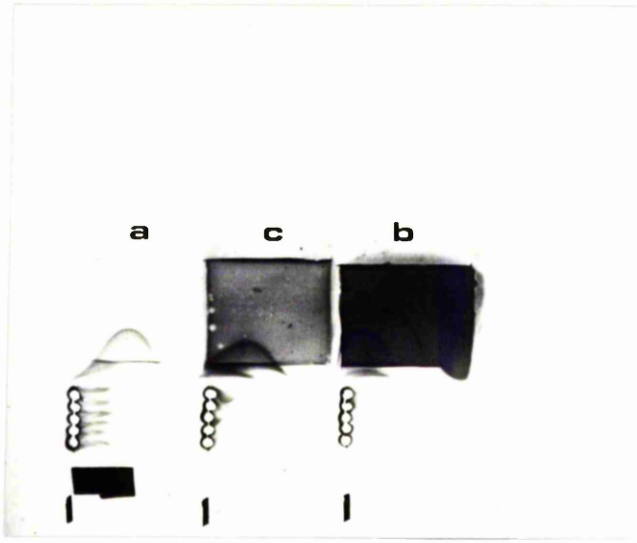
The antisera are:

(a) 5% commercial anti-SP1

(b) 15% incompletely adsorbed rabbit antiserum to protein dissociated on a XM 100A membrane in 2 M KI from multiparous sera

(c) Addition of (a) and (b).

Note that the larger precipitin arc in (c) is smaller than the single arc in (a) and the larger arc in (b). The smaller arc in (c), on the other hand, is identical to the smaller arc in (b).


a
c
b

CHAPTER 3 THE USE OF ANTIBODY AFFINITY CHROMATOGRAPHY IN THE SEARCH FOR PLACENTAL-SPECIFIC PROTEINS

1. INTRODUCTION

This chapter presents the use of negative antibody affinity chromatography (NAAC: see Chapter 1, Section 5) in the search for human placental-specific antigens in the soluble extract of the placenta. In the first experimental section attention is focussed on experiments aimed at the removal of adult and fetal-type protein from placental soluble extract by adsorption on columns of Sepharose coupled with anti-adult and anti-fetal human protein antibodies. This is followed in the second experimental section by an analysis and comparison of the antisera raised in rabbits to unfractionated placental soluble extract and to NAAC products of placental soluble extract.

2. NAAC FRACTIONATIONS OF THE PLACENTAL SOLUBLE EXTRACT

2.1 Introduction

This section describes a series of NAAC fractionations of placental soluble extract using columns of Sepharose coupled with sheep anti-adult human serum protein antibodies alone or in conjunction with columns of Sepharose coupled with sheep anti-adult human tissue protein antibodies and columns of Sepharose coupled with rabbit anti-human amniotic fluid protein antibodies.

2.2 Materials and Methods

Tissues

Thirty-six placentae were obtained at the time of term delivery of normal infants, rinsed with cold PBS, homogenised and the soluble extract harvested. A portion of adult human lung was also

homogenised and the soluble extract in PBS harvested.

Sera and Antisera

Standard human serum was purchased from Behringwerke A. G., West Germany. Adult human male serum serum was obtained from a volunteer.

Rabbit antisera against human serum albumin, human ferritin, human adult haemoglobin, HPL, and SP1 were purchased from Behringwerke A. G., West Germany. Rabbit antiserum specific for PAPP-A was kindly donated by Drs. Lin and Halbert. Sheep antisera against adult male human serum (Sutcliffe, 1976) and adult human lung soluble extract were raised in our laboratory. Rabbit antiserum to NAAC - treated human amniotic fluid was raised in our laboratory by Sutcliffe et al. (1978). It had been raised to the protein fraction of amniotic fluid (from normal second trimester pregnancies) which passed unretarded through a NAAC column of Sepharose coupled with sheep anti-adult male human serum protein antibodies.

Analysis of the Unfractionated and NAAC-fractionated Placental Soluble Extract

The placental soluble extracts and the various NAAC products of the placental soluble extract were analysed for total protein, serum albumin, haemoblobin and heat-stable alkaline phosphatase content. The total protein content was determined by the method of Lowry et al. (1951). Serum albumin content was determined by measuring the area under the precipitin arc formed by albumin on one-dimensional AACE using anti-human serum albumin antiserum. A standard curve for albumin was prepared using Behringwerke standard human serum (albumin content: 52·6 mg. per ml.) and anti-human serum albumin antiserum. The haemoglobin content was assayed by Louise Nicholson using the cyanmethaemoglobin method of Drabkin and Austin (1932). The specific activity due to

placental heat-stable alkaline phosphatase was determined by the method of Hunter (1969). In addition the NAAC products of the placental soluble extract were analysed for HPL and SP1 content by one-dimensional AACE using anti-HPL and anti-SP1 antiserum respectively. Prior to the analysis of the HPL and SP1 contents the protein concentrations of the NAAC products were adjusted to that of the placental soluble extract. Each millilitre of the placental soluble extract was arbitrarily designated as containing 100 U HPL and 100 U SP1 and used to prepare standard curves for HPL and SP1.

Immunoelectrophoretic Methods

One- and two- dimensional antibody-antigen crossed electrophoresis (AACE) according to Laurell (1966, 1972) and immunoelectrophoresis according to Hirschfield (1959, 1960, 1962) were carried out in 1% agarose gels. Double immunodiffusion was performed according to Ouchterlony (1968) and allowed to proceed for 24 hours.

SDS Slab Gel Electrophoresis

SDS slab gel electrophoresis was carried out by the method of Marsden et al. (1976). The spacer gel was 4·75% acrylamide and the running gel was a gradient of 7 - 15% acrylamide.

Preparation and Use of NAAC Columns

NAAC column 1: Anti-adult human male serum protein column

20 g. of immunoglobulin purified by ammonium sulphate precipitation from sheep antisera against adult human male serum was conjugated to 1·9 kg. Sepharose 4B by the CNBr reaction (Porath et al., 1976). The material was packed into a 15 cm. diameter Wright Scientific column (Sutcliffe, 1976).

NAAC column 2: Anti-adult human lung tissue protein column

600 mg. of immunoglobulin purified ammonium sulphate

precipitation from sheep antiserum against adult lung soluble extract was conjugated to 100 ml. Sepharose 4B by the CNBr reaction. The material was packed into a 3 cm. diameter Wright Scientific column.

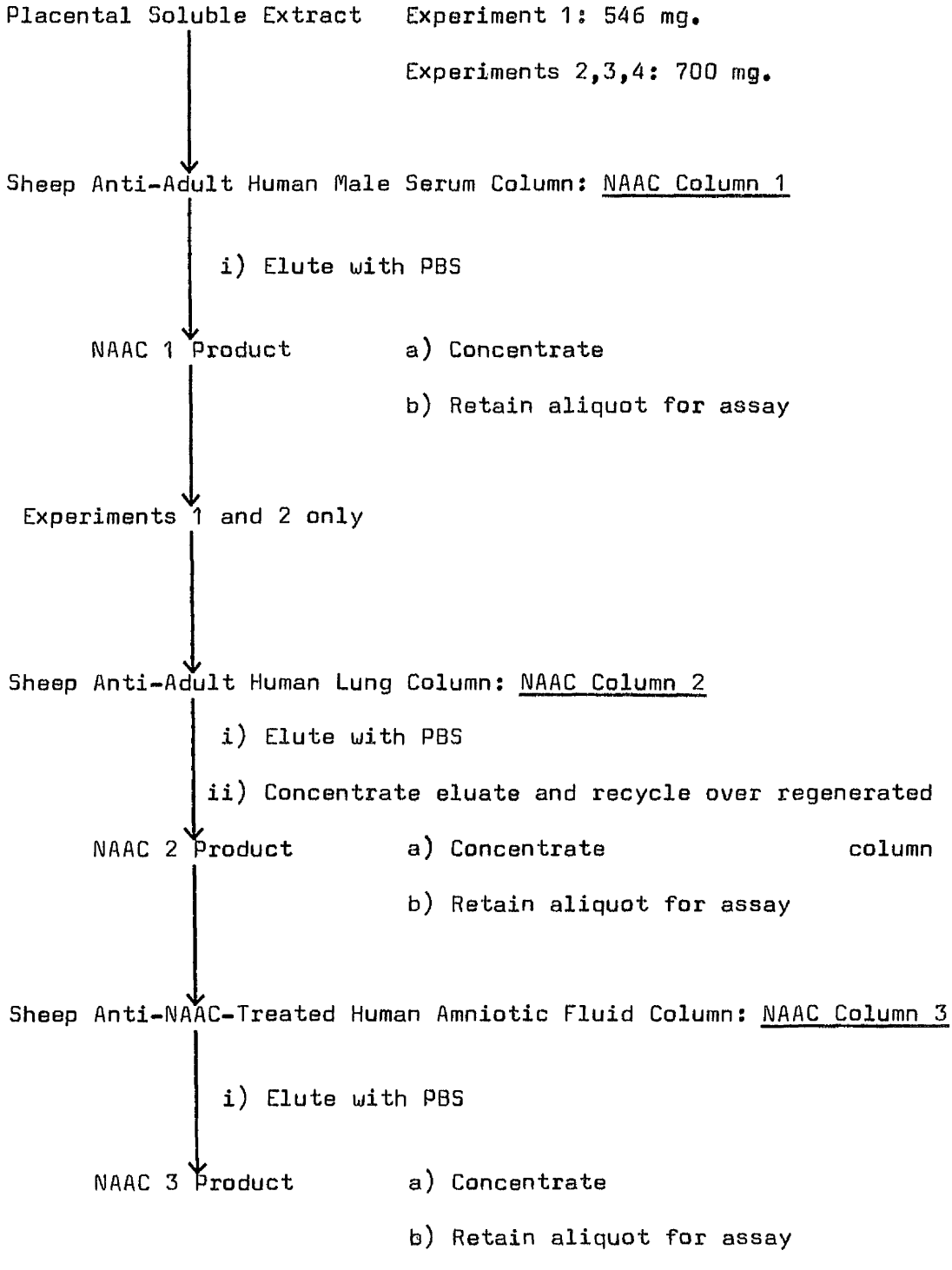
NAAC column 3: Anti-human amniotic fluid protein column

300 mg. of immunoglobulin purified from rabbit antiserum to NAAC-treated human amniotic fluid was conjugated to 60 ml. Sepharose 4B by the CNBr reaction. The material was packed into a 3 cm. diameter Wright Scientific column.

NAAC fractionation of the placental soluble extract was attempted using the protocol outlined in Figure 3.1. Placental soluble extract was passed through NAAC column 1 in PBS in aliquots of 546 mg. (NAAC fractionation experiment 1) and 700 mg. (NAAC fractionation experiments 2,3,4) and at a rate of 400 ml. per hour.Material which failed to adsorb to the NAAC column (NAAC 1 product) was concentrated to 10 ml. by ultrafiltration (Amicon PM10) and analysed for total protein, serum albumin (experiments 1 - 4), haemoglobin, HPL and SP1 (experiments 1 - 3). The NAAC 1 products were also analysed on two-dimensional AACE using sheep anti-adult human male serum antiserum. In experiments 1 and 2 an aliquot of the NAAC 1 product was passed through the NAAC 2 column in PBS at a rate of 80 ml. per hour. In each experiment the material which failed to adsorb to this NAAC column was concentrated to 10 ml. by ultrafiltration, analysed for total protein and recycled over the regenerated column. The unadsorbed material which was the product of the recycling (NAAC 2 product) was concentrated to 10 ml., analysed for total protein, haemoglobin, HPL and SP1. 90% of the NAAC 2 product (experiments 1 and 2 only) was next passed through NAAC column 3 at a rate of 80 ml. per hour. The remaining 10% was retained for assay. The material which was not adsorbed (NAAC 3 product) was concentrated to 10 ml. by ultrafiltration

FIGURE 3.1

Protocol for NAAC Fractionations of Placental Soluble Extract



and analysed for total protein, haemoglobin, HPL, Sp1 and placental alkaline phosphatase.

Desorption of NAAC columns 1 - 3 was performed using 2 M KI, pH 7•0; the columns were then regenerated in PBS. The material desorbed from NAAC column 3 in 2 M KI was collected, concentrated by ultrafiltration (Amicon PM10), separated from the KI by dialysis against PBS, lyophilised and redissolved in 1 ml. distilled water. The concentrated material was then tested for cross-reactivity with antigens in humandecidual extract, placental soluble extract and lung soluble extract on two-dimensional AACE using antiserum to NAAC-treated human amniotic fluid.

The NAAC 1 products and the NAAC 3 products from experiments 1 and 2 were analysed by SDS slab gel electrophoresis along with the soluble placental extract, adult human male serum, adult human haemoglobin and the gamma fraction of sheep antiserum against adult human male serum purified by ammonium sulphate precipitation.

2.3 Results

The total protein concentration of the placental soluble extract was 20 mg. per ml. and it was found to be composed of 18% serum albumin, 43% haemoglobin and 39% other protein.

The results of the various steps of the NAAC fractionation experiments are summarised in Table 3.1. No precipitin arcs were formed by the NAAC 1 products of experiments 1 - 3 on two-dimensional AACE with sheep anti-adult human male serum antiserum.

Examination of the SDS slab gel (Figure 3.2) revealed that at least twelve of the fourteen visible bands formed by the NAAC 1 product from experiment 1 corresponded in position to the bands formed

TABLE 3.1

The Recovery of Total Protein and Individual Proteins from NAAC Experiments with Placental Soluble Extract

I Fractionations over the anti-adult male serum column:

NAAC Column 1

Experiment	Total Protein Applied to column (mg.)	Total Protein Unadsorbed (%)	% Recovery of Individual Proteins[a] Albumin	Haemoglobin	HPL	SP1
1	546	47•0	0•0	83•7	14	13
2	700	54•3	0•1	70•5	25	19
3	700	57•5	0•0	68•0	20	16
3	700	49•0	0•0	ND	ND	ND

ND: not determined

II Fractionations over the anti-adult lung column:

NAAC Column 2

Experiment	Total Protein Applied to column (mg.)	Removed during first adsorption (%)	Removed during second adsorption (%)	Unadsorbed (%)	% Recovery of Individual Proteins[ab] Haemoglobin	HPL
1	245	20•2	7•2	72•6	62	47
2	293	26•0	11•3	62•7	58	38

III Fractioations over the anti-NAAC-treated amniotic fluid column:

NAAC Column 3

Experiment	Total Protein Applied to column (mg.)	Total Protein Unadsorbed (%)	% Recovery of Individual Proteins[ac] Haemoglobin	Placental alkaline phosphatase[d]
1	160	90•4	100	9•2
2	166	79•0	94	5•0

a calculated: $\frac{\text{Protein in unadsorbed fraction}}{\text{Protein applied to NAAC column}} \times 100$

b SP1 detected in NAAC products from both experiments but quantitation was not possible on AACE

d calculated: $\frac{\text{Activity in unadsorbed fraction}}{\text{Activity in material applied to NAAC column}} \times 100$

c SP1 and HPL detected in NAAC products from both experiments but quantitation was not possible on AACE

by the NAAC 1 product from experiment 2, in which a total of nineteen bands was counted. Nine of these common bands were still present in the NAAC 3 products from experiments 1 and 2; the other three were missing from both. The NAAC 3 product from experiment 1 formed a total of ten bands, while the NAAC 3 product from experiment 2 formed a total of fifteen bands. Two of the bands formed on SDS gel by all the NAAC products examined corresponded in position to the two bands formed by adult haemoglobin. None of the bands formed by the NAAC products corresponded in position to the two main bands formed by the gamma fraction of rabbit antiserum. The patterns formed by all the NAAC products differed from that formed by adult male human serum.

Analysis of the material desorbed from NAAC column 3 in 2 M KI on two-dimensional AACE using antiserum to NAAC-treated human amniotic fluid revealed the presence of a single antigen of alpha-mobility which cross-reacted with an alpha-antigen found also in human decidua. The presence of this antigen in placental soluble extract or in lung soluble extract could not be demonstrated on two-dimensional AACE.

2.4 Discussion

Inspection of Table 3.1 reveals that there is considerable similarity between the different fractionations over each NAAC column with respect to the percentage recoveries of total protein and individual specific proteins.

Although extremely low recoveries of serum albumin (trace amount in experiment 2, undetectable by AACE in experiments 1, 2 and 3) and relatively high recoveries of haemoglobin (68·0% - 83·7%) from NAAC column 1 were expected, the recoveries of placental proteins HPL

and SP1 were unexpectedly low at 14% - 25% for HPL and 13% - 19% for SP1. However, relatively low recovery values for HPL and SP1, ranging from 17% - 42% for HPL and from 20% - 38% for SP1 were also observed when aliquots of term maternal serum were fractionated over the same column (Sutcliffe et al., 1979). On that occasion high recoveries of AFP (89% - 116%) suggested little non-specific adsorption of protein to the NAAC column. Although occurence of non-specific adsorption of other proteins cannot be ruled out, more likely mechanisms responsible for low recoveries of individual proteins might be minor or cross-reacting antibody specificities on the column or specific interactions of proteins with the column not involving the binding by specific antibodies. Loss of some proteins could also be due to their instability.

In experiments 1 and 2 second adsorption over the NAAC column 2 was found to remove between $\frac{1}{3}$ - $\frac{1}{2}$ as much protein as the first adsorption over the column. Further adsorption over the column was not therefore judged to be worthwhile, especially as it would increase problems connected with storage of fractionated material and might have resulted in non-specific adsorption of placental-specific proteins on the column. Recoveries of haemoglobin from the NAAC column 2 were slightly lower at 58% - 62% than from the NAAC column 1; this may have been due to the presence of anti-haemoglobin antibodies on the NAAC column 2. HPL recoveries from the NAAC column 2 on the other hand, were slightly higher at 38% - 47% than from the NAAC column 1; this difference could have been due to a number of factors, including the presence of cross-reacting antibodies on the NAAC column 1.

Although recoveries of haemoglobin from the NAAC column

3 were high at 94% - 100%, neither HPL nor SP1 were present in sufficient quantities to allow their quantitation on AACE. However, the amount of both these proteins in the NAAC 2 products applied to the NAAC column 3 were already small. The anti-NAAC-treated amniotic fluid antiserum used to make the NAAC column 3 has been shown by Sutcliffe et al. (1978) to have antibody specificities detectable on AACE directed at several ubiquitous adult tissue antigens, two fetal skin antigens and an alpha-mobility decidual antigen, AUP. Desorption of the NAAC column 3 with 2 M KI was carried out after fractionation of the NAAC 2 products over the column and the concentrated desorbed material was analysed on AACE. An alpha-mobility antigen was detected in the desorbed material on two-dimensional AACE using antiserum to NAAC-treated amniotic fluid. This antigen cross-reacted with an alpha-mobility decidual antigen, AUP. No cross-reacting alpha-antigen could be detected on AACE in the lung soluble extract or in the placental soluble extract. It appears therefore that AUP, present in amounts undetectable by AACE in the placental soluble extract had been specifically concentrated on the column. Absence of the tissue antigens in the 2 M KI eluate suggests that they may have already been removed from the placental extract. The fetal skin antigens had not previously been detected in placenta. The presence of trace amounts of several placental-specific proteins in amniotic fluid has been reported (see Chapter 1, Section 3.1) and some minor antibody specificities directed at placental-specific proteins may therefore have been present on the NAAC column 3. It was for this reason that adsorption over this column was limited to a single passage.

The findings from SDS gel electrophoresis of a large

proportion of protein components in the NAAC 1 products from experiments 1 and 2 common to both and the preservation of two-thirds of these common components during adsorption over the NAAC columns 2 and 3 constitute further evidence of non-random removal of proteins from the placental soluble extract by the NAAC columns. The SDS gel electrophoresis results also provided no evidence of any significant contribution to the protein in the NAAC 1 products from the antibodies immobilised on the column.

In conclusion, the adsorption of aliquots of placental soluble extract over the NAAC column 1 removed virtually all of the quantitatively most important serum protein and albumin and presumably also the bulk of other serum proteins, since none could be detected in the NAAC 1 product on two-dimensional AACE using anti-adult human male serum. However, the adsorption over NAAC column 1 also removed a substantial proportion of HPL and SP1 from the placental soluble extract although relatively high recoveries of haemoglobin suggested that there was little non-specific adsorption of protein to the NAAC column. Subsequent adsorptions over NAAC columns 2 and 3 aimed at adsorption of ubiquitous tissue antigens and decidual antigens appear to have been effective in removing a number of proteins, many of them different to those removed by NAAC column 1, but also led to further removal of HPL and SP1 from the placental extract. The recovery of placental heat-stable alkaline phosphatase from the three column adsorption process was also low. However trace amounts of heat-stable placental alkaline phosphatase, HPL and SP1 have been found in amniotic fluid (see Chapter 1, Section 3.1; Sutcliffe, 1975) and it is therefore conceivable that antibodies to these proteins were present on the NAAC column 3. As has been previously stated, the recovery of individual

proteins could be governed by a number of different factors and it is therefore not possible to determine with certainty the reasons for the relatively low recoveries of placental proteins observed or to predict the fate of other placental proteins during adsorption over the different NAAC columns.

3. RESPONSES TO IMMUNISATION WITH THE PLACENTAL SOLUBLE EXTRACT AND NAAC PRODUCTS OF PLACENTAL SOLUBLE EXTRACT

3.1 Introduction

This section presents the results obtained when the placental soluble extract and the NAAC products of placental soluble extract were injected into rabbits and the antisera analysed by AACE.

3.2 Materials and Methods

Sera and Antisera

See Section 2.2 of this chapter.

Preparation of antisera to the soluble extract of placenta

Two aliquots of placental soluble extract used in the experiments described in Section 2 of this chapter, containing 20 mg. of protein each, of which 18% was serum albumin and 43% was haemoglobin, were lyophilised, dispersed in 1 ml. of Freund's complete adjuvant each and used to immunise two New Zealand white rabbits (rabbits 1 and 2).

10 mg. of protein in Freund's complete adjuvant was used to boost each rabbit four weeks later.

Preparation of Antisera to the NAAC 1 Product of Placental Soluble Extract

Four aliquots of the NAAC 1 product from experiment 3

(see Table 3.1) each containing 110 mg. of protein of which 50•9% was haemoglobin were lyophilised, dipersed in 1 ml. Freund's complete asjuvant each and used to immunise four New Zealand white rabbits (rabbits 3,4,5 and 6).

70 mg. of protein from the NAAC 1 product from experiment 4 (Table 3.1) in Freund's complete adjuvant was used to boost each rabbit four weeks later.

Preparation of Antisera to the NAAC 3 Product of Placental Soluble Extract

Two aliquots of the NAAC 3 product from experiment 1 (Table 3.1) containing 30 mg. of protein of which 72% was haemoglobin were lyophilised, dispersed in Freund's complete adjuvant and used to immunise two New Zealand white rabbits (rabbits 7 and 8).

30 mg. of protein from the NAAC 3 product from experiment 2 (Table 3.1) of which 0•03% was albumin and 68% was haemoglobin was used to boost each rabbit four weeks later.

Antisera were obtained from rabbits on the seventh day following boosting. Aliquots of antisera were adsorbed in solution with pooled adult serum. Aliquots of antisera raised to the NAAC 1 product were in addition adsorbed with protein from soluble extract of lung which had been polymerised with a final concentration of 0•4% glutaraldehyde and then dispersed into fine beads by homogenisation and washing in PBS (Avrameas and Ternynck, 1969).

Analysis of Antisera

Unadsorbed as well as adult serum-adsorbed antisera were tested on two-dimensional AACE with:

(1) Pooled adult serum.

(2) Pooled maternal serum.

(3) Placental soluble extract.

(4) Adult lung soluble extract.

Adult serum-adsorbed antisera were also tested on immunoelectrophoresis with:

(1) Pooled maternal serum

and (2) Placental soluble extract.

Adult lung-adsorbed antisera to the NAAC 1 product were tested on two-dimensional AACE with:

(1) Placental soluble extract

and (2) Adult lung soluble extract.

Adult serum-adsorbed antisera were tested for cross-reactivity with:

(a) Anti-HPL, anti-SP1 and anti-PAPP-A antisera using pooled maternal serum as the source of antigens

and (b) Anti-human ferritin and anti-adult haemoglobin antisera using placental soluble extract as the source of antigens.

Antigens in pooled maternal serum and in placental soluble extract reacting with the antisera to placental soluble extract were also tested for cross-reactivity.

3.3 Results

Table 3.2 shows the number of AACE precipitation arcs formed by rabbit antisera against placental soluble extract with antigens from different sources. In a number of cases the AACE curves formed by the antisera were faint or overlapped with each other and the exact number present could not be determined with certainty. The minimum number of arcs counted is indicated in such cases.

It was found that complete adsorption of antisera raised in

TABLE 3.2

Responses to Immunisation with Placental Soluble Extract and NAAC Products of Placental Soluble Extract

Source of Antigens in AACE	Rabbit Antisera Tested on AACE								
	Anti-placental soluble extract			Anti-NAAC 1 product of placental soluble extract			Anti-NAAC 3 product of placental soluble extract		
	Rabbit	Number of precipitin arcs	Antigen identities	Rabbit	Number precipitin arcs	Antigen identities	Rabbit	Number precipitin arcs	Antigen identities
Adult Serum[a]	1, 2	10 or more	ND	3	(1)[c]	ND	7, 8	3(+1)[c] or more	ND
				4, 5, 6	3 (+1)[c] or more	ND			
Maternal Serum[b]	1	1	HPL	3	1	PAPP-A	7	2(+1)[e]	HPL, PAPP-A, (Agb[e])
	2	2	HPL, SP1	4, 5	2	PAPP-A, HPL	8	1	HPL
				6	2	PAPP-A, SP1			
Placental Soluble Extract[b]	1	2	Hb, HPL	3	1[d]	Hb.	7	3[d](+1)[e]	Hb, HPL, Aga, (Agb[e])
	2	3	Hb, HPL, SP1	4, 5, 6	9 or more	Hb, ferritin, rest ND	8	4	(Hb, HPL, (ferritin, Aga
Lung Soluble Extract[b]	1, 2	1	Hb.	3		Hb.	7	1	Hb.
				4, 5, 6	5 or more	Hb, ferritin, rest ND	8	2	Hb, ferritin

a tested with unadsorbed antisera
b tested with adult serum-adsorbed antisera
c incomplete arc ND = not determined
d PAPP-A was not easily detected in placental soluble extract
e Agb was detected on immunoelectrophoresis, but not on AACE: for details see text

rabbits 4, 5 and 6 to the NAAC product of placental soluble extract with solid-phase lung protein frequently could not be accomplished in a single step and the antisera had to be readsorbed several times. This led to a dilution of the antibody titre and the resultant precipitin arcs produced on AACE were very faint. Fully adsorbed antisera formed a small number of precipitin arcs on AACE with placental soluble extract.

Antibodies to an antigen (antigen a) found in placental soluble extract but not in pooled maternal serum nor lung soluble extract which did not cross-react with HPL, SP1 or PAPP-A were produced by rabbits 7 and 8 immunised with the NAAC 3 product of placental soluble extract. The arc formed by the antigen on two-dimensional AACE was large and encircled the area in the antibody bed from behind the antigen well to just behind anodal end of the arc formed by ferritin (Figure 3.3). Its electrophoretic mobility was found to be between that of haemoglobin (Beta-1) and ferritin (alpha-2). Figure 3.3 shows also the AACE arcs produced by rabbit 8 antiserum with lung soluble extract and with placental soluble extract and lung soluble extract applied to the same antigen well. The two antigens in common with the extracts of placenta and lung were found to cross-react with haemoglobin and ferritin on double immunodiffusion: the other antigen of alpha-2 mobility found only in placental soluble extract was found to cross-react with HPL.

When antiserum to the NAAC 3 product of placental soluble extract produced by rabbit 7 was analysed on immunoelectrophoresis the presence of an additional specificity against an antigen (antigen b) in pooled maternal serum and in placental soluble extract was revealed (Figure 3.4). The antigen b system required at least 24 hours for development. The electrophoretic mobility of antigen b was much lower

than that of SP1 (Beta-1) analysed on the same immunoelectrophoretic plate. The only other arc formed on immunoelectrophoresis by rabbit 7 antiserum with both pooled maternal serum and placental soluble extract was formed by an antigen with electrophoretic mobility similar to that of HPL (alpha-2) tested in parallel. With placental soluble extract an arc corresponding in position to the precipitin arc formed by haemoglobin was also detectable. Anti-antigen b specificity was not detected on the same immunoelectrophoretic plate in the antiserum produced by rabbit 8. When rabbit 7 antiserum was tested on a separate immunoelectrophoretic plate with pooled adult serum and lung soluble extract no arcs were formed with the former and a single arc corresponding in position to haemoglobin was formed with the latter.

Figure 3.5 shows the reaction of the following unadsorbed antisera with pooled adult serum:

(1) anti-placental soluble extract antiserum raised in rabbit 1

and (2) anti-NAAC 3 product of placental soluble extract raised in rabbit 7.

Figure 3.6 shows the reaction of adult-serum adsorbed antisera to NAAC 1 product of placental soluble extract raised in rabbits 4, 5 and 6 with placental soluble extract. Figure 3.7 shows cross-reactivity of antiserum to NAAC 1 product of placental soluble extract raised in rabbit 3 with rabbit antiserum to NAAC 1 product of term umbilical cord serum raised independently in our laboratory, which in turn shows reactions of identity with anti-PAPP-A antiserum raised by Lin and Halbert.

3.4 Discussion/

3.4 Discussion

The data summarised in Table 3.2 suggest consistent differences in the response of rabbits to adult serum protein, maternal serum protein, placental and ubiquitous tissue protein between groups immunised with different placental preparations and assayed by gel precipitation methods, despite some differences in the responses of individual rabbits.

The very limited response to adult serum proteins produced by those rabbits immunised with NAAC 1 and the NAAC 3 products of placental soluble extract contrasts with the much stronger and more extensive anti-adult serum response produced by rabbits immunised with unfractionated placental extract. These findings suggest that the removal of adult serum protein by NAAC column 1 had been effective but not complete and subsequent adsorptions over NAAC columns 2 and 3 did not remove the remaining adult serum specificities. The incomplete AACE arc produced by the antisera to the NAAC 1 and the NAAC 3 products may indicate partial denaturation of protein during the fractionation procedure.

Precipitating antibodies to the placental proteins found in maternal serum - HPL and SP1 - were produced by some of the rabbits within those groups immunised with unfractionated placental extract and NAAC 1 product. Antibodies to HPL but not SP1 were also produced by rabbits immunised with NAAC 3 product.

However the most significant difference in the response to proteins in maternal serum observed with different groups of rabbits was the production of anti-PAPP-A antibodies by all the rabbits immunised with the NAAC 1 product and one of the two rabbits immunised with the NAAC 3 product but not by the two rabbits immunised with unfractionated

placental extract. Response to PAPP-A was induced independently in our laboratory by the immunisation of rabbits with both term umbilical cord serum and term maternal serum fractionated over the NAAC column 1 (Sutcliffe, 1979). The recoveries of PAPP-A in maternal serum fractionated over the NAAC 1 column were found to be consistently higher than either that of HPL or SP1 (Sutcliffe et al., 1979). Recoveries of both HPL and SP1 in placental soluble extract fractionated over the NAAC column 1 were also found to be low and further adsorption over the NAAC columns 2 and 3 resulted in still smaller recoveries of HPL and SP1. The low recovery of SP1 may partly account for the absence of anti-SP1 antibodies in rabbits immunised with the NAAC 3 product.

On the basis of the findings presented here as well as those reported by Sutcliffe et al. (1979) it may be concluded that adsorption of adult serum from PAPP-A -containing preparations on NAAC columns is effective in inducing anti-PAPP-A response in rabbits immunised with the NAAC product. Response to tissue antigens other than ferritin was only detected in antisera produced by three out of four rabbits immunised with NAAC 2 product: however many of the antigens were also detectable in adult lung soluble extract and only a few remained detectable in placental soluble extract after adsorption of antisera with solid-phase lung tissue protein. These results show that immunisation of rabbits with placental soluble extract from which contaminating adult serum protein had been adsorbed over a NAAC column induced only a limited response to antigens found in placental tissue but not in adult lung tissue.

Absence of any detectable response to ubiquitous tissue protein greatly simplified analysis of the two antisera raised to the NAAC 3 product and enabled detection on AACE of an antigen (antigen a;

Figure 3.3) in placental soluble extract undetectable in maternal serum or in lung soluble extract. Another unidentified antigen (antigen b; Figure 3.4) was detected on immunoelectrophoresis in both placental soluble extract and maternal serum with one of the two anti-NAAC 3 antisera. These findings suggest usefulness of NAAC fractionation methodology combining removal of adult serum protein with removal of tissue and decidual protein in the search for placental-specific antigens. However, this conclusion is tentative as further studies to determine the nature and specificity of the two antigens detected with antisera to the NAAC 3 product have not been possible due to lack of antisera.

Two factors preclude the drawing of generalised conclusions as to the usefulness of different forms of NAAC technology in producing immunogens capable of evoking specific immune response in rabbits : (1) observed variations in the response of individual rabbits to immunisation with the same immunogen and (2) the use of gel precipitation to assay the response. This method cannot detect antibodies against monovalent antigens and may also not detect antibodies against antigens present at a very low concentration in maternal serum or in tissue extracts. Nevertheless, the production of anti-PAPP-A specificities by all the rabbits immunised with the NAAC products of placental soluble extract provides a clear example of the value of NAAC technology. Preliminary studies carried out by Lin and his associates have indicated that this protein is specific for the placenta (see Chapter 1, Section 3.1). Specific antisera to PAPP-A were raised independently in our laboratory by immunising rabbits with NAAC products of term umbilical cord serum and maternal serum. Thus a pool of anti-PAPP-A antisera became available and enabled me to make

PAPP-A a subject of further studies described in subsequent chapters.

FIGURE 3.2

SDS Slab Gel Electrophoresis of:

(1) Placental soluble extract

(2) Adult male serum

(3) Gamma-fraction of rabbit antisera to adult male serum

(4) NAAC 1 product from Experiment 1

(5) NAAC 1 product from Experiment 2

(6) NAAC 3 product from Experiment 1

(7) NAAC 3 product from Experiment 2

(8) Adult haemoglobin.

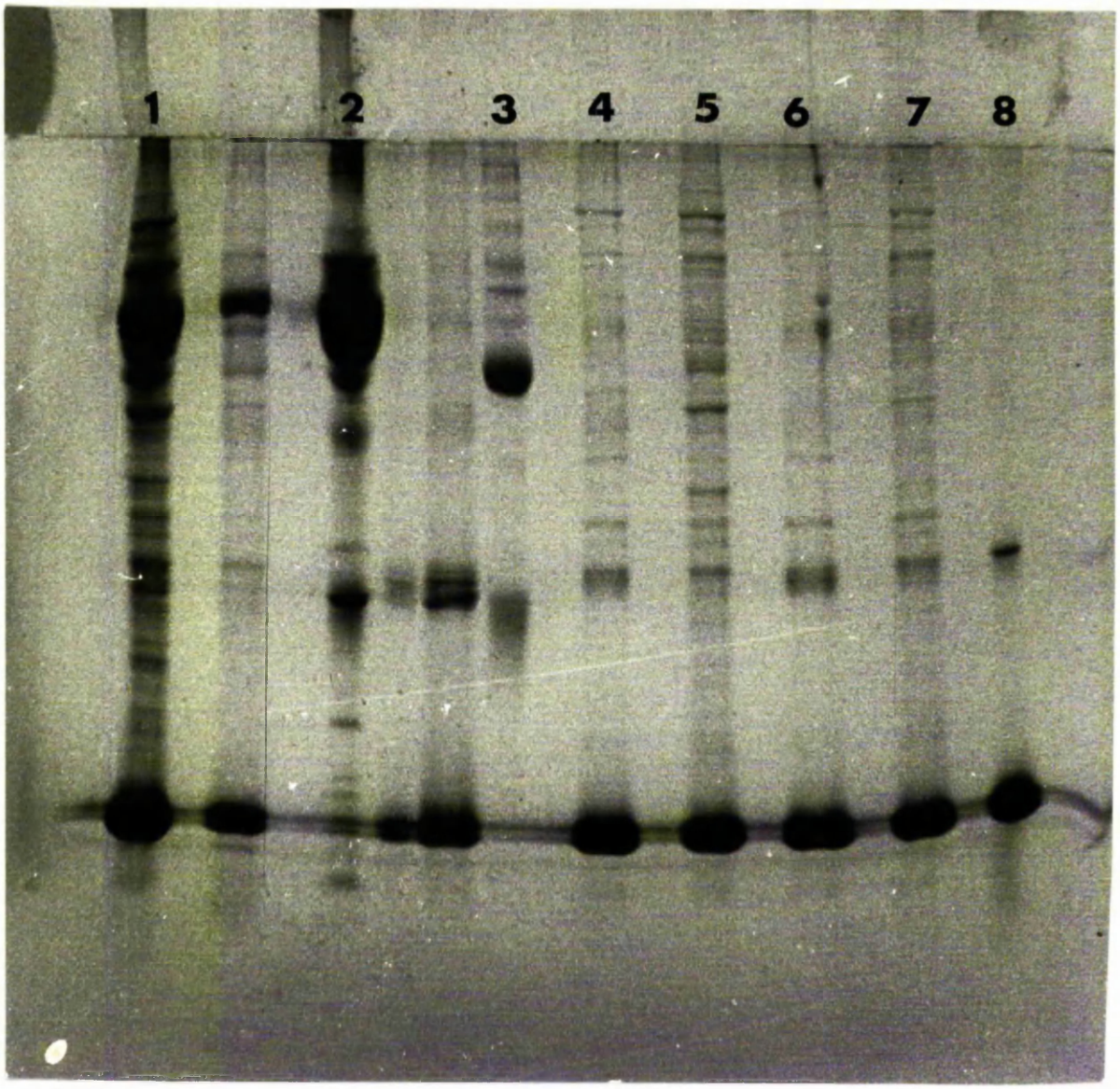

1
2
3
4
5
6
7
8

FIGURE 3.3 Two-Dimensional Antibody-Antigen Crossed Electrophoresis Showing Antigen a.

Here, as in Figures 3.4, 3.6 and 3.7, the ovoid antigen wells are to the left of each run and the circular mark to the right of each antigen well represents the mobility of albumin in the first dimension electrophoresis.

The antiserum is adult serum-adsorbed anti-NAAC 3 product of placental soluble extract raised in rabbit 8. The concentration of antiserum is 20%.

The antigens are:

(1)6 microlitre placental soluble extract at protein concentration of 30 mg. per ml.

(2) Adult lung soluble extract at protein concentration of 30 mg. per ml.

(3) Addition of (1) and (2).

a = precipitin arc formed by antigen a (see Table 3.2)

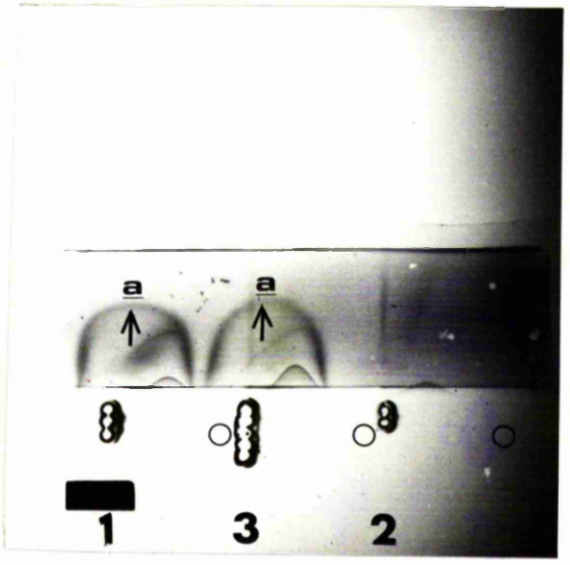

a
a
1
3
2

Figure 3.4

Immunoelectrophoresis Showing Antigen b.

The circular mark above each marked antigen well represents the electrophoretic mobility of albumin.

The antisera are:

(a) Anti-HPL

(b) Adult serum adsorbed anti-NAAC 3 product of placental soluble extract raised in rabbit 8

(c) Adult serum adsorbed anti-NAAC 3 product of placental soluble extract raised in rabbit 7

(d) Anti-adult haemoglobin

(e) Anti-SP1.

The antigens are:

(1) 12 microlitre pooled maternal serum at protein concentration of 68 mg. per ml.

(2) 12 microlitre placental soluble extract at protein concentration of 35 mg. per ml.

Development time: 24 hours.

b = precipitin arcs formed by antigen b (see Table 3.2).

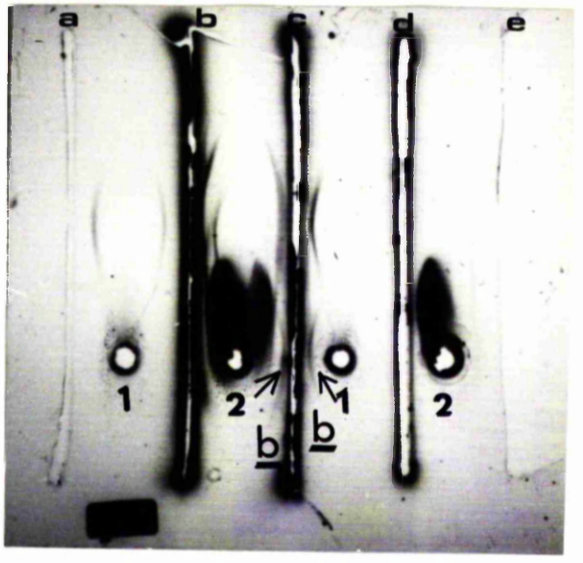

a
b
c
d
e
1
2
b
1
2

FIGURE 3.5 One- and Two-Dimensional Antibody-Antigen Crossed Electrophoresis Showing Serum Antigens Detectable with Unadsorbed Rabbit Antisera to (i) Placental Soluble Extract and (ii) NAAC 3 Product of Placental Soluble Extract.

I Shows the reaction of 10 microlitre pooled adult serum with unadsorbed antiserum against placental soluble extract raised in rabbit 1 on:

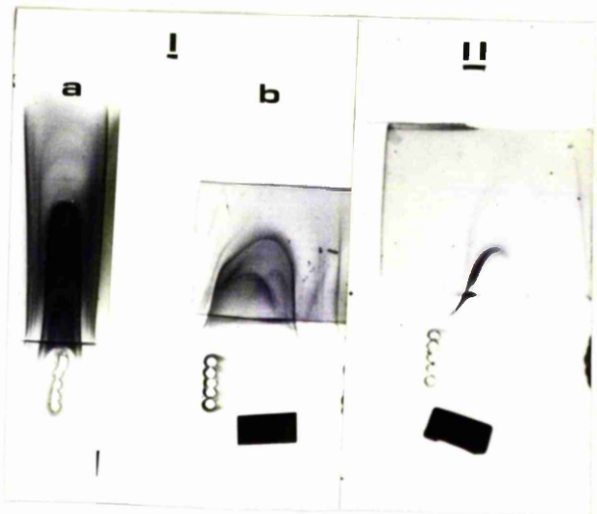
(a) One-dimensional

and (b) Two-dimensional AACE.

II Shows the reaction of 10 microlitre pooled adult serum with unadsorbed antiserum against NAAC 3 product of placental soluble extract raised in rabbit 7.

The concentration of antisera in I and II is 20%.

The protein concentration of pooled adult serum is 69 mg. per ml.


I
II
a
b

c- Immunofluorescence Assay for Detection of Antibody-Antigen Complexes

Immunofluorescence is a technique that uses fluorescent dyes to detect the presence of antibodies or antigens in a sample. The basic principle of immunofluorescence is that a fluorescent dye is attached to an antibody or antigen, and then the sample is exposed to light. If the antibody or antigen is present in the sample, it will bind to the fluorescent dye and emit light.

There are two main types of immunofluorescence assays: direct and indirect. In a direct immunofluorescence assay, the fluorescent dye is attached to the antibody that is specific for the antigen of interest. In an indirect immunofluorescence assay, the fluorescent dye is attached to a secondary antibody that binds to the primary antibody.

Immunofluorescence assays are used in a variety of applications, including:

- Diagnosing infectious diseases
- Detecting autoimmune diseases
- Identifying cancer cells
- Studying the immune system

Immunofluorescence assays are a powerful tool for detecting antibodies and antigens in a sample. They are relatively easy to perform and can be used to detect a wide range of antibodies and antigens.

FIGURE 3.6 Two-Dimensional Antibody-Antigen Crossed Electrophoresis Showing Placental Antigens Detectable with Serum-Adsorbed Rabbit Antiserum to NAAC 3 Product of Placental Soluble Extract.

(a), (b) and (c) show the reaction of 10 microlitre placental soluble extract with adult serum adsorbed antisera against NAAC 3 product of placental soluble extract raised in rabbits 4, 5 and 6 respectively.

The concentration of each antiserum is 20%.

The protein concentration of placental soluble extract is 103 mg. per ml.

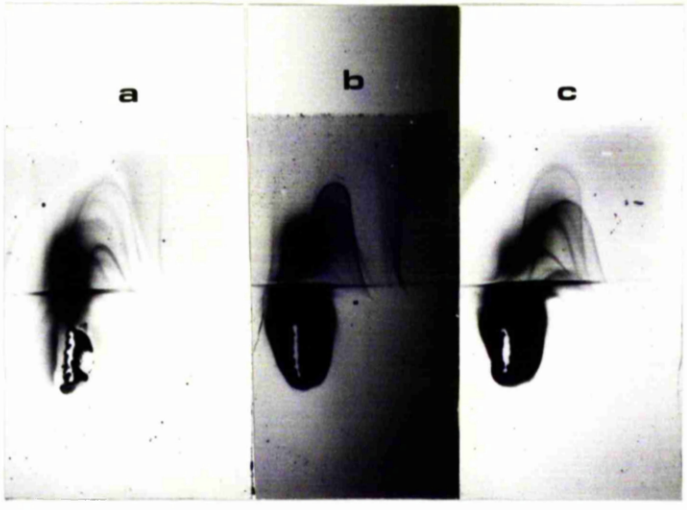

a
b
c

FIGURE 3.7 I. Two-Dimensional Antibody-Antigen Crossed Electrophoresis Showing Cross-Reactivity Between Rabbit Antiserum to NAAC 1 Product of Placental Soluble Extract and Rabbit Antiserum to NAAC 1 Product of Term Umbilical Cord Serum (Sutcliffe et al. 1976).

II. Double Immunodiffusion Showing Cross-Reactivity Between Rabbit Antiserum to NAAC 1 Product of Term Umbilical Cord Serum (Sutcliffe et al. 1976) and Rabbit Anti-PAPP-A Antiserum of Lin and Halbert.

I Two-Dimensional Antibody-Antigen Crossed Electrophoresis.

The antisera are:

(a) Adult serum-adsorbed anti-NAAC 1 product of placental soluble extract raised in rabbit 3

(b) Adult serum-adsorbed anti-NAAC 1 product of term umbilical cord serum (Sutcliffe et al., 1976)

(c) Addition of (a) and (b).

The concentration of each antiserum is 10%.

Antigen (1) is 10 microlitre pooled maternal serum at protein concentration of 68 mg. per ml.

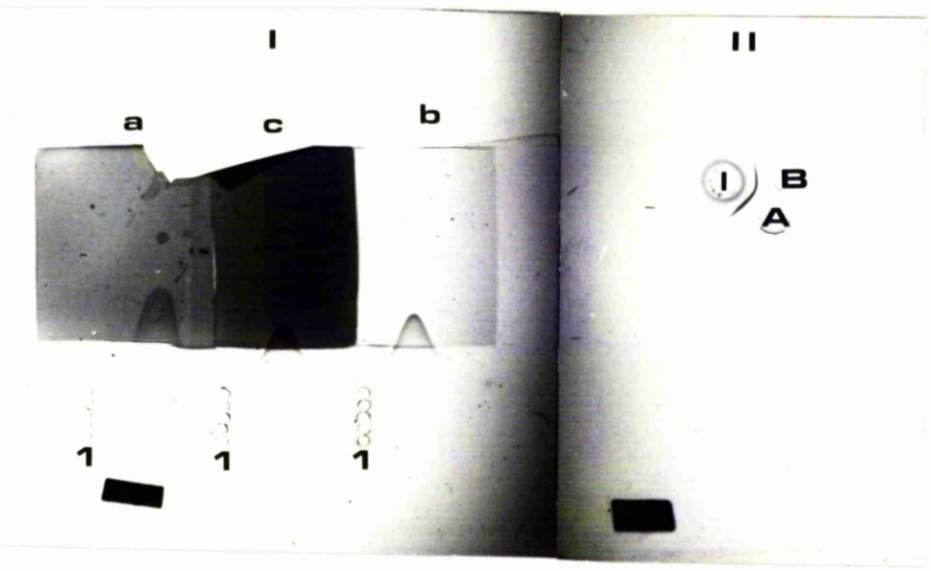
II Double Immunodiffusion

Antisera are:

(a) Adult serum adsorbed anti-NAAC 1 product of term umbilical cord serum (Sutcliffe et al., 1976)

(b) Anti-PAPP-A (Lin and Halbert).

Antigen (1) is 10 microlitre pooled maternal serum at a protein concentration 68 mg. per ml.


I
II
a
c
b
I
B
A
1
1
1

CHAPTER 4 THE SENSITIVITY OF PREGNANCY-ASSOCIATED AND SERUM PROTEINS TO DISSOCIANTS OF ANTIBODY-ANTIGEN COMPLEXES

1. INTRODUCTION

The studies described in this chapter were designed to establish how the use of antibody affinity chromatography might be extended to high molecular weight serum proteins. Antibody affinity purification might in their case be difficult since it requires dissociation of the antigen-antibody complex without denaturation of the antigen *per se*, which, being of a large molecular weight, is likely to be a complex structure of subunits and domains. Recently a number of high molecular weight protein antigens have been reported to occur in pregnancy sera - for example, PAPP-A, PAPP-B, PZP, PP6, SP1; see Chapter 1, Section 3.1 - and there is a need to assess their sensitivity to dissociants of antigen-antibody complexes before attempting to purify them by affinity chromatography. However, since little is known about the quaternary structure and biological function of these pregnancy proteins the assessment of their sensitivity to dissociants of antigen-antibody complexes depends largely upon the gel precipitation assays which were originally used to define them (i.e. double immune diffusion and antibody-antigen crossed electrophoresis).

An examination was made of the susceptibilities of a sample of large and average-sized serum proteins - including certain pregnancy proteins - to several dissociation agents commonly used in affinity chromatography to disrupt non-covalently bonded antibody-antigen complexes. The dissociation agents included in this study were: the chaotropic ions - thiocyanate and iodide, each at 3 M, 2·5 M, 2 M and 1·5 M concentrations (Dandliker *et al.*, 1967; Dandliker *et al.*, 1968; de Saussure and Dandliker, 1969; Avrameas and Ternynck, 1969), 5 M guanidine-HCl (Dandliker *et al.*, 1968), 8 M urea (Slobin and Sela, 1965),

pH 2·8 glycine-HCl buffer (Avrameas and Ternynck, pH 11 glycine-NaOH buffer (modified from Omenn et al., 1970, see also Hoag et al., 1975). After treatment with a dissociating agent the extent of antigen denaturation was assessed by changes in the antigen's precipitating behaviour on one-dimensional antibody-antigen crossed electrophoresis (AACE). The results of these studies were then applied to the partial purification of pregnancy associated plasma protein A (PAPP-A; molecular weight 750,000; Lin et al., 1974b) by antibody affinity chromatography.

2. MATERIALS AND METHODS

Human Materials

Pools of serum collected from ten normal pregnant women at 38-42 weeks' gestation were used as sources of antigens for AACE and for affinity chromatography. Uterine decidua from a termination of pregnancy of 11 weeks' gestation was homogenised in PBS to a final protein concentration of 0·72 mg. per ml. and used as a source of alpha-uterine protein (AUP) (Sutcliffe et al., 1978).

Antisera

Rabbit anti-PAPP-A, anti-steroid-binding globulin (SP2) (Bohn, 1974d) and anti-AUP antisera were prepared as previously described (Sutcliffe, 1976; Sutcliffe et al., 1978). The anti-PAPP-A antiserum showed reactions of identity with that raised by Lin and Halbert (personal communication) (Fig. 4.1). Rabbit antisera against alpha-1-lipoprotein, alpha-2-macroglobulin, beta-1-lipoprotein, plasminogen, pregnancy-specific beta-1-glycoprotein (SP1), albumin and human placental lectogen (HPL) were purchased from Behringwerke AG, W. Germany. Rabbit antiserum specific for pregnancy zone protein (PZP) was kindly donated

by Drs. Lin and Halbert.

Preparation of Dissociation Agents

The final concentrations of the dissociation agents to which the human proteins were exposed were as follows:

(i) KSCN-PBS and KI-PBS, each at 3 M, 2•5 M, 2 M and 1•5 M.

(ii) 5 M guanidine-HCl-PBS.

(iii) 8 M urea-PBS.

(iv) 0•2 M glycine-HCl pH 2•8.

(v) 0•2 M glycine-NaOH pH 11.

These solutions were prepared as 2-fold concentrates for subsequent 2-fold dilution in the experiments except in the case of guanidine-HCl which was prepared as a 7•5 M solution, urea which was 10•66 M and KI which was 5 M.

Except for the low and high pH buffers the pHs of all solutions were adjusted to neutrality. The pH of the pH 2•8 and pH 11 buffers was altered to pH 2•5 and pH 11•4 respectively in order to produce the desired pH when added to the serum sample which acted as the source of the antigen to be tested.

Treatment of Proteins with Dissociation Agents

Into separate 1 volume aliquots of maternal serum were vigorously mixed equal volumes of the individual dissociation solutions except in the case of the control aliquot, into which was added 1 volume of PBS. The exceptions in this procedure were the addition of 1•5 volumes of 5 M KI (toyield 3 M KI), the addition of 2 volumes of guanidine-HCl and 3 volumes of 10•66 M urea. All serum samples treated with KSCN, KI and 5 M guanidine-HCl were incubated for 1 hour at 18 deg. Centigrade to avoid crystallisation. Samples in pH 2•8 and pH 11 buffers were neutralised with pretitrated volumes of NaOH and HCl respectively after

30 minutes' incubation at 4 deg. Centigrade. In addition, one sample in pH 2•8 buffer was neutralised after 15 minutes incubation at 4 deg. Centigrade. The volume of each sample was adjusted to four times the original serum volume with PBS at the end of the incubation period, dialysed for 18 hours against three changes of PBS and tested for the presence of intact antigen on one-dimensional AACE.

Parallel studies on human AUP were carried out in the same way using homogenised decidua as source material.

Immunoelectrophoretic Methods

Double immunodiffusion was performed according to Ouchterlony (1968) and allowed to proceed for 24 hours. One-dimensional AACE (antibody-antigen crossed electrophoresis) according to Laurell (1966; 1972) was carried out in 1% agarose gels. The treated samples , with controls on each plate, were subjected to electrophoresis for up to 15 hours at 1•5 Volts per cm. After electrophoresis plates were washed, dried and stained with Coomassie Brilliant Blue.

Polyacrylamide Tube Gel Electrophoresis

Polyacrylamide tube gel electrophoresis was carried out in 10% acrylamide using a tris-glycine tank buffer pH 8•9 (Maizel, 1971).

Preparation and Use of Antibody Affinity Column for PAPP-A

Activation and substitution of Sepharose 4B were performed according to the method of Porath et al. (1976).

Rabbit anti-PAPP-A serum was first adsorbed by passage over a column of Sepharose coupled with human adult serum protein until no anti-adult serum antibodies were detectable by AACE. The immunoglobulin G (IgG) fraction was prepared by DEAE ion exchange chromatography (Morris and Morris, 1976) and coupled to Sepharose 4B. The IgG-Sepharose (5 ml. in PBS) was then packed into a 1•5 cm. diameter chromatography column,

any residual protein-reactive groups were blocked with 0•1 M ethanolamine pH 8•0 and non-covalently bound protein was eluted as recommended by Pharmacia. This anti-PAPP-A column was then linked to a 1•5 cm. diameter column containing 25 ml. Sephadex G25 in PBS and the eluate from the G-25 column was monitored with a Uvicord II.

Antibody affinity chromatography was performed at 4 deg. Centigrade using 10 ml. of pooled maternal serum as the source of PAPP-A. The serum was washed through the column with PBS (approx. 40 ml.) until the eluate from the G-25 column gave a zero reading at 280 nm. The column was then desorbed using 4 ml. of 2 M KI-PBS pH 7 as dissociating agent. The desorbed material was rapidly separated from the KI by filtration in PBS on Sephadex G25 and the resultant fractions were assayed for PAPP-A by AACE.

Preparation of Antiserum to Affinity-Purified PAPP-A

PAPP-A prepared by affinity chromatography from 10 ml. of pooled maternal serum was subjected to polyacrylamide tube gel electrophoresis. Each tube gel was then sliced into 60 pieces, eluted with 100 microlitres PBS and tesyed for the presence of PAPP-A by AACE. Fractions containing PAPP-A were pooled and lyophilised. One half of the lyophilised material was dissolved in 1 ml. Freund's complete adjuvant and used to immunise a rabbit. The other half in Freund's incomplete adjuvant was used as a booster 4 weeks later. Antiserum was obtained on the 7th day following boosting. Adsorption of antisera was carried out using normal adult human serum which had been polymerised with a final concentration of 0•4% glutaraldehyde (BDH) and then dispersed into fine beads by homogenisation and washing in PBS (Avrameas and Ternynck, 1969).

3./

3. RESULTS

Effect of Dissociating Agents

The effects of dissociating agents on AACE precipitin arc formation by the proteins ranged from no apparent modifications of normal arc morphology through various degrees of alteration of normal up to a total loss of precipitation arcs. The various types of altered arcs fell into the following fairly discrete categories:

(a) Arcs split into a 'baseline' precipitate and a second blurred arc or trail.

(b) Arcs ending in a blurred trail.

(c) Flying arcs.

(d) Tail arcs.

and (e) Blurred arcs.

These categories are represented in Table 4.1 by the letters a to e (for examples, see Fig. 4.2). Letters in parenthesis indicate that a less extreme alteration in arc morphology was observed but that the pattern could still be classified according to the above scheme. Where no alteration in the shape of the precipitin arc was detected (designated "+") the height of the arc was the same as that of the control sample run on the same AACE plate. From Table 4.1 it is evident that increases in the strength or the duration of the treatment result in larger alterations in precipitin arc morphology.

When the effect of dissociating agents on beta-1-lipoprotein and alpha-2-macroglobulin was in addition assessed by double immunodiffusion (Fig. 4.3), loss of the precipitating ability was observed where it had also been indicated by AACE. However, where a slight or gross alteration of precipitating behaviour had been detected by AACE,

TABLE 1

Effect of dissociating agents on precipitin arc formation[a] by proteins as tested and compared by AACE

Protein	Molecular Weight	Dissociation agent: KCSN 3M	KCSN 2.5M	KCSN 2M	KCSN 1.5M	KI 3M	KI 2.5M	KI 2M	KI 1.5M	5M Guanidine -HCl	8M Urea	pH 2.8 15 min	pH 2.8 30 min
β_1-lipoprotein	$2.75\text{-}4.8\times10^6$ [b]	a	(a)	+	+	+	+	+	+	d,e,c	±[x]	(a)	a
α_2-macroglobulin	7.8×10^5 [c]	a	a	a	+	b	(b)	+	+	NR	d,e[y]	(a)	a
pregnancy associated plasma protein-A (PAPP-A)	7.5×10^5 [d]	NR	c,e	(e)	+	c,e	(e)	+	+	NR	NR	NR	NR
pregnancy zone protein (PZP)	3.6×10^5 [e]	c,e	c,e	(e)	(e)	(e)	(e)	(e)	+	NR	NR	c,e	NR
α_1-lipoprotein	$1.75\text{-}3.6\times10^5$ [b]	+	+	+	+	+	+	+	+	b	b	+	(b)
plasminogen	9.2×10^4 [f]	(b)	(b)	(b)	(b)	(b)	(b)	(b)	+	b	(b)	(b)	b
pregnancy-specific β_1-glycoprotein (SP1)	$9.0\pm0.5\times10^4$ [g]	b	(b)	(b)	(b)	(b)	(b)	+	+	b	(b)	(b)	b
albumin	6.6×10^4 [h]	+	+	+	+	+	+	+	+	+	+	+	+
sex hormone-binding globulin (SP2)	6.5×10^4 [i]	e	(e)	(e)	+	(e)	+	+	+	NR	NR	(e)	NR
α-uterine protein (AUP)	5.0×10^4 [j]	+	+	+	+	+	+	+	+	+	+	+	+
human placental lactogen (HPL)	2.0×10^4 [k]	+	+	+	+	+	+	+	+	+	+	+	+

[a] + no alteration of precipitin arc characteristics
(letters): slight alteration of precipitin arc characteristics
letters: gross alteration of precipitin arc characteristics
NR no precipitin arc formed
Details of altered arc morphologies (denoted by letters a,b,c,d,e).
a arc split into 'base-line' precipitate and a second blurred arc or trail
b arc ending in a blurred trail
c flying arc
d tall arc
e blurred arc

[x] a-type arc formed at pH 8.8
[y] No reaction at pH 8.8

[b] Scanu et al., (1975)
[c] Frénoy et al., (1977)
[d] Lin et al., (1974b)
[e] Bohn & Winckler (1976)
[f] Sjöholm et al., (1973)
[g] Bohn (1974b)
[h] Peters (1975)
[i] Bohn (1974a)
[j] Sutcliffe et al., (1978)
[k] Friesen (1973)

no such alterations could usually be detected by double immunodiffusion, which instead showed evidence of cross-reactivity between the treated and native antigens. One exception was found for alpha-2-macroglobulin. After treatment at pH 2•8 for 30 minutes, this protein formed two precipitin arcs both on AACE and on double immunodiffusion.

Preparation of PAPP-A by Antibody Chromatography

PAPP-A was susceptible to a greater range of dissociating agents than the other proteins in this study (Table 4.1 and Fig. 4.4). It was, however, resistant to 1•5 M KSCN and 1•5 M and 2 M KI. Antibody affinity chromatography followed by G-25 filtration of the 2 M KI-PBS desorbed material resulted in the recovery of desorbed protein which was free of KI. When the PAPP-A eluted from the column was tested by one-dimensional AACE, it gave a precipitin arc which was morphologically indistinguishable from that observed when testing unfractionated maternal serum. The eluted PAPP-A also gave reactions of identity with PAPP-A in unfractionated maternal serum (Fig. 4.1 and Fig. 4.5). Anti-serum raised against this affinity product showed cross-reactivity with antiserum raised to maternal serum PAPP-A (Fig. 4.1).

When 3 M KSCN was substituted for 2 M KI in the affinity chromatography procedure and the fractions containing the KSCN-free eluate were tested for the prescence of PAPP-A on AACE, no precipitin arcs were detected.

4. DISCUSSION

The effectiveness of various dissociating agents has been evaluated by Johnson and Garvey (1977) for the purification of anti-BSA using BSA-Sepharose. They claimed that chaotropic ions at low pH

dissociated maximum reactive antibody. However, these findings cannot be extended to the affinity purification of other antigens without first considering the stability of antigens in such dissociants. In the present study the susceptibility of proteins to denaturation by dissociating agents was assessed by one-dimensional AACE which revealed a variety of minor changes in the morphology and the intensity of the precipitin arcs as well as showing when a total loss of precipitable antigen had occurred. Similar types of altered AACE peaks were observed by Bjerrum and Bog-Hansen (1975) for proteins subjected to controlled enzymatic degradation. The results with double immunodiffusion, although limited, suggest that this technique is a less sensitive means of comparing the antigenicities of native and purified proteins, since it did not reveal most of the minor changes detected by AACE.

As assayed by AACE, all proteins tested proved resistant to pH 11, 1•5 M KI, and in many cases 1•5 M KSCN and 2 M KI. In general the order of effectiveness of different agents in inducing alterations in the antigenicity of susceptible proteins reflected the order found for denaturing ability, that is, guanidine-HCl greater than urea and chaotropic ions (Habeeb, 1977). Also, the effectiveness of chaotropic ions in inducing loss of normal antigenicity increased in the order I^- less than SCN^- which is the order found for the disruption of the tertiary structure of proteins (von Hippel and Wong, 1964). For many of the proteins (Table 4.1) increased concentrations of I^- and SCN^- resulted in more extreme changes in precipitin arc morphology. An increase in the time of exposure to low pH from 15 to 30 minutes led to more extensive denaturation of antigens. In one case (alpha-1-lipoprotein) denaturation only became apparent after exposure to low pH for thirty minutes.

The sample size in Table 4.1 is too small to seek a general

relationship between molecular size and sensitivity to denaturation. However, the data is compatible with the expectation that large size results in increased sensitivity. The results show that the very large glycoproteins - alpha-2-macroglobulin (composed of four dimeric subunits - Roberts and Hall, 1974; Frenoy et al., 1977), PZP (composed of two di-sulphide bonded chains - Bohn and Winckler, 1976) and PAPP-A - were most susceptible to denaturants. Some of the other proteins were found to be susceptible to lesser degrees. Alpha-1-lipoprotein and beta-1-lipiprotein both have complex structures involving several di-sulphide and non-covalently bonded, lipid-associated polypeptide chains (Scanu et al., 1975). However, alpha-1-lipoprotein did not appear to be susceptible to the action of chaotropic ions, which exert their effects mainly by destabilising hydrophobic bonds (Dandliker et al., 1967). This may be because the antigenicity of alpha-1-lipoprotein molecule has been found to be dependent on the presence of polar phospholipid head groups, which may not involve hydrophobic bonding (Chapman and Goldstein, 1976). Three relatively small proteins - albumin, alpha-uterine protein and HPL - were found to be completely resistant to the whole range of denaturants investigated. Albumin is a monomer containing no bound carbohydrate and is exceptional in its conformational adaptability (Peters, 1975). Similarly, HPL is a monomer containing no bound carbohydrate (Friesen, 1973). The structure of AUP is not established but it appears to be a dimer composed of two identical subunits (Sutcliffe et al., 1980).

Considering the detailed alterations observed in morphologies of precipitin arcs, these tended to be typical of the antigen concerned rather than the denaturant employed. The finding of multiple precipitin arcs (types a and b; see Table 4.1) suggest that denaturation may involve the production of more than one class of altered antigen. Although these

alterations could represent different ways in which the original antigen has unfolded (due perhaps to initial antigenic heterogeneity) it might instead reflect different patterns and extents of antigen denaturation after removal of the denaturing conditions.

In all cases where no alteration in the shape of the precipitin arc was detected the height of the arc was the same as that of the control PBS-treated sample, indicating an antigen recovery of approaching 100% with each dissociant. However, the recovery of antigen from affinity chromatography will depend on a number of additional factors, the main one being the avidity of the immobilised antibody used and should therefore be determined with each particular antigen-antibody system for each of the dissociants found to give full antigen recoveries in the AACE test. Where antibodies of high avidity are to be used the highest concentration of a dissociating agent to which the antigen is resistant may well have to be employed.

The most susceptible of the proteins tested, PAPP-A, has previously only been partially purified by physico-chemical methods (Lin et al., 1974a). Application of the present findings to its partial purification for the first time by affinity chromatography resulted in its recovery from the affinity column in an apparently normal state by elution with 2M KI, but not with 3M KSCN, supporting the findings in Table 4.1. Subsequent immunisation of a rabbit with this affinity product yielded antiserum which cross-reacted with an antiserum raised against PAPP-A which had not been exposed to dissociants.

These experiments provide evidence that the sensitivities to denaturation summarised in Table 4.1 can be applied to affinity chromatography. Thus it may be that the purification of SP1 would be better achieved by dissociation with KI rather than by dissociation at

low pH (as used by Bohn et al., 1976). Fitsche and Mach (1977) used 3M NaSCN as the dissociating agent in a study designed to compare the physico-chemical and immunological properties of carcinoembryonic antigens obtained from normal and malignant colon. They studied affinity-purified material by double immunodiffusion and radioimmunoassay. Although carcinoembryonic antigen is a monomer it could be that a milder dissociating agent would have revealed more subtle differences between the carcino-embryonic antigens of normal and malignant tissue. Interestingly, two of the monomers reported in the present study were sensitive to 3 M KSCN (viz: plasminogen and SP1).

The present findings apply to the purification of proteins of unknown quaternary structure and biological function by antibody affinity chromatography for subsequent use in immunoassays or as immunogens for raising specific antisera. The search for new and probably weak antigens may require the use of affinity purification of antigens early in the studies in order to develop sufficient antisera to assay antigens during other, possibly more conventional purification techniques. Under such circumstances and especially if the initial antisera are of non-precipitating type, it may be advisable to choose 1.5 M or 2 M KI-PBS as the dissociating agent.

FIGURE 4.1

Double immunodiffusion plate showing cross-reactivities between:

(i) Rabbit anti-PAPP-A antiserum of Sutcliffe et al. (1976) and rabbit anti-PAPP-A antiserum of Lin and Halbert.

(ii) Rabbit anti-PAPP-A antiserum of Sutcliffe et al. (1976) and rabbit anti-affinity purified PAPP-A antiserum.

and (iii) Maternal serum PAPP-A and affinity purified PAPP-A.

The antisera are:

(a) Anti-PAPP-A (Sutcliffe et al., 1976)

(b) Anti-PAPP-A (Lin and Halbert)

(c) Rabbit anti-affinity purified PAPP-A.

Antigens are:

(1) Pooled maternal serum

(2) Affinity purified PAPP-A

(3) Pooled adult serum

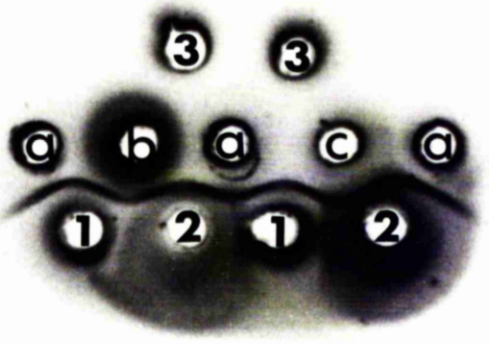

3 3
a b a c a
1 2 1 2

FIGURE 4.2

One-dimensional AACE patterns of native and 3 M KSCN-treated proteins.

The antibody beds are:

(a) Anti-beta-1-lipoprotein

(b) Anti-alpha-2-macroglobulin

(c) Anti-SP1

(d) Anti-PZP.

The antigens are:

(1) Native protein in serum

(2) 3 M KSCN-treated protein

Note the various types of aberrations produced, i.e.

(i) Arc split into 'base-line' precipitate and a second blurred arc or trail (a2,b2)

(ii) Arc ending in a blurred trail (c2)

and (iii) Incomplete 'flying' blurred arc (d2).

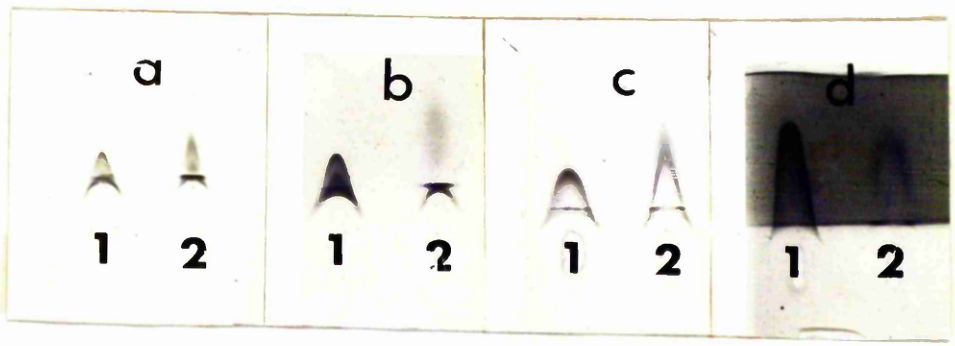

a
b
c
d
1
2
1
2
1
2
1
2

FIGURE 4.3

Double immunodiffusion patterns of Beta-1-lipoprotein and Alpha-2-macroglobulin treated with dissociation agents.

The antisera are:

(a) Anti-Beta-1-lipoprotein

(b) Anti-Alpha-2-macroglobulin.

Antigens are:

(1) Native protein in serum

(2) - (13) Treated proteins. Treatments are:

(2) 3 M KSCN

(3) 2·5 M KSCN

(4) 2 M KSCN

(5) 1·5 M KSCN

(6) 3 M KI

(7) 2·5 M KI

(8) 2 M KI

(9) 1·5 M KI

(10) 5 M guanidine-HCl

(11) 8 M urea, pH 8·8

(12) 0·1 M glycine-HCl, pH 2·8, for 15 minutes

(13) 0·1 M glycine-HCl, pH 2·8, for 30 minutes.

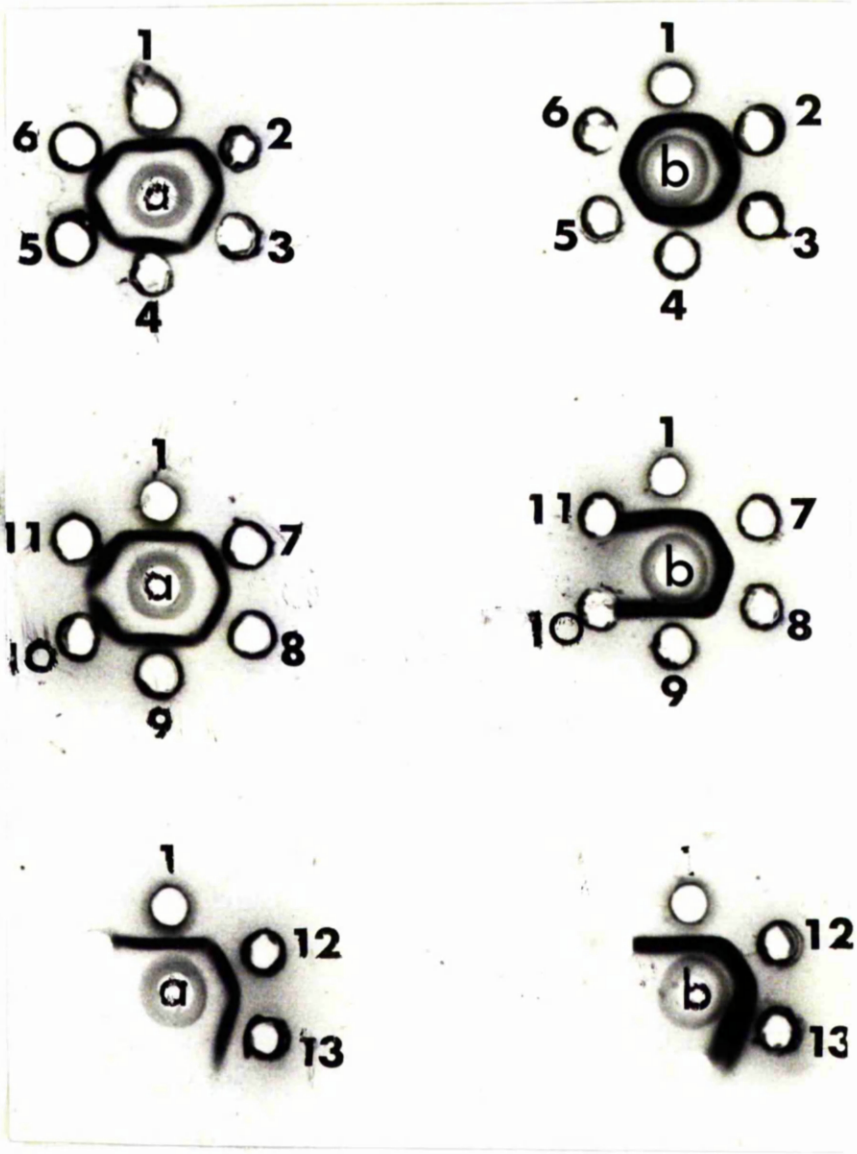

1
6
2
a
5
3
4
1
6
2
b
5
3
4
1
11
7
a
10
8
9
1
11
7
b
10
8
9
1
12
a
13
12
b
13

FIGURE 4.4

One-dimensional AACE patterns of PAPP-A treated with dissociation agents.

The antiserum is anti-PAPP-A (Sutcliffe et al., 1976).

The antigens are:

(1) Native protein in serum

(2) - (15) Treated proteins. Treatments are:

(2) 3 M KSCN

(3) 2·5 M KSCN

(4) 2 M KSCN

(5) 1·5 M KSCN

(6) 3 M KI

(7) 2·5 M KI

(8) 2 M KI

(9) 1·5 M KI

(10) 5 M guanidine-HCl

(11) 8 M urea

(12) 0·14 M mercaptoethanol

(13) 0·1 M glycine-HCl, pH 2·8, for 15 minutes

(14) 0·1 M glycine-HCl, pH 2·8, for 30 minutes

(15) 0·1 M glycine-NaOH, pH 11.

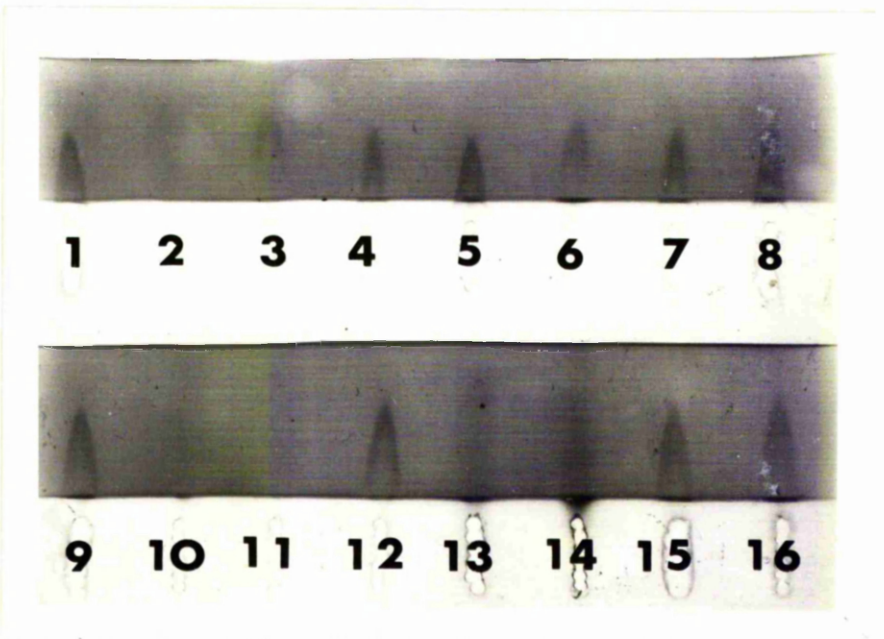

1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16

FIGURE 4.5

One-dimensional AACE patterns of native and affinity-purified PAPP-A. Demonstration of cross-reactivity.

The antiserum is anti-PAPP-A (Sutcliffe et al., 1976)

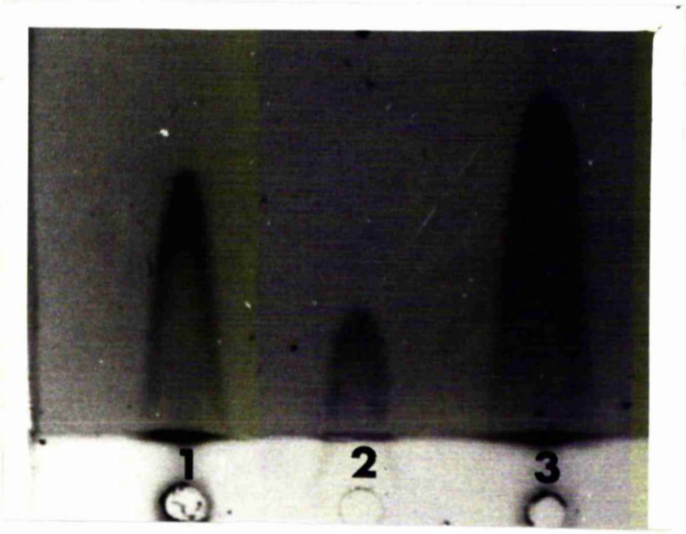
Antigens are:

(1) Native protein in serum

(2) Affinity-purified protein

(3) Addition of (1) and (2).

Note that (3) is a single arc larger than either (1) or (2).


1
2
3

CHAPTER 5 THE PARTIAL PURIFICATION OF PAPP-A BY ANTIBODY AFFINITY CHROMATOGRAPHY

1. THE EFFECT OF TIME OF EXPOSURE TO 1•5 M AND 2 M KI ON THE IMMUNOCHEMICAL REACTIVITY OF PAPP-A

1.1 Introduction

In Chapter 4 the effects of dissociants of antibody-antigen complexes on the immunochemical reactivity of PAPP-A was assessed. PAPP-A was found to be resistant to 1•5 M and 2 M KI and 2 M KI was subsequently used with success in the partial purification of PAPP-A by small-scale affinity chromatography.

In order to assess the suitability of 1•5 M and 2 M KI for large as well as small-scale purification of PAPP-A by affinity chromatography the effect on PAPP-A of exposure to these dissociants for extended periods of time must be known. This is investigated in the present experimental section.

1.2 Materials and Methods

A full description of the experimental method is provided in Chapter 4, Section 2.2. The differences in the present set-up were as follows:

(1) Only two dissociating agents were used - 1•5 M and 2 M KI.

(2) Eight serum samples were treated with individual dissociating agents. Seven of these were incubated for periods of 1 - 7 hours: the remaining sample was incubated for 18 hours.

(3) Following dialysis, samples were only tested for the presence of intact PAPP-A antigen on one-dimensional AACE.

1.3 Results/

1.3 Results

The effects of the dissociating agents on AACE precipitin arc formation by PAPP-A are shown in Table 5.1. Prolonged exposure to 1•5 M KI for periods up to 18 hours failed to produce any alterations in the precipitating behaviour on AACE of PAPP-A. Prolonged exposure to 2 M KI produced alterations which first became apparent at 7 hours exposure.

1.4 Discussion

The extent of the 2 M KI-induced irreversible denaturation of PAPP-A was found to depend on how long it has been allowed to remain under denaturing conditions. This finding is in agreement with the general conclusion reached in Chapter 4 based on observations of the effect of the exposure of various antigens to low pH for periods of 15 and 30 minutes. The similarity of the 2 M KI-induced alterations in PAPP-A AACE arc morphologies with those induced by 1 hour exposure of PAPP-A to 2•5 M and 3 M KI (Chapter 4, Table 4.1) provides further support for the conclusion reached in Chapter 4 which was that such alterations tend not to be random, but are typical of the antigen concerned.

The immunochemical reactivity of PAPP-A (as assessed by AACE) was unaffected by exposure to 1•5 M KI for periods up to at least 18 hours. This implies that 1•5 M rather than 2 M KI should be used whenever prolonged exposure of PAPP-A to a dissociating agent is unavoidable, as is likely to be the case during large-scale affinity chromatography purification procedures.

TABLE 5.1

Effect of Dissociating Agents on Precipitin Arc Formation[a] by PAPP-A Protein as Tested and Compared by AACE

Dissociation Agent	Exposure Time (hour)								
	1	2	3	4	5	6	7	8	18
1•5 M KI	+	+	+	+	+	+	+	+	+
2•0 M KI	+	+	+	+	+	+	(e)	(c,e)	c,e

a for explanation of symbols refer to legend to Table 4.1

2a. PURIFICATION OF PAPP-A FROM TERM MATERNAL SERUM

2a.1 Introduction

Purification of PAPP-A from term maternal serum was undertaken in two stages. Initially partially purified PAPP-A prepared by the small-scale antibody affinity chromatography (Chapter 4) was used to immunise sheep and the antisera thus obtained used to prepare an affinity column for the large-scale purification of PAPP-A.

The affinity column was then employed in the first stage of the purification procedure. Selection of further purification steps was guided by the knowledge of the biochemical characterisations of PAPP-A defined by the earlier work of Lin and his associates (Lin et al., 1974a; Lin et al., 1974b). According to these workers, PAPP-A has a pI of around 4·1. This enabled the selection of a suitable matrix for ion-exchange chromatography for use in the second step for purification: an anion exchanger, DEAE-cellulose. The molecular weight of PAPP-A as defined by Lin and his associates is 750,000 daltons: this enabled selection of a suitable matrix for gel filtration for use in the third and final purification step: Sepharose 6B which has an exclusion limit for proteins of $2 - 3 \times 10^6$.

2a.2 Materials and Methods

Materials

A pool of term maternal serum (300 ml.) was made from blood collected per-vaginam at normal deliveries. The protein concentration of the pooled serum was 32·4 mg. per ml.

Normal human serum was obtained from adult male volunteers.

Methods/

Methods

Preparation of Sheep Antiserum to Affinity-Purified PAPP-A

Preparation of partially-purified PAPP-A by small-scale antibody affinity chromatography and preparation of rabbit antiserum to this affinity-purified PAPP-A were described in Chapter 4. Sheep antiserum to affinity-purified PAPP-A was prepared in a similar way, except that antibody affinity chromatography as described in Section 2 of Chapter 4 was performed twice. The products from the two adsorptions over the affinity column were pooled prior to polyacrylamide tube gel electrophoresis. Antisera collected between the 7th and the 21st day following boosting were pooled. Adsorption of pooled sheep antiserum to affinity-purified PAPP-A was carried out using adult human male serum which had been polymerised with a final concentration of 0•4% gluteraldehyde and then dispersed into fine beads by homogenisation and washing in PBS (Avrameas and Ternyck, 1969). The extent of adsorption was tested by two-dimensional AACE using adult human serum as the source of antigens. Adsorption was judged to be complete when no precipitin lines could be detected on AACE. The adult serum-adsorbed sheep anti-PAPP-A antiserum was checked for cross-reactivity with anti-PAPP-A antiserum kindly provided by Drs Lin and Halbert on double immunodiffusion.

Preparation and Use of Sheep Antibody Affinity Column for PAPP-A

The immunoglobulin G (IgG) fraction was precipitated from 560 ml. of adult serum-adsorbed sheep anti-PAPP-A antiserum with 33% $(NH_4)_2SO_4$ by the method of Kekwick *et al.* (1942). 1•65 gm. of IgG thus purified was conjugated to 170 gm. Sepharose 4B by the CNBr reaction (Porath *et al.*, 1976). Before use the material was packed into a 3 cm. diameter Wright Scientific Column and washed

with three volumes of 2 M KI in PBS at 4°C and then equilibriated with PBS.

Antibody affinity chromatography was performed at 4°C using 300 ml. of pooled maternal serum as the source of PAPP-A. The serum was washed through the column with PBS at a rate of 80 ml. per hour until the eluate from the affinity column gave a zero reading at 280 nm. Material which failed to adsorb to the column was concentrated by ultrafiltration on a PM 10 membrane and analysed for PAPP-A content on one-dimensional AACE. The column was desorbed with 240 ml. 1·5 M KI-PBS, pH 7·0, as the dissociating agent at a flow rate of 100 ml. per hour. The eluate was diluted in PBS to below 0·8 M KI, as judged by conductance, concentrated to 90 ml. by ultrafiltration on a PM 10 membrane and dialysed for 48 hours against three changes of a 20-fold excess of 10 mM KPO_4, pH 7·5.

Preparation and Use of DEAE-cellulose Ion-exchange Chromatography Column

DE-52 microgranular DEAE-cellulose (Whatman, New York) was packed into a 2·6 cm. diameter Pharmacia column to give a column volume of 100 ml. The column was equilibriated with 10 mM KPO_4, pH 7·5 buffer (Buffer A) according to the instructions of Whatman. The material from the affinity column in a total volume of 90 ml. Buffer A was applied to the column at 4°C and washed through the column with Buffer at a rate of 48 ml. per hour. That material which failed to adsorb on the column was concentrated by ultrafiltration on a PM 10 membrane and analysed for PAPP-A content on one-dimensional AACE. Elution of the column was carried out with 500 ml. of a linear NaCl gradient, starting with 10 mM KPO_4, pH 7·5 and ending with 10 mM KPO_4, 0·5 M NaCl, pH 6·5, at a flow rate of 48 ml. per hour. The column was finally flushed with 50 ml. of a limiting buffer. Eluates were collected at 3·7 ml. per tube. The eluate was

monitored at 280 nm. with a Uvicord II analyser (LKB Instruments). The linearity of the gradient was checked by measuring the specific conductivity of the eluate. The presence of PAPP-A was assessed by testing each of the fractions by one-dimensional AACE. Those PAPP-A-containing fractions least contaminated with serum proteins were pooled, concentrated to 3 ml. by ultrafugation on a PM 10 membrane and used in the subsequent gel filtration purification step.

Preparation and Use of the Sepharose 6B Filtration Column

CL-Sepharose 6B (Pharmacia N. J.) was prepared in PBS and packed into a 1•5 cm. by 90 cm. Pharmacia column. The void volume was measured with dextran blue 2,000 (Pharmacia). Material recovered from the ion-exchange chromatography in a total volume of 3 ml. PBS was applied to the column, the column was washed in PBS at the rate of 20 ml. per hour and the eluates collected 3•3 ml. per tube at 4^{o}C. The eluate from the column was monitored at 280 nm. with a Uvicord II. The presence of PAPP-A was assessed by testing each fraction by one-dimensional AACE. In a separate experiment 3 ml. of pooled term maternal serum was fractionated over the same column following the experimental procedure outlined above.

The product of each purification stage was analysed for total protein content and for PAPP-A content. The total protein content was determined by the method of Lowry et al. (1951). The PAPP-A content was determined by measuring the area of the arc formed by PAPP-A on one-dimensional AACE using adult serum adsorbed sheep anti-PAPP-A. Each millilitre of the pooled term maternal serum was arbitrarily designated as containing 100 U PAPP-A and the pool was used to prepare a standard curve for PAPP-A.

2a.3 Results

Sheep Anti-PAPP-A Column Chromatography

Sheep anti-affinity purified PAPP-A antiserum used to construct an antibody affinity column for PAPP-A showed cross-reactivity with anti-PAPP-A antiserum of Lin and Halbert on double immunodiffusion (Figure 5.1).

The material from term maternal serum which failed to adsorb to the affinity column was tested on one-dimensional AACE for the presence of PAPP-A. It was found to contain none. The concentration of protein and the amount of PAPP-A recovered in the material eluted from the column with 1·5 M KI-PBS was determined and used to calculate the degree of purification of PAPP-A. The reults are shown in Table 5.2. Dialysis of the concentrated eluate from the affinity column resulted in the precipitation of protein which had to be removed by centrifugation.

DEAE-Cellulose Ion Exchange Chromatography

The material which failed to adsorb to the affinity column was tested on one-dimensional AACE for the presence of PAPP-A. It was found to contain none. Those fractions eluted from the column with a salt gradient were analysed for protein and PAPP-A content. The results are shown in Figure 5.2. Three protein peaks were resolved. Elution of PAPP-A occurred over a wide NaCl concentration range (0·175 - 0·26 M NaCl) with a maximum at 2 M NaCl which corresponded to the middle portion of the descending limb of the second protein peak. It eluted in the same volume as two of the three protein peaks. The PAPP-A peak was asymmetric: it had an extended descending limb with a shoulder on it. Inspection of the distribution of the protein and PAPP-A peaks indicated that the least serum protein-contaminated fractions were likely to be those forming the descending limb of the PAPP-A peak and three of those

fractions A,B and C were pooled and used in the subsequent purification step: see Figure 5.1 for the location of A,B and C. The concentration of protein and the amount of PAPP-A recovered in (i) pooled PAPP-A - containing fractions eluted from the column with a salt gradient and (ii) pooled fractions A,B,C were determined and used to calculate the degree of purification of PAPP-A in each. The results are shown in Table 5.2.

CL-Sepharose 6B Gel Filtration

Fractions A,B,C resulting from the ion exchange chromatography were fractionated over the Sepharose 6B column. In a separate experiment pooled term maternal serum was fractionated over the same column. The fractions eluted from the column were analysed for protein and PAPP-A content. The results are shown in Figure 5.3 and Figure 5.4c. PAPP-A in fractions A,B,C eluted in 63·5 ml. elution volume; PAPP-A in term maternal serum eluted in 63·5 ml. elution volume. The concentration of protein and the amount of PAPP-A recovered in (i) pooled PAPP-A-containing eluate fractions and (ii) the eluate fraction containing the maximum amount of PAPP-A (Fraction 5, Figure 5.4c: designated 'PAPP-A purification product') were determined and used to calculate the degree of purification of PAPP-A in each. The results are shown in Table 5.2.

Morphology of AACE Arcs Formed by PAPP-A in Term Maternal Serum and in Partially-Purified Products

PAPP-A in purification products at different stages during the purification procedure formed two precipitin arcs, one within and parallel to the other one on AACE but not on double immunodiffusion. In the case of PAPP-A eluted from DEAE ion-exchange column the presence of a third arc within and parallel to the other two was discerned (Figure

TABLE 5.2

The Recovery and Degree of Purification of PAPP-A After Each Purification Step Described in Section 2a

Purification step	Material tested	Total Protein (mg.)	Recovery of PAPP-A (%)	Purification of PAPP-A (-fold)
Start	300 ml. term maternal serum	9720.00	100.0	1
Anti-PAPP-A affinity chromatography	1.5 M KI eluate	49.20	20.8	41.1
DE-52 ion-exchange chromatography	PAPP-A-containing fractions	34.40	5.1	14.4
	Fractions A,B,C[1]	1.80	2.4	129.6
Sepharose 6B gel filtration	PAPP-A-containing fractions	0.52	2.4	448.8
	Peak PAPP-A fraction[2]	0.13	0.6	426.9

1 see Figure 5.2

2 'PAPP-A purification product': see text

5.4). As can be seen from Figure 5.4 the proteins which formed these arcs eluted together or close together from the DEAE-Sephadex ion-exchange and Sepharose 6B gel filtration columns.

The double-arc phenomenon was also frequently observed with PAPP-A in term maternal serum before any purification although the area inside the large arc tended to stain darkly and the second arc within was usually less discernable.

2b. PREPARATION AND ANALYSIS OF ^{125}I-LABELLED PAPP-A PURIFICATION PRODUCT

2b.1 Introduction

The final product of the purification protocol described in Section 2a - "PAPP-A purification product" - was iodinated and subjected to further studies. In order to achieve iodination with least risk of damage to the proteins being iodinated a gentle iodination method employing lactoperoxidase was chosen (Bolton, 1977). The identification of the ^{125}I-labelled species in the purification product was carried out using AACE. Anti-PAPP-A antiserum was used to detect labelled PAPP-A and anti-adult serum to detect any labelled serum proteins. The biochemical characteristics of ^{125}I-PAPP-A were then investigated by Sepharose CL 6B gel filtration and two-dimensional AACE. PAPP-A was identified in both systems by immune precipitation and its behaviour compared with that of PAPP-A from term maternal serum.

2b.2 Materials and Methods

Experimental Protocols for the Iodination of the PAPP-A Purification Product

(i) The enzymatic method using soluble lactoperoxidase (see Bolton, 1977). Iodination of 5 microgrammes of protein from the PAPP-A purification product (see Section 2a.3) was carried out using the soluble lactoperoxidase technique. 5 microgrammes of protein from pooled non-pregnant adult serum - the product of ten male and non-pregnant female sera - was also labelled by this method. In addition a blank iodination was performed in which no protein was added to the reaction mixture containing lactoperoxidase so that identification of the labelled species in the PAPP-A purification product due to labelled

lactoperoxidase would be possible.

The experimental method was based on the method described by Bolton (1977).

Materials

$Na^{125}I$: 100 mCi per ml.

0·5 M potassium phosphate buffer, pH 7·5.

1 mg. per ml. lactoperoxidase in 0·1 M potassium phosphate buffer, pH 7·5

20 microgram. per ml. hydrogen peroxide diluted in 0·05 M potassium phosphate buffer, pH 7·5.

20 mg. per ml. tyrosine in 0·05 M potassium phosphate buffer, pH 7·5.

20 mg. per ml. KI solution in gelatin-phosphate buffer (0·05 M potassium phosphate buffer, pH 7·5, containing 0·2% gelatin).

Protein to be iodinated: 0·5 mg. per ml. in 0·05 M potassium phosphate buffer, pH 7·5.

12% horse serum-phosphate buffer (0·05 M potassium phosphate buffer, pH 7·5, containing 12% Wellcome type V horse serum).

100 mg. per ml. solution of bovine serum albumin (Armour Pharmaceutical Company Limited).

Lactoperoxidase (B grade) was purchased from Calbiochem. It was diluted from a 10 mg. per ml. stock solution (stored frozen) immediately before use.

The buffers contained no sodium azide as preservative.

Iodination

0·5 mCi. (5 microlitre) of $Na^{125}I$ was dispensed into a polystyrene tube and 20 microlitre of 0·5 M potassium phosphate buffer was added. The solution was continuously agitated and the other reagents were added in the following order:

(1) 10 microlitre (5 microgram.) of protein solution

(2) 10 microlitre (10 microgram.) lactoperoxidase solution

(3) 10 microlitre (200 ng.) hydrogen peroxide solution.

The reaction mixture was incubated at room temperature for seven minutes and a further 10 microlitres of hydrogenperoxide solution was added. After a further 7 minutes a third 10 microlitre aliquot of hydrogen peroxide was added, and then after a further 7 minutes' incubation, 50 microlitres (1mg.) tyrosine was added to quench the reaction. After one minute, 50 microlitres of the carrier KI solution was added to the reaction mixture and the radioactivity in the iodination tube was counted in a LKB Ultragamma counter.

Separation of Labelled Protein from Unreacted Iodide

The Sephadex G-25 gel filtration medium was equilibriated in gelatin-phosphate buffer (0•05 M sodium phosphate buffer containing 0•2% gelatin) and packed into a small column. The column was saturated with bovine serum albumin and all free albumin was washed off the column with gelatin-phosphate buffer. The iodination mixture was then transferred to the prepared column. The column was eluted with gelatin-phosphat buffer and 0•1 ml. fractions were collected into polystyrene tubes (of the same dimensions as the iodination reaction tube) containing 25 microlitres of 12% horse serum-phosphate buffer. The column was run until both the protein and small molecular weight peaks had been eluted and the radioactivity in all the fractions was measured. The tube with the protein peak was taken for use as a tracer.

(ii) The enzymatic method using solid-phase lactoperoxidase (David, 1972; David and Reisfeld, 1974). Iodination of 5 microgrammes of protein from the PAPP-A purification product was carried out at a later stage using lactoperoxidase covelently coupled to Sepharose 6B. Conjugation of lactoperoxidase to Sepharose 6B was carried out by the

CNBr reaction Porath et al., 1976) using 5 mg. lactoperoxidase per 1 ml. packed Sepharose. 20 microlitre of Sepharose slurry in 0·05 M potassium phosphate buffer, pH 7·5 was added to the iodination mixture instead of 10 microlitre lactoperoxidase solution in the method described in (i) above. Addition of tyrosine to quench the iodination reaction reaction was not necessary.

Identification of Labelled Species in the Purification Product

(a) One-dimensional AACE using anti-PAPP-A antiserum

10 microlitre aliquots of PAPP-A purification product labelled by the soluble lactoperoxidase method (in 50 mM K_2PO_4, pH 7·5; 35,000 c.p.s.) were added to wells 1 - 3 on an AACE plate. Term maternal serum (10 microlitres) was added as a source of carrier PAPP-A to well 2. Pooled non-pregnant adult serum (10 microlitres) was added to well 3. The antiserum used was 3% sheep anti-PAPP-A. A similar set of wells were used in a separate control plate which contained 3% non-immune serum. After electrophoresis and two days of washing to remove non-precipitated counts, the gels were subjected to autoradiography with Kodirex (Kodak) X-ray film. The areas of intense radioactivity in the gel (and the corresponding areas in the control plate) were excised with a scalpel and counted in a LKB Ultragamma counter.

(b) Two-dimensional AACE using anti-adult serum antiserum

A 10 microlitre aliquot of serum protein labelled by the soluble lactoperoxidase method (38,000 c.p.s.) and a 10 microlitre aliquot of the PAPP-A purification product labelled by the soluble lactoperoxidase method (35,000 c.p.s.) (both in 50 mM K_2PO_4, pH 7·5) were added to wells 1 and 2 respectively. Well 1 was at the extreme left and well 2 in the middle of an AACE plate. 1·5 microlitres of pooled non-pregnant adult serum was also added to wells 1 and 2. The

samples were subjected to electrophoresis in the first dimension over the same distance on the AACE plate. Second dimension electrophoresis was then carried out into gel containing 10% sheep anti-human male serum antiserum (Sutcliffe, 1976). After electrophoresis and two days of washing to remove non-precipitated counts the gels were subjected to autoradiography with Kodirex X-ray film.

Analysis of ^{125}I-PAPP-A in the Purification Product

(a) Sepharose CL 6B gel filtration

The material resulting from iodination of the PAPP-A purification product by the soluble lactoperoxidase method and that resulting from blank iodination by the soluble lactoperoxidase method was each subjected to gel filtration over a Sepharose 6B column. The column used was the same as that used in the purification procedure: see Section 2a.2. In each fractionation the material to be fractionated was applied to the column, the column washed in PBS at the rate of 20 ml. per hour and the eluates collected at 2·55 ml. per tube. The radioactivity in the fractions resulting from each fractionation was counted in a LKB Ultragamma counter. The percentage of counts due to ^{125}I-PAPP-A in the fractions resulting from the fractionation of the labelled purification product was determined by solid-phase immune precipitation: see below.

Solid-Phase Immune Precipitation of ^{125}I-PAPP-A: 10 microlitre aliquots of Sepharose 6B fractions were added to tubes containing 50 microlitres of sheep anti-PAPP-A-Sepharose diluted 1:10 in 2% horse serum-phosphate buffer (0·05 M potassium phosphate buffer, pH 7·5, containing 2% horse serum). For details of the preparation of anti-PAPP-A-Sepharose see Section 2a.2. The radioactivity in each tube was measured in a LKB Ultragamma counter. The tubes were left on a gyratory shaker overnight

at room temperature. The tube contents were then centrifuged and the Sepharose washed three times with 2% horse serum-phosphate buffer, pH 7·5: each time the supernatant was removed by centrifugation. Finally the radioactivity in each tube was measured.

(b) Two-dimensional AACE using anti-PAPP-A antiserum

To each of two wells, located one directly underneath the other in agarose gel on a 8 cm. by 8 cm. plate, were added 5 microlitre of purification product labelled by the solid lactoperoxidase method (10 microlitre in 50 mM K_2PO_4, pH 7·5; 5,600 c.p.s.) together with 5 microlitres of term maternal serum containing Bromophenol Blue. The samples were subjected to electrophoresis along parallel tracks on the gel. When first dimension electrophoresis was completed the lower part of the gel containing one of the two electrophoresed samples was excised with a scalpel and cut into 30 pieces, each 0·25 cm. long. The radioactivity in each piece of gel was measured in a LKB Ultragamma counter. The other electrophoresed sample was subjected to electrophoresis in the second dimension into 20% sheep anti-PAPP-A antiserum-containing gel: for details of the antiserum preparation see Section 2a.2. After electrophoresis and two days of washing the gel was subjected to autoradiography with Kodirex X-ray film and stained for protein with Coomassie Blue.

2b.3 Results

Identification of Labelled Species in the Purification Product

(a) One-dimensional AACE using anti-PAPP-A antigens

Detection of immunoreactive ^{125}I-PAPP-A in the labelled purification product was attempted on one-dimensional AACE plates containing anti-PAPP-A antiserum: see Figure 5.5. Immunoreactive

^{125}I-PAPP-A was detected as an intensely radioactive arc. The control plate containing non-immune serum showed only faint activity around the margins of the antigen wells. The radioactivity in the ^{125}I-PAPP-A AACE arcs and the corresponding areas in the control plate were counted and the results, expressed as a percentage of the counts applied, are shown in Table 5,3.

(b) Two-dimensional AACE using anti-adult serum antiserum

The detection of immunoreactive ^{125}I-serum proteins in the labelled pooled non-pregnant adult serum and in the labelled purification product was attempted on two-dimensional AACE plates containing anti-adult serum antiserum: see Figure 5.6. The incorporation of a large number of immunoreactive serum proteins into the AACE arcs was observed when labelled when labelled non-pregnant adult serum was used as the antigen source. Incorporation of very few immunoreactive serum proteins into AACE arcs was observed when labelled PAPP-A purification product was used as the antigen source and the bulk of the radioactive material was found in the area close to the origin after electrophoresis.

Analysis of ^{125}I-PAPP-A in the Purification Product

(a) Sepharose CL 6B gel filtration

The material resulting from iodination of the purification product by the soluble lactoperoxidase method and that resulting from blank iodination by the soluble lactoperoxidase method was each fractionated over the Sepharose 6B column. Those fractions eluted from the column were counted in a gamma-counter and the results are shown in Figure 5.7.

Fractionation of the labelled purification product gave rise to four radioactive peaks, three of which corresponded in position

TABLE 5.3

Retention of Counts in the Labelled PAPP-A Purification Product on AACE Plates Containing (1) Sheep Anti-PAPP-A Antiserum and (2) Non-Immune Serum.

	Antigen	Percentage of counts retained in AACE arcs or corresponding areas	
		Plate 1	Plate 2
		3% anti-PAPP-A	3% non-immune serum
a	^{125}I-labelled purification product	45	2·3
b	^{125}I-labelled purification product added to term marernal serum	49	2·6
c	^{125}I-labelled purification product added to non-pregnant adult serum	46	-

to the three peaks which resulted from the fractionation of the product of blank iodination. The first of these three common peaks, fractions 14 - 22 in Figure 5.7, corresponds to the void volume of the column. The second peak, fractions 30 - 57, had a shoulder on the descending limb. The third peak, fractions 57 - 68, was the smallest of the three. A fourth peak, which was not observed in the eluate resulting from the fractionation of the product of the blank iodination, was detected in fractions 22 - 30.

The percentage of counts due to ^{125}I-PAPP-A in those fractions resulting from the fractionation of the labelled purification product was determined by solid-phase immuno precipitation and the results are also shown in Figure 5.7. The elution volume of the ^{125}I-PAPP-A peak thus detected was 63·1 ml.

(b) Two-dimensional AACE using anti-PAPP-A antiserum

Two aliquots of material resulting from the iodination of the purification product by the solid-phase lactoperoxidase method were subjected to electrophoresis along parallel tracks on agarose gel. The distribution of radioactivity over the gel was determined by counting slices of the gel containing one of the electrophoresed samples in a gamma-counter. The results are shown in Figure 5.8. Three radioactive peaks were resolved. The first on corresponded to the position of the antigen well. The second peak corresponded to the position of the ^{125}I-PAPP-A precipitin arc detected by autoradiography: see the following paragraph. The third peak corresponded to the position of the Bromophenol Blue band.

The other electrophoresed sample was subjected to second dimension electrophoresis into anti-PAPP-A antiserum containing gel. The presence of a single immunoreactive arc on the AACE plate was

revealed by autoradiography and protein staining: Figure 5.9. The AACE arc detected by protein staining corresponded in position to the most intensely radioactive part of the AACE arc detected by autoradiography: however, the latter arc extended further towards the cathodal end of the gel.

2a,b.4 Discussion

Prior to the present work being undertaken Lin et al. (1974a) claimed to have acheived 115-fold purification of PAPP-A from third trimester maternal serum using a purification procedure which involved the combined use of DEAE ion-exchange chromatography and gel filtration. However they provided no physicochemical evidence of purity and their PAPP-A preparation contained easily detectable contaminating serum proteins. The goal of the purification procedure described in the present chapter was to obtain a highly purified preparation of PAPP-A suitable for the preliminary structural studies described in Chapter 6. The procedure incorporated an antibody affinity chromatography step followed by DEAE ion-exchange and gel filtration steps and has resulted in 448-fold purification of PAPP-A from term maternal serum.

Interpretation of yields and recovery data from the first two purification steps is difficult as precipitation is known to have occurred during dialysis of the concentrated eluate from the affinity column and the possibility that further precipitation may have occurred during application of the dialysed sample to the ion-exchange column cannot be excluded. Nevertheless, the anti-PAPP-A affinity chromatography step resulted in a considerable purification of PAPP-A (41-fold) with a yield of 20·8%. The second purification step, DEAE ion-exchange chromatography, resulted in a decreased (14-fold) degree of purification of PAPP-A with a yield of 5%. However 130-fold purified PAPP-A was found in the ion exchange fractions selected for further purification. The reason for the failure of this column to effect further purification of PAPP-A becomes apparent from inspection of Figure 5.2. PAPP-A was found to elute from the column over a relatively wide NaCl concentration range, namely 0·1 - 0·2 M, as a single peak with an

extended descending limb containing a shoulder possibly due to a second peak. Since the linearity of the NaCl gradient had been confirmed by measuring the specific conductivity of the eluate this pattern is unlikely to be due to discontinuities in the gradient but instead may suggest the presence of at least two species of PAPP-A in the material recovered from the affinity chromatography with different affinities for the ion-exchange column, the major species eluting as a peak with a maximum at 0•2 M NaCl. PAPP-A eluted in the same volume as two of the three protein peaks detected by adsorption at OD_{280} whose maxima did not correspond to the maximum of the PAPP-A peak. Since the major objective was to obtain a highly purified and a most representative sample of the PAPP-A population with less regard being given to yield the highest purity fractions i.e. those corresponding in position to the lower half of the descendimg limb of the second protein peak were pooled for use in the next purification step. The elution profile of PAPP-A from the third and final purification column - Sepharose 6B gel filtration column - was more straightforward: PAPP-A eluted as a single peak which corresponded in position to one of the two protein peaks detected by absorption at OD_{280}. The other protein peak eluted in a volume corresponding to the void volume of the column. The purified PAPP-A eluted in the same volume as the PAPP-A from term maternal serum fractionated previously over the same column: it can therefore be inferred that it did not suffer a gross alteration in molecular size during the process of purification. The Sepharose 6B column improved further the purity of the PAPP-A preparation: at the end of the purification procedure PAPP-A was found to have been purified 448-fold. The degree of purification was similar (427-fold) in the peak PAPP-A fraction used for iodination.

The significance of the multiple AACE arcs formed by partially-purified PAPP-A and by PAPP-A in term maternal serum before any purification is unknown but suggests the existence of at least two overlapping immune reactions and therefore a degree of antigenic heterogeneity in the protein. The second arc within the main arc was easier to detect when partially purified preparations rather than term maternal serum were used as the source of PAPP-A: this appears to be due to a lighter background within the arc formed by the partially-purified PAPP-A due possibly to decreased contamination of the area within the arc by unrelated proteins. The antigenic variants which gave rise to these arcs eluted together or close together from the chromatography columns and it may therefore be concluded that they are not separable by DEAE-cellulose ion-exchange or Sepharose 6B gel filtration.

An enzymatic method employing lactoperoxidase has been chosen to radio-iodinate the purification product as it has been found to minimise damage to labelled proteins, maintaining their structural integrity (Miyachi and Chrambach, 1972) and resulting in preparations with a high retention of immunological and other biological properties (Miyachi et al., 1972; Krohn and Welch, 1974). Immunoreactive ^{125}I-PAPP-A (identified as an AACE arc) accounted for almost 50% of the counts in the PAPP-A purification product labelled by the soluble lactoperoxidase method. Incorporation of very few immunoreactive serum proteins from this tracer into the AACE arcs was observed and the bulk of the contaminating material was of very slow electrophoretic mobility. A further iodination of the PAPP-A purification product was carried out by the soluble lactoperoxidase method. The elution pattern produced by this labelled purification product was very similar to that produced by the labelled lactoperoxidase, the product of the blank iodination, on

Sepharose 6B gel filtration except for one radioactive peak present in the former eluate which was not detected in the latter. This peak was identified as PAPP-A by solid-phase immune precipitation. Immunoreactive ^{125}I-PAPP-A eluted from the Sepharose 6B column in the same volume as PAPP-A from term maternal serum and it may therefore be concluded that it did not suffer any gross alteration in molecular size during iodination. The identity of the labelled species in the void in both fractionations has not been determined but it appears likely they are proteins damaged by the process of iodination, which have formed aggregates: several proteins are prone to this form of damage (see Bolton, 1977). In the case of the labelled purification product these aggregates may consist of lactoperoxidase, PAPP-A or contaminating serum proteins; which may be the same as the low electrophoretic mobility material detected on anti-serum AACE. The identity of the remaining peaks eluted from the Sepharose 6B column has not been established but as they are due to low molecular weight species it is most likely that they represent labelled lactoperoxidase (molecular weight 77,500 daltons: Rombauts et al., 1967) and free ^{125}Iodine. In order to reduce contamination evidently resulting from the labelling of the lactoperoxidase molecules subsequent radio-iodinations employed lactoperoxidase covalently coupled to an insoluble matrix.

Analysis of the purification product labelled by the solid-phase lactoperoxidase method by electrophoresis on agarose gel revealed the presence of only three species. The species displaying the slowest mobility may have been detected previously in the purification product labelled by the soluble lactoperoxidase method as the slow electrophoretic mobility component on anti-serum protein AACE and the high molecular weight component (postulated to be due to aggregates) in

the void from Sepharose 6B gel filtration. The identity of the fastest moving species associated with the Bromophenol Blue band has not been established but a strong possibility is free ^{125}Iodine non-covalently bound to albumin in the carrier term maternal serum. The third species of intermediate electrophoretic mobilitywere demonstrated to contain immunoreactive ^{125}I-PAPP-A. The part of the arc formed by it on AACE and detected autoradiographically which contained the maximum number of counts and appeared most intense coincided with the arc detected by protein staining: however the former arc extended slightly further towards the cathodal end of the gel than the latter arc. This difference is probably due to the greater sensitivity of autoradiography and its ability to detect the presence of a small number of ^{125}I-PAPP-A molecules. It may, however, indicate the presence of a small number of ^{125}I-PAPP-A molecules of decreased electrophoretic mobility, too small to be detected by protein staining alone. Since only a single precipitin line was observed such molecules appear to be cross-reactive. Iodination damage resulting in decreased electrophoretic mobility of a proportion of molecules but an unaltered molecular size, as determined by Sepharose 6B gel filtration, and immunochemical reactivity, as determined by AACE, could be one reason for the presence of such molecules.

FIGURE 5.1

Double Immunodiffusion.

The immunodiffusion plate shows cross-reactivities between:

(a) Sheep anti-affinity purified PAPP-A antiserum

and (b) Anti-PAPP-A antiserum of Lin and Halbert.

The antigens are:

(1) Pooled maternal serum

(2) Pooled adult serum

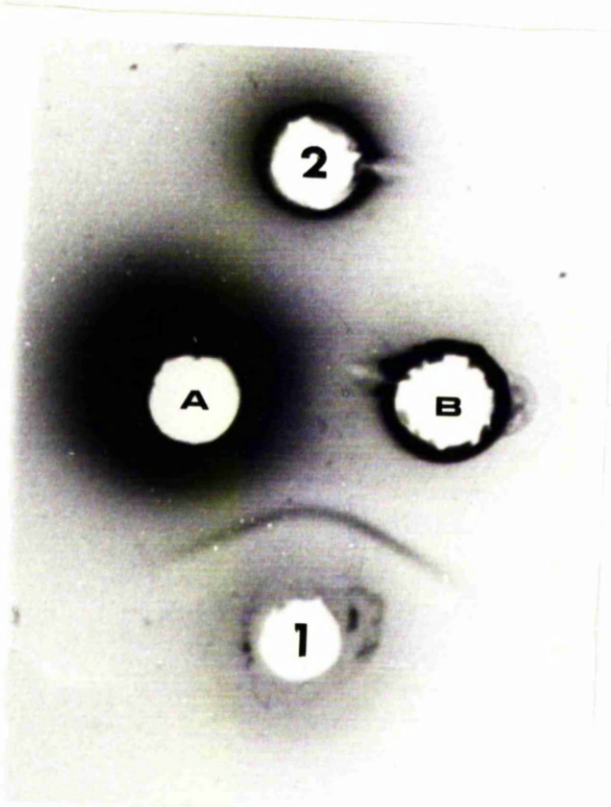

2
A
B
1

FIGURE 5.2

Elution Profile of Protein and PAPP-A from DEAE-Cellulose Column.

For details of column preparation and use see text.

The optical density due to the proteins was measured using a Uvicord II at 280 nm. and the trace is shown as a heavy fluctuating line.

The specific conductivity of the eluate is indicated by a broken line: arrows indicate NaCl concentrations.

The set of points joined by a line indicates the heights of the PAPP-A precipitin arcs produced on one-dimensional AACE by PAPP-A-containing eluate fractions.

The letters A,B,C indicate those fractions selected for the subsequent purification step: see text.

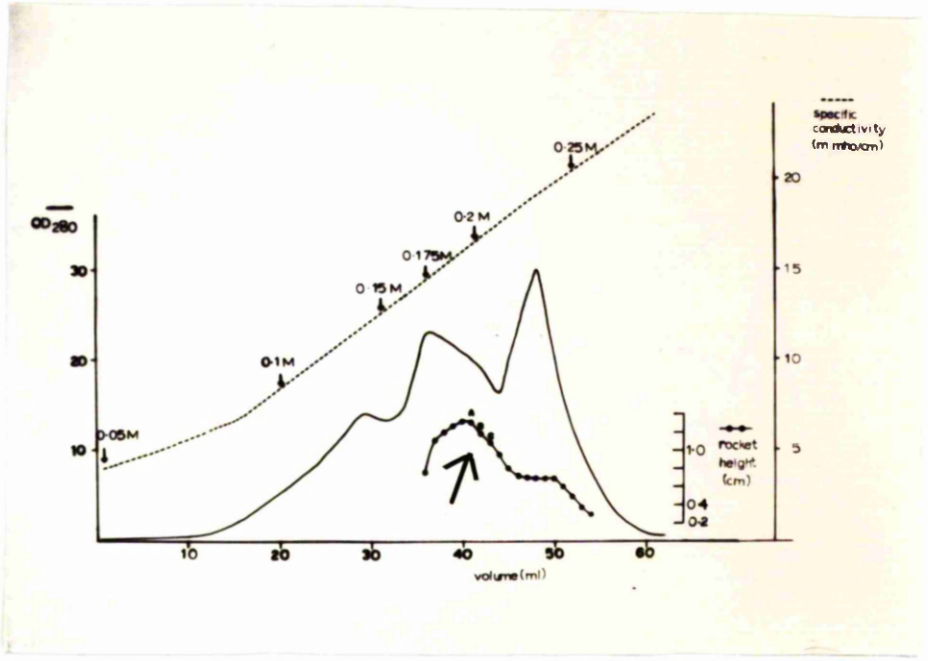

OD280
30
20
10
0·05M
0·1M
0·15M
0·175M
0·2M
0·25M
1·0
0·4
0·2
rocket height (cm)
20
15
10
5
specific conductivity (m mho/cm)
10
20
30
40
50
60
volume (ml)

FIGURE 5.3

Elution Profile of Proteins and PAPP-A from the Sepharose CL 6B Column.

For details of column preparation and use see text.

The optical density due proteins was measured using a Uvicord II at 280 nm. on eluate reulting from:

(1) The fractionation of term maternal serum

and (2) Fractions A,B,C from ion-exchange chromatography: see Figure 5.2.

Their tracers are shown as:

(1) A heavy fluctuating line

and (2) A broken fluctuating line -------

respectively.

The first protein peak in each profile corresponds to the void volume of the column.

The position of PAPP-A-containing fractions in eluates is also shown. The rocket height on the right abscissa refers to the he heights of the rockets produced by PAPP-A in eluate fractions on one-dimensional AACE. In all cases 20 microlitre aliquots of eluate fractions were tested on AACE.

Curve (3) is the optical density tracer which resulted from fractionation of the product of a pilot PAPP-A purification experiment employing antibody affinity chromatography.

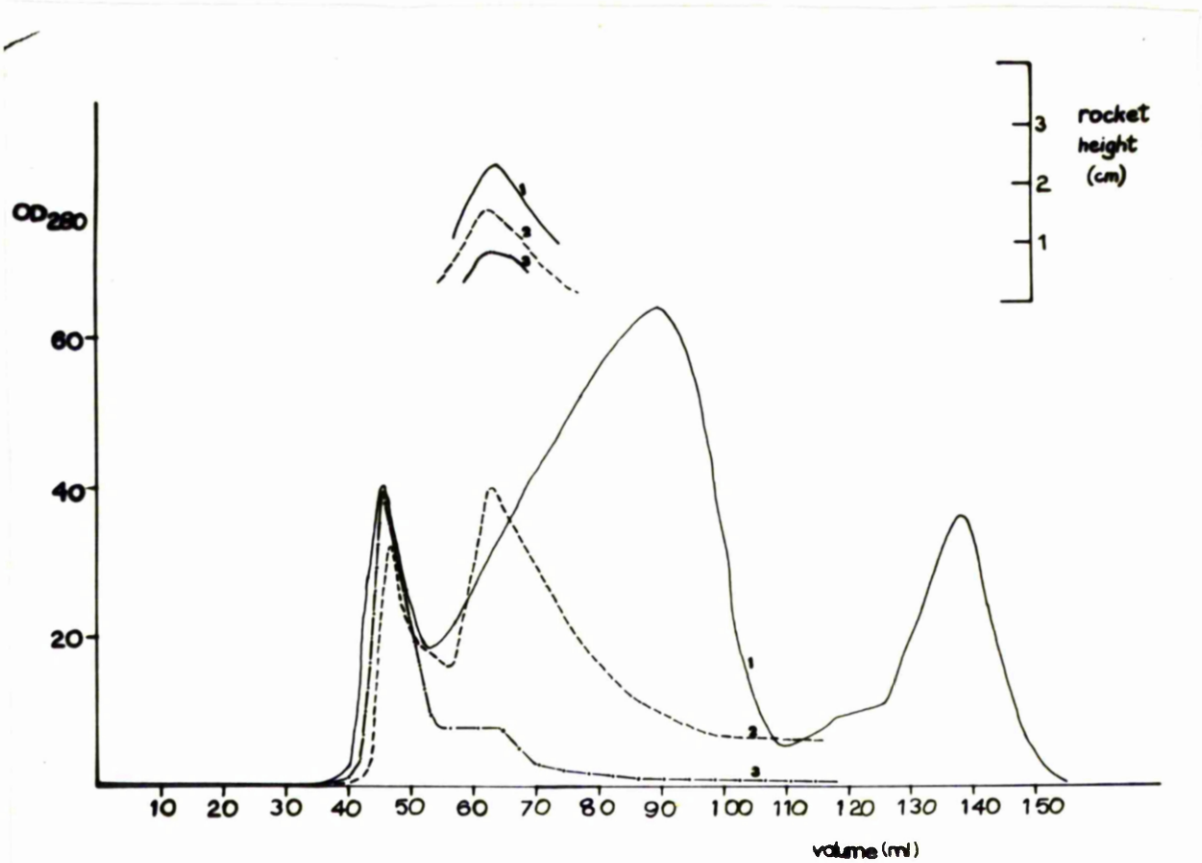

rocket height (cm)
3
2
1
OD280
60
40
20
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
volume (ml)

FIGURE 5.4

Morphology of AACE Arcs Formed by Partially-Purified PAPP-A and PAPP-A in Pooled Term Maternal Serum.

The antigens on Plate a are:

(1)-(5) PAPP-A in eluates from consecutive slices of polyacrylamide tube gels obtained after electrophoresis of the small-scale purification product.

The antigens on Plate b are:

(MS) PAPP-A in pooled term maternal serum

(1)-(9) PAPP-A in consecutive eluate fractions from DEAE-Sephadex ion-exchange chromatography column: see Figure 5.2.

Fractions 6,7,8 correspond to fractions A,B,C in Table 5.2

The antigens on Plate c are:

(MS) PAPP-A in pooled term maternal serum

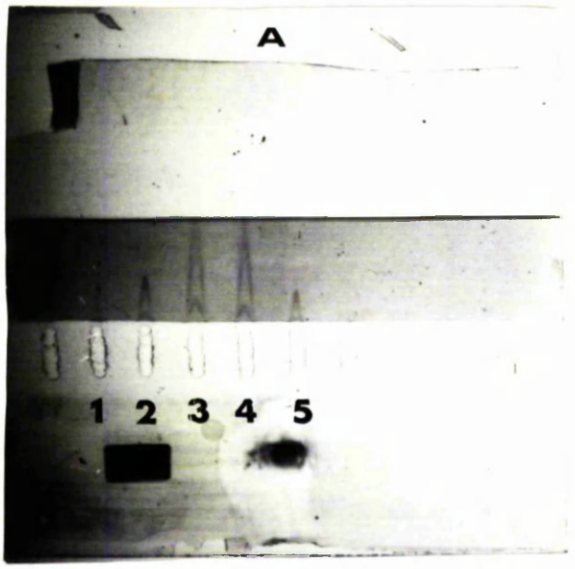
(1)-(7) PAPP-A in consecutive PAPP-A-containing eluate fractions from Sepharose CL-6B gel filtration column: see Figure 5.3.

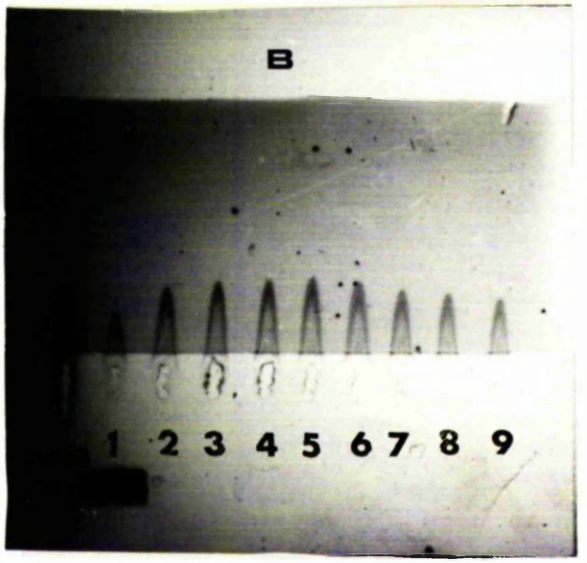
Fraction 5 is the peak PAPP-A fraction in Table 5.2.

The antisera are:

Plate a - 10% rabbit anti-PAPP-A

Plates b & c - 10% sheep anti-PAPP-A.


A
1 2 3 4 5


B
1 2 3 4 5 6 7 8 9

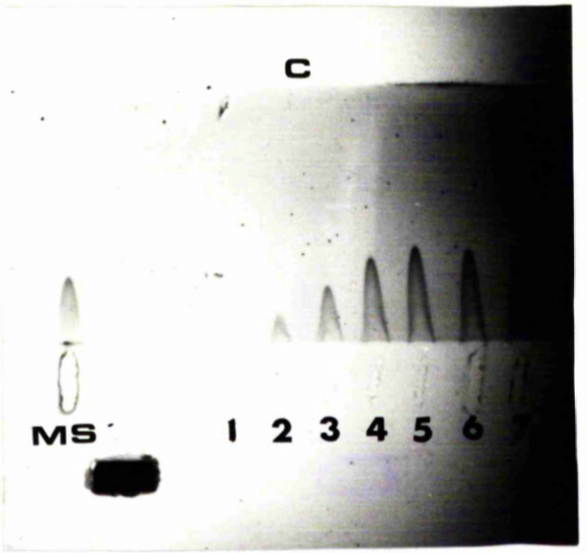

C
MS
1 2 3 4 5 6

FIGURE 5.5

Autoradiograph of One-Dimensional AACE Arcs Formed by ^{125}I-PAPP-A in the Purification Product Labelled by the Soluble LPO Method.

The antiserum is 3% sheep anti-PAPP-A.

The antigens are:

(1) 10 microlitre ^{125}I-labelled purification product

(2) 10 microlitre ^{125}I-labelled purification product added to 10 microlitre pooled term maternal serum

(3) 10 microlitre ^{125}I-labelled purification product added to 10 microlitre pooled non-pregnant adult serum.

The autoradiograph was developed for $\frac{1}{2}$ hour.


1 2 3

FIGURE 5.6

Autoradiograph of Two-Dimensional AACE Pattern Formed by I^{125}-serum Proteins in Non-Pregnant Adult Serum and in the Purification Product Labelled by the Soluble LPO Method.

The antigen wells are marked a and b. The circular mark to the right of each antigen well represents the mobility of albumin in the first dimension electrophoresis.

The antigens are:

(a) 10 microlitre ^{125}I-labelled pooled non-pregnant adult serum added to 1·5 microlitre pooled non-pregnant adult serum

(b) 10 microlitre ^{125}I-labelled purification product added to 1·5 microlitre pooled non-pregnant adult serum.

The antiserum is 10% sheep anti-human serum.

The autoradiograph was developed for 3 hours.

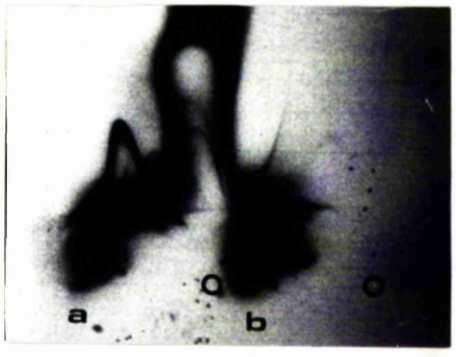

a
b

FIGURE 5.7

Sepharose CL 6B Gel Filtration of the Purification Product Labelled by the Soluble LPO Method and Material Resulting from Blank Iodination by the Soluble LPO Method.

For details of column use see text.

Legend:

●–● cps in fractions resulting from fractionation of the labelled purification product.

○–○ cps in fractions resulting from fractionation of the product of blank iodination.

▲–▲ % cps bound to anti-PAPP-A Sepharose in solid phase immune precipitation.

Fraction size: 2·55 ml. per tube.

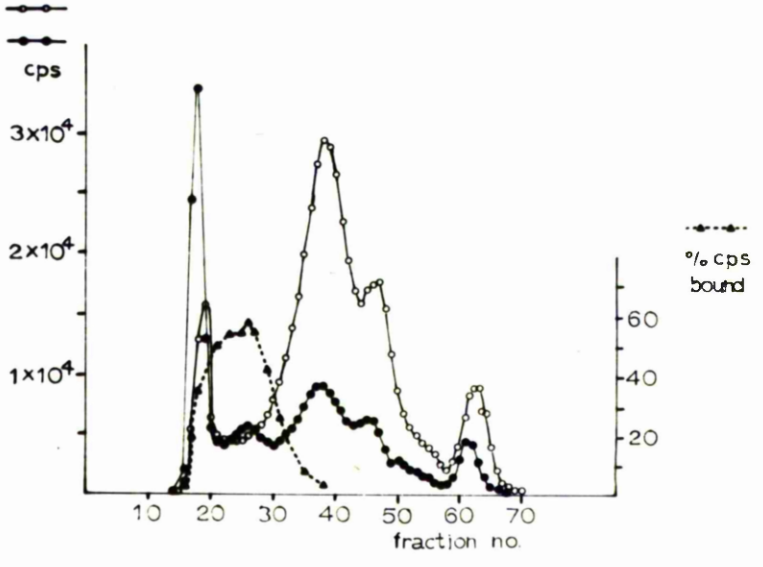

cps
3x10⁴
2x10⁴
1x10⁴
10
20
30
40
50
60
70
fraction no.
60
40
20
% cps bound

FIGURE 5.8

Electrophoretic Separation of the Material Resulting from Iodination of the Purification Product by the Solid-Phase LPO Method.

For experimental details see text.

The first radioactive peak (gel slice 7) corresponds to the position of the antigen well.

1 indicates the position of the ^{125}I-PAPP-A precipitin arc detected by autoradiography (see Figure 5.9).

2 indicates the position of the Bromophenol Blue band.

Correction: Gel slice 7 contained $1{\cdot}42 \times 10^4$ c.p.100s. not $4{\cdot}2 \times 10^4$ c.p.100s. as indicated on the graph.

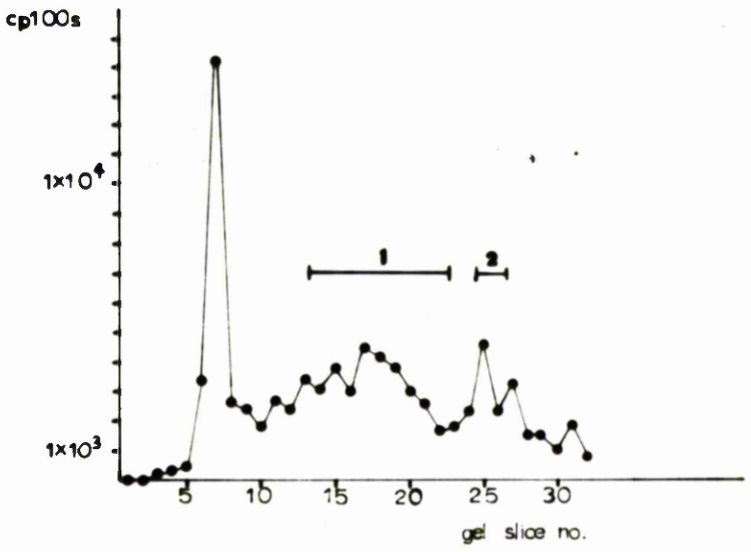

cpl00s
1x10⁴
1x10³
1
2
5
10
15
20
25
30
gel slice no.

FIGURE 5.9

Autoradiograph of the Two-Dimensional AACE Pattern Formed by ^{125}I-PAPP-A in the Purification Product Labelled by the Solid-Phase LPO Method

The antiserum is 20% sheep anti-PAPP-A.

The antigen is 5 microlitre PAPP-A purification product labelled by the solid-phase LPO method added to 5 microlitre pooled term maternal serum.

The tracing of the precipitin arc detected on the AACE plate stained for protein has been superimposed over the autoradiograph.

O = albumin

CHAPTER 6 THE STUDY OF PAPP-A PURIFIED BY ANTIBODY AFFINITY CHROMATOGRAPHY

1.1 Introduction

The purification and iodination of PAPP-A from term maternal serum was described in Chapter 5. The molecular weight of PAPP-A has been estimated as 750,000 daltons by Lin et al. (1974) but no information relating to its subunit structure was available prior to the present study being undertaken. Studies were therefore carried out on labelled PAPP-A in the purification product whose production was described in Chapter 5 in order to gain an insight into its subunit structure.

Labelled PAPP-A in the purification product was analysed by SDS polyacrylamide gel electrophoresis. Initially analyses were performed on immune precipitated PAPP-A from the PAPP-A purification product labelled by the soluble lactoperoxidase method. These experiments employed 10% SDS polyacrylamide tube gels and a 7% SDS polyacrylamide slab gel. The information gained from these analyses was used to select more suitable concentrations of polyacrylamide for the analysis of PAPP-A from the purification product labelled by the solid phase lactoperoxidase method.

1.2 Materials and Methods

A. Analysis of ^{125}I-PAPP-A from the Purification Product Labelled by the Soluble Lactoperoxidase Method

10% SDS Polyacrylamide Tube Gel Electrophoresis

^{125}I-PAPP-A from the labelled purification product was immuno-precipitated on an AACE plate containing sheep anti-PAPP-A antiserum: see Chapter 5, Section 2b.2. The areas of the gel from the AACE plate containing precipitin arcs formed by ^{125}I-PAPP-A in the purification product which had been applied to the AACE plate alone

(arc 1, Figure 5.5) or together with either pooled maternal serum (arc 2, Figure 5.5) or pooled non-pregnant adult serum (arc 3, Figure 5.5) were excised with a scalpel. The radioactivity in each precipitin-containing piece of gel was measured in an LKB Ultragamma counter and the gel was boiled for 5 minutes in 50 microlitres of $\frac{1}{3}$ dilution of the standard SDS extraction buffer (Maizel, 1971; Marsden et al., 1976). The molten agarose-SDS-tracer mixture was pipetted onto tube gels where it cooled and set before the upper reservoir buffer was added. 5 microlitres of the same labelled purification product (17,900 c.p.s.) was added to 25 microlitres of tank buffer and 15 microlitres of the standard SDS extraction buffer and boiled for 5 minutes. All four samples were subjected to SDS polyacrylamide gel electrophoresis (method according to Maizel, 1971) in 10% polyacrylamide gels. After electrophoresis gels were snap-frozen in liquid nitrogen and sliced into 65 fractions using a Mickle gel slicer (Gomshall, Surrey). The radioactivity in each fraction was measured in a LKB Ultragamma counter.

7% SDS Polyacrylamide Slab Gel Electrophoresis

^{125}I-PAPP-A was extracted from the labelled purification product by solid phase immuno-precipitation. 10 microlitres of the purification product (35,800 c.p.s.) was added to a tube containing 100 microlitres of 10% slurry of anti-PAPP-A-Sepharose in 2% horse serum-phosphate buffer (50 mM K_2PO_4 buffer, pH 7·5, containing 2% Wellcome Type 5 horse serum). For details of the anti-PAPP-A-Sepharose preparation see Chapter 5, Section 2a.2. The tube was incubated overnight on a gyratory shaker at 4°C. After centrifugation to remove the supernatant the Sepharose was washed four times with 2% horse serum-phosphate buffer, the supernatant being removed each time by centrifugation. At the end of the washing cycle the supernatant was removed

replaced with 40 microlitres of 2% horse serum-phosphate buffer. 25 microlitres of the standard SDS extraction buffer was then added to the Sepharose and the resultant slurry boiled for 5 minutes. The slurry was then centrifuged and the supernatant analysed by SDS polyacrylamide slab gel electrophoresis. A set of five calibrated viral polypeptides was applied to a separate track on the same SDS polyacrylamide slab gel. The SDS polyacrylamide slab gel was run according to the method of Marsden et al. (1976) but using a uniform 7% concentration of acrylamide in the resolving gel. Following electrophoresis the slab gel was dried and subjected to autoradiography using Kodirex (Kodak) X-ray film.

B. Analysis of ^{125}I-PAPP-A from the Purification Product Labelled by the Solid-Phase Lactoperoxidase Method

5% and 3% SDS Polyacrylamide Tube Gel Electrophoresis

^{125}I-PAPP-A was extracted from the labelled purification product by solid-phase immunoprecipitation. 50 microlitre aliquots of the labelled purification product were added to each of the following four tubes: two tubes containing 25 microlitres of 50% slurry of anti-PAPP-A-Sepharose in 2% horse serum-phosphate buffer each and two control tubes containing 25 microlitres of 50% slurry of horse serum-Sepharose in 2% horse serum-phosphate buffer each. For details of the anti-PAPP-A-Sepharose preparation see Chapter 5, Section 2a.2. Horse serum had been conjugated to Sepharose 4B by the CNBr reaction (Porath et al., 1976). A control set of four tubes containing 50 microlitre aliquots of the product of "blank" iodination by the soluble lactoperoxidase method instead of the labelled PAPP-A purification product was also set up. The tubes were incubated overnight on a gyratory shaker. The radioactivity in each tube was measured in an LKB Ultra-

gamma counter. After centrifugation to remove the supernatant the Sepharose in each tube was washed four times with 2% horse serum-phosphate buffer, the supernatant being removed each time by centrifugation. At the end of the washing cycle the supernatant was removed and the radioactivity in each tube measured in a LKB Ultragamma counter.

Cross-linked bovine serum albumin (BSA) and cross-linked aldolase for use as molecular weight standards were kindly provided by Dr John R. Coggins (Department of Biochemistry, University of Glasgow). Cross-linked BSA contained the following molecular weight species: 68,000; 136,000; 204,000; 272,000; 340,000; 408,000 daltons. Cross-linked aldolase contained the following molecular weight species: 40,000; 80,000; 120,000; 160,000 daltons.

Boiling mix for use in this experiment was made up of 20 mM dithiothreitol (DTT) and 2% SDS in 10mM Na_2PO_4, pH 7·5. 200 microlitres of this boiling mix was added to anti-PAPP-A-Sepharose which had been incubated with the labelled PAPP-A purification product (combined contents of tubes 1 and 2; Table 6.1). The resultant slurry was incubated overnight on a gyratory shaker at room temperature, following which the sample was boiled for 5 minutes. The slurry was centrifuged and a 5 microlitre aliquot of the supernatant counted in a LKB Ultragamma counter. 85 microlitre aliquots of the supernatant were applied to 5% and 3% SDS polyacrylamide tube gels together with 10 microlitres of 0·1% bromophenol blue and 10 microlitres of glycerol. 100 microlitre aliquots of cross-linked BSA and cross-linked aldolase solutions were each added to 50 microlitres of boiling mix and boiled for 5 minutes. 20 microlitres of the aldolase-boiling mix solution was then applied to a 5% polyacrylamide tube gel and 40 microlitres of the

BSA-boiling mix solution was applied to a 3% polyacrylamide tube gel. In addition, 10 microlitres of 0•1% bromophenol blue and 10 microlitres of glycerol were also added to each gel. SDS polyacrylamide tube gel electrophoresis was carried out as described by Maizel (1971).

After electrophoresis the tube gels containing molecular weight markers were stained with 0•2% Coomassie blue and scanned at 600 nm. in a Gilford spectrophotometer fitted with a Model 252 gel scanner. The mobility of any bands present was determined as:

$$R_f = \frac{\text{distance of band from origin}}{\text{distance of bromophenol blue band from origin}}$$

Using the densitometer traces plots were made of R_f vs. logarithm of molecular weight for each series of cross-linked markers. The tube gels containing radioactive samples were snap-frozen in liquid nitrogen and sliced into approximately 20 fractions each using a Mickle gel slicer. The radioactivity in each fraction was measured in a LKB Ultragamma counter and the R_f of any radioactive band present determined.

1.3 Results

A. Analysis of ^{125}I-PAPP-A from the Purification Product Labelled by the Soluble Lactoperoxidase Method

10% SDS Polyacrylamide Tube Gel Electrophoresis

The material extracted from each of the three AACE precipitin arcs formed by ^{125}I-PAPP-A in the purification product resolved into a single radioactive band on 10% SDS polyacrylamide gel electrophoresis. This band was situated near the top of each gel. The level of radioactivity in the area of the gel containing the bromophenol blue marker was slightly raised above the baseline in each gel.

^{125}I-PAPP-A from the same labelled purification product which had not been precipitated on AACE resolved into two radioactive bands on 10% SDS polyacrylamide tube gel electrophoresis; a major band at the top of the gel and a minor band in the upper quarter of the gel. As in all the other gels, the level of radioactivity in the area of the gel containing the bromophenol blue marker was slightly raised above the baseline. The results are shown in Figure 6.1.

7% SDS Polyacrylamide Slab Gel Electrophoresis

The material immuno precipitated from the labelled PAPP-A purification product on anti-PAPP-A-Sepharose and subjected to SDS polyacrylamide slab gel electrophoresis formed an area of intense radioactivity at the junction of the loading and the running gels. The largest of the viral polypeptides examined - 156,000 daltons molecular weight - was found in the running gel: see Figure 6.2.

In the experiments just described penetration of the major radioactive component in the immune precipitated ^{125}I-PAPP-A into 10% SDS polyacrylamide tube gels and a 7% SDS polyacrylamide slab gel was insufficient to allow molecular weight determination. Polyacrylamide gels with a smaller degree of cross-linking - 5% and 3% - were therefore chosen for the purpose of determining the molecular weight of PAPP-A subunits.

B. Analysis of ^{125}I-PAPP-A from the Purification Product Labelled by the Solid Phase Lactoperoxidase Method

5% and 3% SDS Polyacrylamide Tube Gel Electrophoresis

Aliquots of the labelled PAPP-A purification product and the product of "blank" iodination by the soluble lactoperoxidase method were incubated with anti-PAPP-A-Sepharose and horse serum-Sepharose and the percentage of counts immobilized on Sepharose

determined. The results are shown in Table 6.1.

The plots of mobility (R_f) vs. logarithm of molecular weight for cross-linked aldolase and BSA markers were linear over the molecular weight range 40,000 - 160,000 for aldolase and 68,000 - 408,000 for BSA: see Figure 6.3. The distribution of counts in the 5% and 3% SDS polyacrylamide gels containing immune precipitated ^{125}I-PAPP-A is shown in Figure 6.4. Both gels contained two radioactive peaks; in each the second peak was detected as a shoulder on the descending limb of the major peak. The molecular weight of the radioactive components detected on 5% and 3% gels was estimated from plots a and b (Figure 6.3) respectively. The molecular weight of the major protein component detected in the 5% gel (peak 1; Figure 6.4a) was estimated by extrapolation of plot a (Figure 6.3) to be around 180,000. The molecular weight of the minor protein component detected in the 5% gel (peak 2; Figure 6.4a) was estimated directly from plot a (Figure 6.3) as 74,000. The molecular weight of the major protein component detected in the 3% gel (peak 1; Figure 6.4b) was estimated directly from plot b (Figure 6.3) as 180,000. The molecular weight of the minor protein component detected in the 3% gel (peak 2; Figure 6.4b) was estimated directly from plot b (Figure 6.3) as 93,000. These findings are summarised in Table 6.2.

1.4 Discussion

10% SDS polyacrylamide tube gels of total ^{125}I-labelled PAPP-A purification product labelled by the soluble lactoperoxidase method and ^{125}I-PAPP-A extracted from AACE precipitin arcs all contained a radioactive band located very near the top of each gel. It appears likely that this band represents PAPP-A protein. The

TABLE 6.1

Retention of Counts from (1) PAPP-A Purification Product Labelled by the Solid-Phase LPO Method and (2) the Product of 'blank' Iodination by the Soluble LPO Method on Anti-PAPP-A-Sepharose 6B and on Horse Serum-Sepharose 6B.

Incubation mixture	Counts in incubation mixture (c.p.s.)		Counts immobilised on Sepharose (c.p.s.)		
	Tube 1	Tube 2	Tube 1	Tube 2	Average (%)
(i) PAPP-A purification product[a] + anti-PAPP-A-Sepharose	21,954	22,536	6,165	6,744	29·0
(ii) PAPP-A purification product + horse serum-Sepharose	21,778	22,085	852	827	3·8
(iii) 'Blank' product[b] + anti-PAPP-A-Sepharose	21,049	20,957	1,981	1,511	8·3
(iv) 'Blank' product + horse serum-Sepharose	20,210	20,010	1,649	1,630	8·2

a PAPP-A purification product labelled by the solid-phase LPO method: for details see Chapter 5.

b product of 'blank' iodination by the soluble LPO method: for details see Chapter 5.

TABLE 6.2

Molecular Weights of the Species Detected on 5% and 3% SDS-Polyacrylamide Tube Gel Electrophoresis of Immune Precipitated ^{125}I-PAPP-A[a]

Material applied to gels	Molecular weight of species detected as radioactive bands[b] (daltons)			
	5% Gel		3% Gel	
	Band 1	Band 2	Band 1	Band 2
Immune precipitated ^{125}I-PAPP-A	180,000	74,000	180,000	93,000

a estimated from plots a (5% gel) and b (3% gel): see Figure 6.3.

b see Figure 6.4.

origin of the radioactivity associated with the bromophenol blue band in each gel is unknown but it may represent free ^{125}Iodine (as in Figure 5.7). The identity of the minor radioactive band detected only on the SDS polyacrylamide gel of total ^{125}I-labelled PAPP-A purification product is unknown but a strong possibility must be lactoperoxidase protein, since Sepharose 6B gel filtration has already indicated this protein as the main contaminant of the PAPP-A purification product labelled by the soluble lactoperoxidase method (see Figure 5.7). 7% SDS polyacrylamide slab gel electrophoresis of solid phase immune precipitated PAPP-A labelled by the soluble lactoperoxidase method revealed that the main component was a species of slow mobility and molecular weight greater than about 150,000 daltons. It was therefore concluded that the molecular weight of PAPP-A subunits was greater than 150,000 daltons.

Incubation of duplicate samples of : (1) PAPP-A purification product labelled by the solid phase lactoperoxidase method and (2) product of "blank" iodination by the soluble lactoperoxidase method (ie. labelled lactoperoxidase) on anti-PAPP-A-Sepharose and on horse serum-Sepharose produced remarkably consistent results (Table 6.1). The extent of non-specific retention of counts from labelled lactoperoxidase was fairly high and constant (8•2%--8•3%) on both anti-PAPP-A-Sepharose and horse serum-Sepharose. The extent of non-specific retention of counts from labelled PAPP-A purification product on horse serum-Sepharose was much smaller (3•8%) and a significant difference was observed in the amount of counts retained from PAPP-A purification product on anti-PAPP-A-Sepharose (29%). This finding suggests that the bulk of material retained from PAPP-A purification product labelled by the solid phase lactoperoxidase

method on anti-PAPP-A-Sepharose was adsorbed by a specific immunological reaction.

Analysis of solid phase immune precipitated ^{125}I-PAPP-A from the purification product labelled by the solid phase lactoperoxidase method on 5% and 3% SDS polyacrylamide tube gels revealed the presence of two components of different molecular weight on each (Figure 6.4). As predicted, penetration of these components into the gels was sufficient to allow molecular weight determination. The molecular weight of the major component was determined as approximately 180,000 daltons (Table 6.2). The molecular weight of the minor component was estimated as 74,000 daltons on the 5% gel and 93,000 daltons on the 3% gel. The different values obtained for the molecular weight of the minor component may be due to different standards used to calibrate the gels or alternatively it may indicate the presence of two or more closely migrating minor components. The nature of the minor component (or components) is unknown, but one possibility is that it represents a product of poteolysis. One way of testing this possibility would be to examine the degradation products produced by treatment of PAPP-A with trypsin. At present, however, it is not possible to decide on the basis of the available evidence whether the 74,000 - 93,000 component(s) is a subunit of PAPP-A, a degradation product of a subunit of PAPP-A or a contaminating protein polypeptide (for example lactoperoxidase).

On the evidence of the findings described here it may be concluded that PAPP-A, whose molecular weight has been estimated as 750,000 daltons (Lin et al., 1974) contains polypeptide subunits of approximately 180,000 daltons and may therefore be composed of up to four such subunits. In addition it may also contain subunits

of molecular weight between 74,000 - 93,000 daltons. Confirmation of the findings described here and further investigations, such as treatment with urea under reducing and non-reducing conditions (to establish the number of disulphide-linked subunits present) will have to await purification of a sufficiently large quantity of PAPP-A to allow analysis to be carried out directly on the unlabelled protein. Since PAPP-A is a glycoprotein (Lin et al., 1974), final interpretation of the results of SDS polyacrylamide gel electrophoresis will also have to await determination of its carbohydrate content. This is especially so since it is known that glycoproteins containing more than 10% carbohydrate behave anomalously during SDS polyacrylamide gel electrophoresis when compared to standard proteins (Bretscher, 1971; Segrest et al., 1971); they show decreased mobility and a higher apparent molecular weight. Different approaches may therefore be necessary for the estimation of their actual molecular weight (Segrest and Jackson, 1972).

FIGURE 6.1

Analysis of the PAPP-A Purification Product and of Immune-Precipitated ^{125}I-PAPP-A by 10% SDS Polyacrylamide Tube Gel Electrophoresis

The PAPP-A purification product had been labelled by the soluble lactoperoxidase method.

Polyacrylamide gels were sliced following electrophoresis and the radioactivity in each slice measured.

The gels are:

-□-□- (1) Labelled purification product (17,908 c.p.s. applied)

-●-●- (2) Material extracted from the AACE precipitin arc formed by ^{125}I-PAPP-A in the labelled purification product (16,057 c.p.s. applied)

-■-■- (3) Material (2) above together with pooled term maternal serum which had been added to the AACE plate (17,601 c.p.s. applied)

-○-○- (4) Material (2) above which had been added to the AACE plate together with pooled non-pregnant adult serum (16,459 c.p.s. applied).

The smallest radioactive peak near the bottom of each of the four gels corresponds to the position of the Bromophenol Blue band.

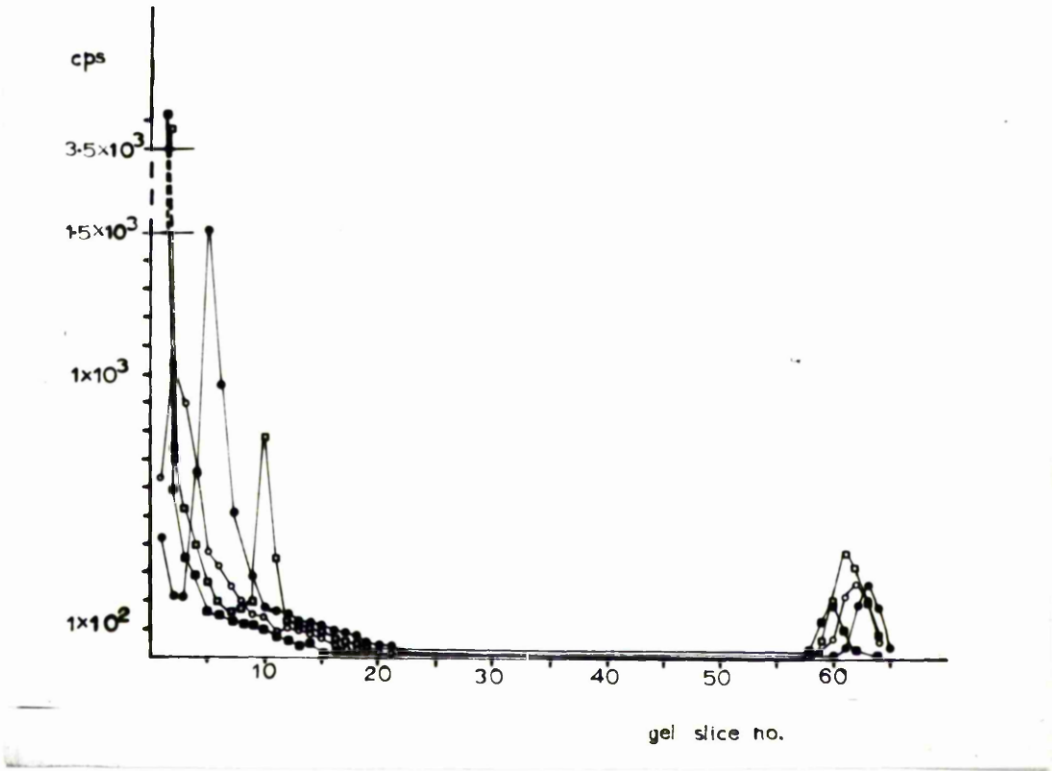

cps
3·5x10³
1·5x10³
1x10³
1x10²
10
20
30
40
50
60
gel slice no.

FIGURE 6.2

Analysis of Immune Precipitated ^{125}I-PAPP-A by 7% SDS Polyacrylamide Slab Gel Electrophoresis

Track (a) shows the mobility of a set of five calibrated viral polypeptides which are marked by black dots to the left of the track. Their molecular weights are 156,000, 136,000, 120,000, 66,000, and 42,000 daltons.

The PAPP-A purification product had been labelled by the soluble lactoperoxidase method.

In track (b) is ^{125}I-labelled PAPP-A which had been dissociated from anti-PAPP-A-Sepharose.

The origin and cathode are at the top of the gel. Part of the loading gel has been excluded from the plate. The horizontal line - marked by a star to the right of the gel - shows the interface between the loading and the running gels.

FIGURE 6.3

Plot of Mobility (R_f) vs. Logarithm of Molecular Weight

(a) Cross-linked aldolase subjected to electrophoresis in 5% SDS polyacrylamide tube gel.

(b) Cross-linked BSA subjected to electrophoresis in 3% SDS polyacrylamide tube gel.

Molecular weight of the species in cross-linked aldolase:

40,000; 80,000; 120,000; 160,000.

Molecular weight of the species in cross-linked BSA:

68,000; 136,000; 204,000; 272,000; 340,000; 408,000.

R_f was calculated as: $\frac{\text{Distance of band from origin}}{\text{Distance of Bromophenol Blue band from origin}}$

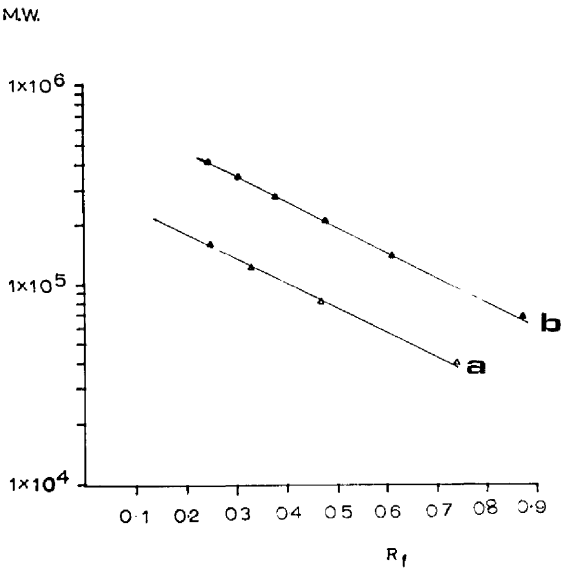
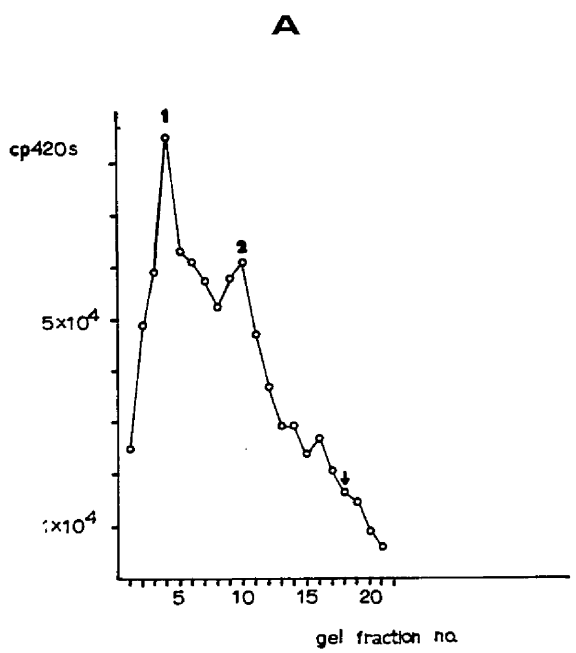

M.W.
1x10^6
1x10^5
1x10^4
0·1 02 03 04 05 06 07 08 0·9
R_f
b
a

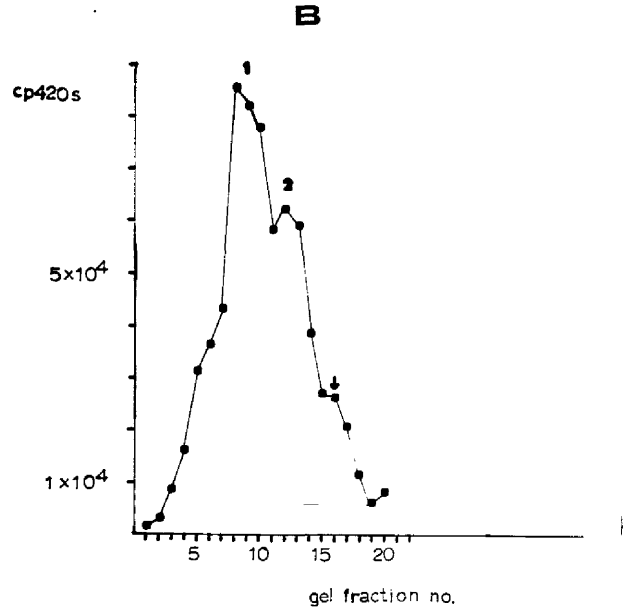
FIGURE 6.4

Radioactivity in Slices of (a) 5% and (b) 3% SDS-Polyacrylamide Tube Gels Following Electrophoresis of Immune-Precipitated ^{125}I-PAPP-A

The PAPP-A purification product had been labelled by the solid-phase lactoperoxidase method. ^{125}I-PAPP-A was dissociated from anti-PAPP-A-Sepharose and 4,220 c.p.s. applied to each gel.

The peaks are numbered (1) and (2). The arrow indicates the position of the bromophenol blue band.


A
cp420s
5x10^4
1x10^4
1
2
5
10
15
20
gel fraction no.


B
cp420s
5x10^4
1x10^4
1
2
5
10
15
20
gel fraction no.

CHAPTER 7 STUDIES ON THE CONCENTRATION OF PAPP-A DURING NORMAL AND COMPLICATED PREGNANCY

1. INTRODUCTION

The possibility that placental-specific proteins might be of use in the diagnosis of certain complications of pregnancy has been examined in Chapter 1 section 4.1. Prior to the present work being undertaken the only published work on the application of PAPP-A to obstetric diagnosis has been a study by Lin and his colleagues (1977). Their findings indicated that PAPP-A concentration may be increased by toxemia, but is not affected in pregnancies complicated with insulin-dependent diabetes. In the present study the concentration of PAPP-A in the blood during normal pregnancies and pregnancies complicated by intrauterine growth retardation, diabetes or a variety of other conditions was assayed by antibody-antigen crossed electrophoresis (AACE). The assay employed the monospecific sheep anti-PAPP-A antiserum which I prepared previously (see Chapter 5 section 2.a.2).

2. MATERIALS AND METHODS

The concentration of PAPP-A in the serum of 53 patients attending the University Hospital in Aberdeen was assayed by the author. Samples of blood collected from these patients were kindly provided by Dr C.W. Horne (Department of Pathology, Department of Medicine, Aberdeen). The composition of this group of patients was as follows: - normal pregnancies (34 patients); pregnancies complicated by intrauterine growth retardation (19 patients). As my research was drawing to a close, a further 294 patients were made available by our clinical colleagues, Dr C.W. Horne (Aberdeen) and Dr A.B. MacLean (formely of Department of Midwifery, Queen Mother Hospital, Glasgow;

currently of Department of Obstetrics and Gynaecology, University of Christchurch, New Zealand). Of these 294 patients, 249 were attending Queen Mother's Hospital, Glasgow; the remaining 45 patients were attending the University Hospital in Aberdeen. The composition of the two groups of patients is summarised in Table 7.1. Noteworthy in particular are the 45 diabetic patients from Aberdeen. Using my antiserum, my supervisor carried out the necessary AACE assays with the technical assistance of S. Gibb.

The clinical status of all of the patients was assessed without knowledge of the concentration of PAPP-A in their blood.

Biochemical and immunochemical methods

In most cases blood was collected, allowed to clot and the serum was kept for PAPP-A analyses. In some cases plasma was obtained from blood treated with lithium-heparin. Data has been presented by Sutcliffe et al. (1981) which shows that the level of PAPP-A measured in blood is not affected by this anti-coagulent. After centrifugation, serum and plasma samples were divided into aliquots and stored at -20^{o} C.

The concentration of PAPP-A was assayed by the one-dimensional antibody-antigen crossed electrophoresis method of Laurell (1966) with the modification that 100 mM-tris was present in the gel and tank buffers. A further modification was that the direction of current flow in the electrophoresis tank was reversed after 3 hours (the orientation of the AACE plates was also reversed). The latter method was adopted by Lin et al. (1974b), since the buffer capacity of the system is too small to support electrophoresis in one direction for 17 hours without changes in the pH occuring. During electrophoresis, Sutcliffe and Gibb found that pH differences of as little as 0·2 pH

TABLE 7.1

Details of the patients included in the study

	Glasgow Series	Aberdeen Series
A. Normal Pregnancies	107	34
B. Pregnancies with Complications:		
Intrauterine growth retardation	18	19
Diabetes		
i. Insulin-treated	-	15
ii. Gestational	-	30
Oedema of the leg/ankle	7	-
Elevated blood pressure		
i. During pregnancy only	10	-
ii. Outwith pregnancy also	4	-
Large infant, weight over 4 kg.	5	-
Abnormal maternal weight gain	9	-
Twins	4	-
Spontaneous abortion	4	-
Threatened abortion	8	-
Premature labour	12	-
Placenta praevia	1	-
Retained placenta	1	-
Polyhydramnios	1	-
Caesarian section for:		
i. Failed induction	3	-
ii. Fetal distress	3	-
iii. Previous section	8	-
Antepartum haeorrhage	11	-
Postpartum haemorrhage	5	-
Others	28	-
Total	249	98

units were accompanied by blurring and elongation of the precipitin arcs. The antiserum used for AACE was monospecific sheep anti-PAPP-A (for details of preparation see Chapter 5 section 2.a.2) at a final concentration 1·2% (v/v). Between 2 and 20 microlitre of patient serum (or plasma) was added to the sample wells. All samples were assayed in duplicate, each duplicate being carried out on two separate occasions. The mean concentration of PAPP-A is expressed in microgram. per ml., based on a primary standard of purified PAPP-A (Sutcliffe et al., 1980). For reliable quantitation, the present assay has a sensitivity of 9·5 micogram. PAPP-A per ml. (13 pmole per ml.).

The determination of PAPP-A concentrations was carried out on coded samples.

Statistical methods

Statistical analysis was performed by A.W. Bowman (Department of Statistics, University of Glasgow) and my supervisor.

Preliminary plotting and data sorting was carried out using the statistical computing package, MINITAB (Ryan et al., 1976). Statistical analyses were carried out on observations made after the twenty-eighths week of gestation only since there was little information on complicated pregnancies before this period. The square root transformation was applied to the data to remove skewedness. Analyses of variance (Scheffe, 1959) were used in three different situations, as follows:

(a) An examination for the control data of the effect of the baby's sex and place of birth (Aberdeen or Glasgow).

(b) For the Aberdeen series, the effect on PAPP-A levels of both the sex of the baby and the presence of maternal complications.

(c) As for (b) but including the Glasgow data as well. In

each case models were fitted using the statistical computing package: GLIM (Baker and Nelder, 1978).

In a considerable number of patients repeated blood samples were obtained at different stages of gestation. This was especially in the case of the Aberdeen series. As this might induce correlations the statistical analyses were repeated after all but one (randomly chosen) sample had been omitted from each of these patients. The conclusions obtained were similar to those resulting from the full data set. After the analyses of variance had shown that there was a significant effect due to complications of pregnancy, the technique of multiple comparisons (Scheffe, 1959) was used to identify which of the complications were associated with significant differences in PAPP-A levels (Wallenstein et al. (1980) have provided a critic of this type of analysis.).These analyses were carried out on data after 28 weeks of pregnancy: when the concentration of PAPP-A was rising. It was therefore a possibility that any apparent difference in mean PAPP-A values between two groups of patients might be due to differences in the distribution of samples during pregnancy, rather than to a real difference in PAPP-A levels at comparable stages of gestation. This possibility was excluded by testing subsets of the results.

3. RESULTS

PAPP-A in the peripheral blood during normal pregnancy

PAPP-A was first detected by AACE from 14 weeks of pregnancy in normal patients. As normal pregnancy proceeded, levels rose (Figure 1) and individual values became scattered with a skewed distribution (Figure 2). This skewedness also occured in some of the

groups of complicated pregnancies (Figure 2). To reduce this skewedness and to permit parametric statistical analyses, the data were transformed by expressing values as the square root of the PAPP-A concentration. For this reason, the results in Figure 1 have been plotted on a square root, not a linear scale.

An analysis of variance revealed no significant difference between the controls for the Aberdeen and Glasgow series. For the Aberdeen series, there was no detectable effect of the babies' sex on PAPP-A values. However, from about 28 weeks of pregnancy until term it seemed that normal pregnancies in the Glasgow series had somewhat greater PAPP-A values if the baby was a male rather than a female. This was confirmed by an analysis of variance.

Sutcliffe and Bowman investigated the possibility of relationships between the weight of the baby at delivery and the concentration of PAPP-A in maternal serum. Male and female babies were distinguished and divided into four groups according to the following periods of pregnancy: 14-20 weeks, 21-30 weeks, 30-36 weeks, 37-42 weeks and 38-41 weeks. In no case did any correlation or even the suggestion of a trend appear. Similarly, no effect of parity was detected.

PAPP-A in peripheral blood of complicated pregnancies

Growth retardation

No significant differences were detected when patients with intrauterine fetal growth retardation were compared with controls (Figure 2). In this group the weight of the baby at delivery ranged from 0·94 to 2·50 Kg.

Diabetes

When insulin-treated diabetics were compared with gestational diabetics and controls (Figures 2, 3 and 4), analysis of variance and

multiple comparisons showed that insulin-treated diabetics had significantly lower levels of PAPP-A in their blood than had controls or gestational diabetics. No significant differences could be detected between gestational diabetics and controls, although the former had a very wide range of values including some of the highest in the study. No effect of the sex of the baby was noted on PAPP-A values.

Other complications

PAPP-A values for all the other complication (see Table 7.1) were plotted out against gestation. No significant differences from control values were observed.

4. DISCUSSION

The concentration of PAPP-A in the blood of patients at term ranged from 0·03 to 0·31 mg. per ml. This compares well with the results of Hughes et al. (1980) who based their data on a standard of pure PAPP-A which was purified in a different manner (Bischof, 1979) to the protein standard used in the present work: see Sutcliffe et al., 1980. In the control data of the Glasgow series, fetal sex was found to significantly effect PAPP-A values with males' levels being somewhat higher than females' levels from 30 weeks of gestation onwards. In the last 10 weeks of gestation in the Glasgow series the mean PAPP-A value for females was 68% of that in males. A similar effect of fetal sex on PAPP-A values was observed by Lin et al. (1974b). In their study during the last month of gestation the mean PAPP-A value for female fetuses was approximately 66% of the value in male fetuses. However in the present work a significant sex difference was not observed in the

other classes of patients and in particular in the Aberdeen controls. This discrepancy between the Aberdeen and Glasgow series is difficult to account for. Both sets of control patients were selected on a random basis from the out-patient clinic. The only difference apart from geographical location seems to be that the Aberdeen controls included many more repeat samples than the Glasgow controls: the relatively fewer individual pregnancies screened may have resulted in poor sampling. One can only conclude that a sex difference may contribute to the variance in PAPP-A values in the latter part of the pregnancy.

Prior to the present study being undertaken there had been no reports in the literature on studies on the effect of intrauterine growth retardation on levels of PAPP-A in maternal blood. Intrauterine growth retardation is a diagnosis made at birth when the baby's weight is in or below the tenth percentile of the mean weight for gestation. These small-for-weight babies are subject to a higher neonatal mortality and morbidity. Since the probable cause of intrauterine growth retardation is fetal malnutrition (Naeye, 1965) attention has in recent years been focussed on the *in utero* diagnosis of this condition. The most common test hitherto employed combines serial ultrasonic cephalometry with serial estriol excretion measurements. This combination of tests has stood favourable comparison with other methods including the measurement of HPL concentration in maternal blood (Robinson *et al.*, 1973, 1974). However as has been previously observed (Chapter 1, Section 4.1) the estrogen excretion test is not completely reliable. The measurement of creatine in amniotic fluid has been found to be useful in assessing fetal maturity (Begneand *et al.*, 1969) but is less convenient to perform

than the measurement of substances in maternal blood or urine. A rapid method for assessing intrauterine growth retardation by radioactive amino acid uptake by the placenta has been described by Garrow and Douglas (1968). This method however suffers from several drawbacks such as low uptake ratios and the need to introduce radioactive substances into the body.

The full potential of assays of placental-specific proteins in maternal blood will not be understood until the physiological function of these proteins and the relationship between their production and other aspects of placental function, such as transport, is known. Nevertheless studies are currently in progress aimed at establishing which, if any, of the placental-specific proteins may be of practical value in the prediction of intrauterine growth retardation: see Chapter 1, Section 4.1. The application of the HPL test to intrauterine growth retardation diagnosis has been the subject of more studies than any other placental-specific protein. These studies have yielded contradictory results (see Chapter 1, Section 4.1; Spellacy et al., 1966; Sciarra et al., 1968; Saxena et al., 1969; England et al., 1974; Lindberg and Nilson, 1974; Kelly et al., 1975) and the test has no established clinical use. Spellacy et al. (1974) in a large series found that the women most likely to have abnormally low HPL concentrations in the third trimester of pregnancy are those in which intrauterine growth retardation arises secondary to other diseases such as pre-eclampsia or chronic renal disease. Low levels of another placental-specific protein, SP1, in the third trimester of pregnancy have been reported to be associated with intrauterine growth retardation (Tatra 1977) and the SP1 test has been claimed to be of greater value than HPL in predicting intrauterine growth retardation (Gordon et al., 1977; Towler et al., 1977; Horne et

al., 1979). All the other placental-specific proteins investigated have proved disappointing in clinical practice. In the present study no significant differences in the levels of PAPP-A in third trimester maternal blood were detected when patients with intrauterine growth retardation were compared with normal pregnant women. An independent study by Hughes et al. (1980) has yielded similar results. These findings indicate that PAPP-A concentration in maternal blood may not be used as an index of placental efficiency and fetal nutrition.

This is one of the first reports on the concentration of PAPP-A in gestational as well as insulin-dependent diabetic pregnancies. Previously Lin et al. (1977) had found no significant difference between 26 insulin-dependent diabetes and controls during the last month of pregnancy. They did not study gestational diabetes. Pregnancy is a diabetogenic experience which increases insulin needs in the established diabetic. Advancing pregnancy imposes a progressive stress on the pancreatic beta cell reserve and, where the effective insulinogenic reserve is inadequate, abnormal carbohydrate metabolism ensues and gestational diabetes. Diagnosis of diabetes in pregnancy may be accomplished by means of several tests of insulinogenic reserve in addition to the standard glucose tolerance tests (Kaplan, 1961; Kyle et al., 1964; Mestman et al., 1971). There appears to be a stage of diabetes which is incapable of identification biochemically at present (Camerini-Davalos et al., 1970). During this unpredictable stage women who will later develop diabetes deliver an unusually high proportion of overweight babies and in the 2 - 3 years immediately preceding overt diabetes there is a raised perinatal mortality rate (Metz, 1975). A test is yet to be described which will directly or indirectly detect the metabolic abnormalities which accompany diabetes and which could be used

in the diagnosis of pre-diabetes.

The well supervised diabetic now has a good chance of a successful pregnancy. The higher perinatal mortality rate associated with diabetic pregnancies (15 - 20%) may be significantly reduced by good maternal metabolic supervision and the proper timing of delivery (Kyle, 1975). It is known that in the diabetic pregnancy the risk of fetal loss *in utero* increases as term approaches. At the same time the risk of neonatal death is increased when pregnancy is terminated before the 37th week of pregnancy (Hagbard, 1961). Early termination is therefore justified only if there is no corresponding increase in fetal loss. This consideration has led to the search for some physiologic measurement which could serve as a means of detecting early fetal stress. Despite its limitations, discussed in Section 4.1 of Chapter 1, the most widespread test hitherto used has been an estimation of estriol or total estrogens in a 24 hour specimen of urine. The fetal death rate associated with diabetic pregnancies has been reduced to 1% by Gabbe *et al*. (1977) who used daily 24 hour estrogen determinations in combination with weekly contraction tests to time the delivery of the baby. Gabbe *et al*., 1977b also reported improved control of the perinatal death rate with the use of plasma estriol assays. However, as has been discussed in Chapter 1, Section 4.1, other workers have not been able to demonstrate the superiority of assays of plasma estriol and estradiol-17 to that of urinary estrogens. In addition, ultrasonography has been used in determining the date of delivery since the size of the fetus is known to affect its chance of survival (Gellis and Hsia, 1959). Of all the placental-specific proteins HPL has been most extensively investigated for its possible value as a marker of fetal welfare. Studies of HPL levels in pregnancy have failed so far to

establish its clinical value. Investigations of other placental-specific proteins such as HCG, heat-stable alkaline phosphatase or histaminase have not so far produced any clinically useful markers of fetal welfare. The clinical value of the other placental-specific proteins such as SP1 has not yet been properly assessed. However, plasma levels of SP1 in pregnancy have been reported to be related to the maternal blood sugar level and carbohydrate metabolism (Tatra et al., 1976a,b; Singh et al., 1979) and there is some evidence to suggest that plasma levels of several other placental-specific proteins - HPL, HCG and PAPP-B - may be altered in pregnancies complicated by insulin-dependent diabetes (see Chapter 1 Section 4.1).

In the present study no significant increase in the mean value could be detected in the concentration of PAPP-A in patients with gestational diabetes compared with controls, although some very high values were found in that type of diabetic. It is not known whether those high values relate to the extent to which the patients' diabetes was controlled. In the group of insulin-controlled diabetics the mean concentration of PAPP-A was reduced to 55% of the mean for gestational diabetics and to 65% of that in the controls. Both of these differences were significant at $p=0{\cdot}05$. It is not known why these findings differ from those of Lin et al. (1977) though differences in patient type and management leave open many possibilities. The present results on insulin-dependent diabetics suggest that plasma levels of PAPP-A may be influenced by maternal carbohydrate metabolism. The significance of these results for placental physiology will require further investigation.

FIGURE 7.1

The Concentration of PAPP-A in Normal Control Patients (N) From the Glasgow (G1) Series Plotted Against Gestation Time (weeks)


Male fetus


Female fetus

The linear axis of PAPP-A concentration is in square root units and the corresponding units in mg. per ml. are shown at the right hand side of the graph.

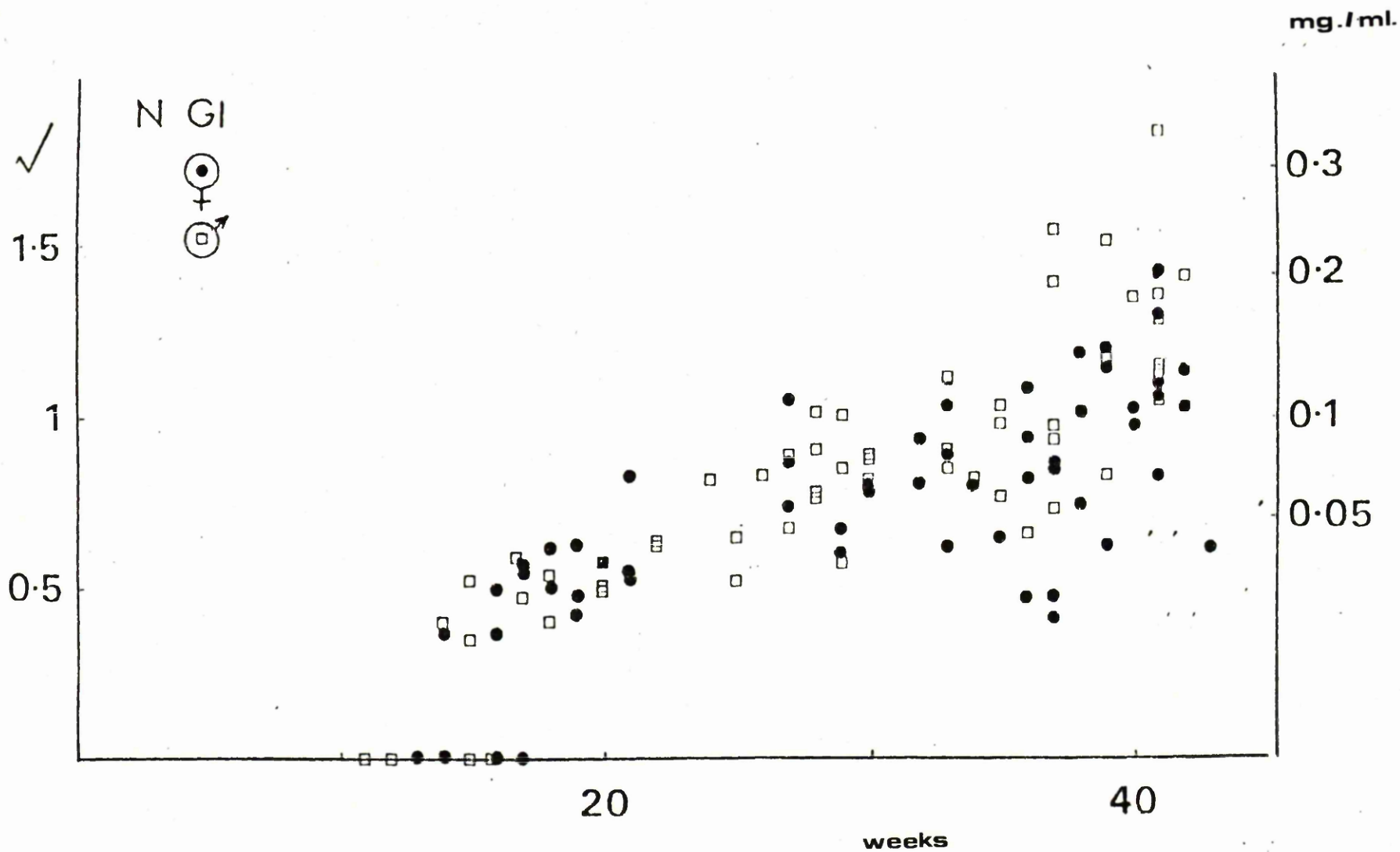

mg./ml.
N GI
√
1·5
1
0·5
0·3
0·2
0·1
0·05
20
40
weeks

FIGURE 7.2

Summary of the PAPP-A Concentration in Maternal Serum from 28 Weeks of Pregnancy to Term

The data groups above the dotted line are from the Glasgow series and those below the line are from the Aberdeen series.

Each data group is divided into:

Male fetus

Female fetus

N - Normal control patients

R - Intrauterine growth retardation

G - Gestational diabetes

I - Insulin-treated diabetic

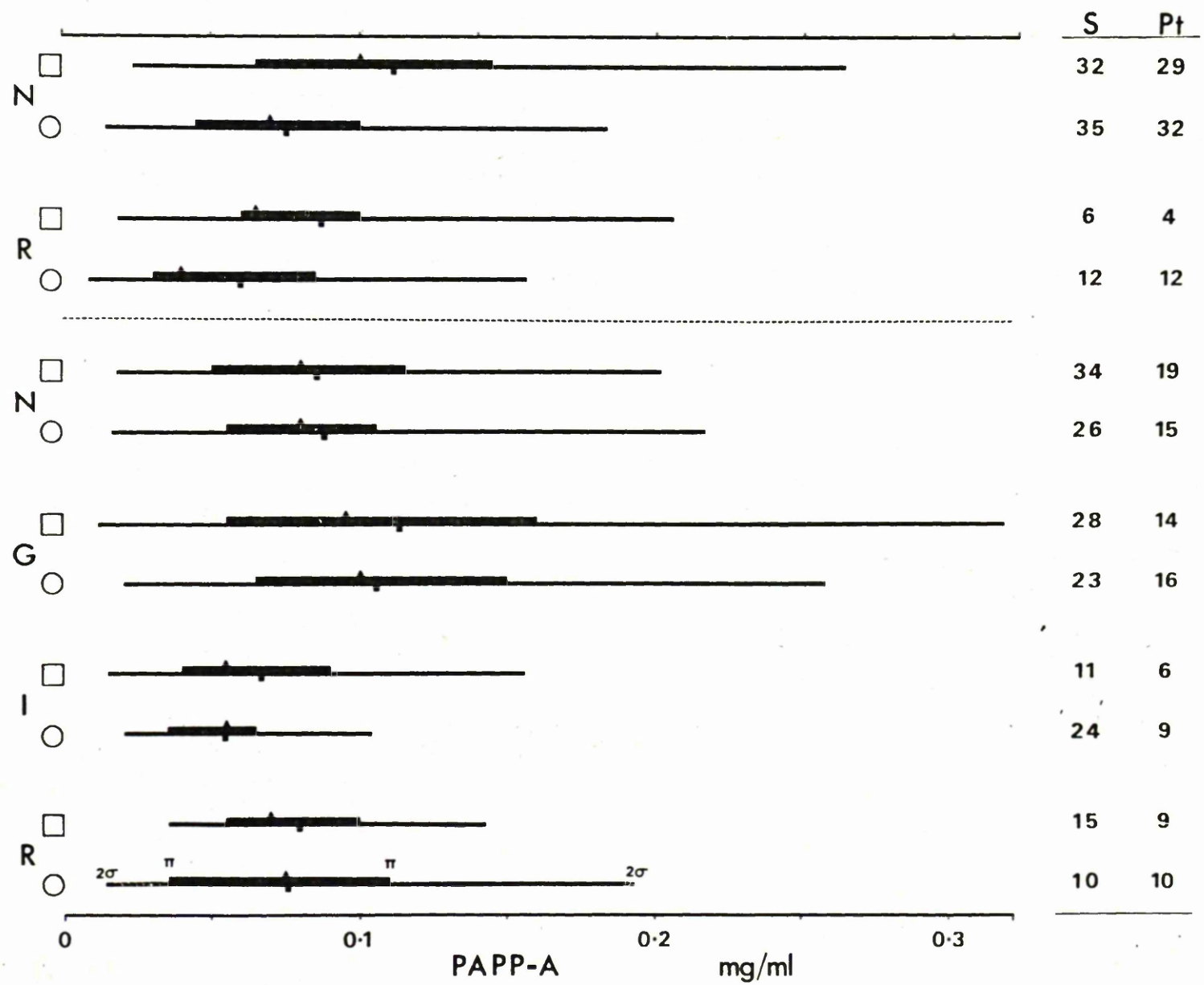
The figures at the right hand side represent the number of patients in each data group (under the heading Pt) and the number of individual blood samples which were assayed in the data group (under the heading S).

As outlined in Section 4, Materials and Methods, most of the Aberdeen patients had more than one blood sample analysed during the course of their pregnancy.

The data bars were drawn as follows (see the bottom data bar in the Figure):

The mode (▲) and 25 percentiles (π) were calculated from the data in mg. PAPP-A per ml.

The mean (■) and $\pm$ 2 standard deviations (2σ) were calculated from the square root transformed data ($mg.^{-2}$ PAPP-A per ml.) and then converted to mg. PAPP-A per ml. for this Figure.



Courtesy of R.G.S. and A.W.B.

FIGURE 7.3

The Concentration of PAPP-A in Mothers Affected with Gestational Diabetes (▲) or Insulin-Treated Diabetes (△)

To avoid confusion, repeat samples have been joined up for the gestational diabetics only: see Figure 7.4 for the insulin-treated cases.

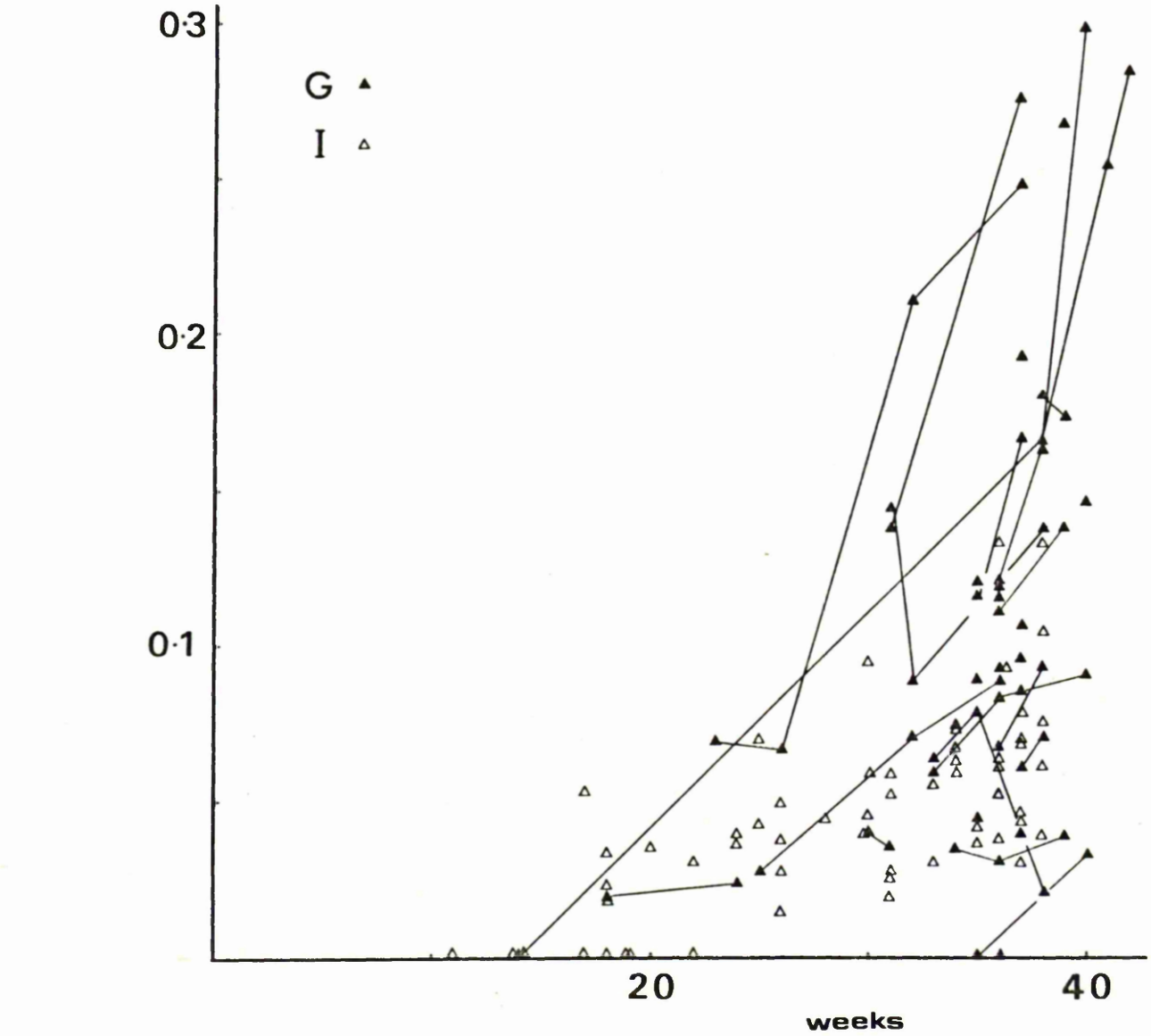
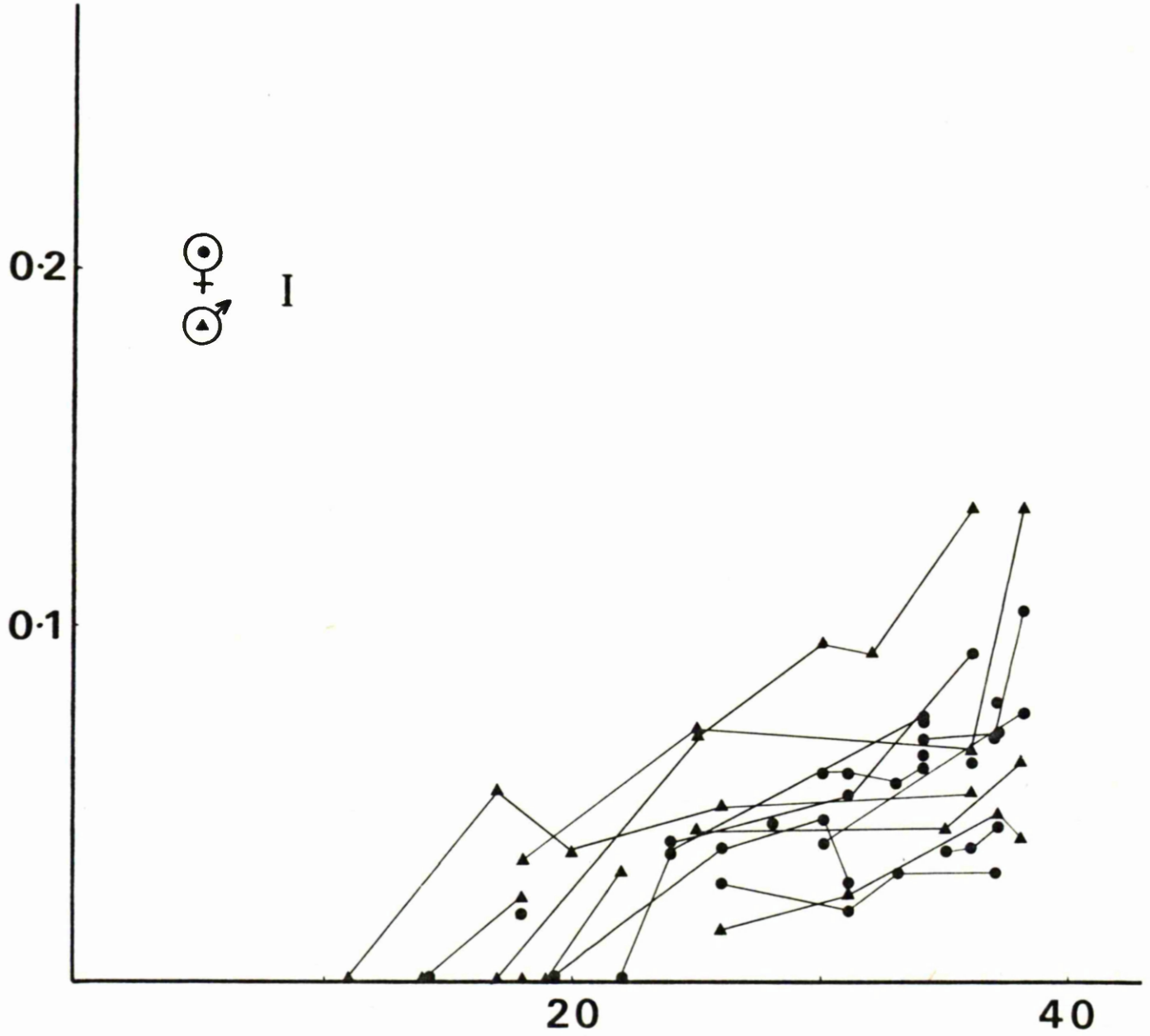

mg./ ml.
0·3
0·2
0·1
G
I
20
40
weeks

FIGURE 7.4

The Concentration of PAPP-A (mg. per ml.) in Cases of Insulin-Treated Diabetes

Males (▲) and females (●) are distinguished.

The lines between the points connect repeated samples from the same patient.


0·2
0·1
I
20
40

CHAPTER 8 CONCLUSIONS AND PROSPECTS

The purpose of this chapter is to review the main conclusions drawn in the preceding chapters, discuss how the aims of this thesis, set out in Chapter 1, Section 6, have been achieved and to suggest directions for further experimental work.

The studies described in Chapter 2 were aimed at detecting antibodies in maternal sera directed against placental-specific antigens. Maternal sensitisation to fetal alloantigens has been well documented but sensitisation to specific products of the placenta has not been uneqivocally demonstrated. It has been suggested that antibodies or soluble antibody-antigen complexes act as specific blocking factors to cellular immunity in pregnancy: see Chapter 1, Section 4.3a. Antibodies to placental-specific proteins were not detected in the present study using techniques which do not depend on the availability of pure antigens i.e. immunofluorescence and gel precipitation. A method has been devised for the dissociation of antigens of molecular weight 10, - 100,000 daltons from soluble antibody-antigen complexes which was successfully applied to the dissociation of albumin from albumin-anti-albumin complexes formed in antigen excess.Application of this method to multiparous sera produced no firm proof for the involvement of any placental antigens of molecular weight 10, - 100,000 daltons in immune complex formation during pregnancy. Although SP1 was present in trace amounts in the dissociated material, its molecular weight (100,000) and the fact that the bulk of it was detectable in the retentate at the end of dissociation argued against a conclusion that it had been dissociated from immune complexes. The involvement of SP1 in immune complex formation during pregnancy nevertheless remains a possibility which will need to be investigated further. The best approach in future studies might be to investigate the presence

of maternal antibodies to individual placental-specific proteins when purified preparations of these proteins become available. Much effort is at present directed at the purification of such proteins. The development of sensitive antigen-specific methods of immune complex detection is also an area of current scientific endeavour.

The main experimental effort described in this thesis has been directed at increasing the state of knowledge of placental-specific proteins. In Chapter 3 the use of negative antibody affinity chromatography (NAAC; the technique which has been described in Chapter 1, Section 5) in the search for specific placental proteins was presented. This approach has revealed several placental proteins, in addition to HPL and SP1, at least three of which appeared to be placental-specific: PAPP-A (see Chapter 1, Section 3.1) and two other antigens whose identity has not been established. These results, which are subject to variations in rabbit responsiveness and the limitations inherent in the gel precipitation methods used to assay the response, nevertheless provide an indication of the value of NAAC technology in the search for new tissue-specific proteins.

Before the purification of PAPP-A for the first time by antibody affinity chromatography was undertaken it was necessary to assess the extent to which a protein was irreversibly denatured by a variety of commonly used dissociants. This was assessed by one-dimensional AACE. Application of this test to a sample of serum proteins showed that exposure to several dissociants frequently resulted in irreversible denaturation of the proteins. This finding draws attention to a relatively neglected aspect in purification protocols; in several published studies no attempt has been made to assess the effect of the dissociants used on the immunoprecipitating behaviour of antigens prior

to purification and the purified antigens were assayed by double immunodiffusion or immunoelectrophoresis rather than AACE (see for example Kostner, 1972; Hoag et al., 1975; Houwen et al., 1975; Bohn et al., 1976; Fitsche and Mach, 1977; Johnson and Garvey, 1977). In the present study AACE was found to be superior to double immunodiffusion for examining the denaturation of antigens. The test allowed the selection of a suitable dissociating agent for use in the purification of PAPP-A by antibody affinity chromatography.

The purification and study of PAPP-A from term maternal serum has been described in Chapters 5 and 6. The purification product, which was shown to contain 427-fold purified PAPP-A when assessed by AACE was iodinated and analysed by AACE, Sepharose 6B gel filtration and SDS polyacrylamide gel electrophoresis. The purification product labelled by soluble lactoperoxidase was found to contain two bands on 10% SDS polyacrylamide gel electrophoresis: (1) a major band which was located very near the top of the gel as was the single band formed by immune-precipitated ^{125}I-PAPP-A; this band was therefore attributed to PAPP-A polypeptide and (2) a minor band not detected in immune-precipitated ^{125}I-PAPP-A and therefore attributed to lactoperoxidase. The purified PAPP-A had a molecular weight and electrophoretic mobility similar to native PAPP-A in maternal serum. Immune-precipitated ^{125}I-PAPP-A was found to contain a major component of approx. 180,000 daltons and a minor component of between 74, - 93,000 daltons. It was concluded that PAPP-A whose molecular weight has been estimated as 750,000 daltons (Lin et al., 1974b) may contain up to four 180,000 daltons polypeptide subunits. The nature of the minor component will need to be investigated further. Final interpretation of these results will also have to await purification of a sufficient quantity of PAPP-A to allow further analysis

such as the determination of carbohydrate content and SDS polyacrylamide gel electrophoresis in the presence and absence of reduction to be carried out directly on the unlabelled protein. In most cases in which a specific placental protein has been thoroughly characterised it has been found to be very similar in physicochemical and sometimes also immunological properties to a protein present in the normal non-pregnant (see Chapter 1, Section 3.1) and the hypothesis has been put forward that all placental proteins are analogues of proteins present in the normal adult (Gordon and Chard, 1979). Proteins showing physicochemical homologies with PAPP-A should therefore be sought. The known molecular weight and electrophoretic mobility of PAPP-A and the finding reported here that it may be composed of subunits of approx. 180,000 daltons and in addition contain subunits, or subunit fragments of 74, - 93,000 daltons suggest that it may be structurally related to $alpha_2$-macroglobulin. The molecular weight of $alpha_2$-macroglobulin has been reported as between 620, - 820,000 daltons (see Hall and Roberts, 1978) and it is known to be a homotetramer (see Swenson and Howard, 1979) consisting of two 370,000 daltons protomers each of which contains two disulphide-linked polypeptides of 185,000 daltons (Frenoy and Bourrillon, 1974). The polypeptides of $alpha_2$-macroglobulin can be cleaved to two 85,000 daltons fragments by a variety of endopeptidases (Harpel, 1973; Swenson and Howard, 1979). The demonstration of physicochemical homology between PAPP-A and $alpha_2$-macroglobulin would give direction to future investigations into the function of PAPP-A, since one function of $alpha_2$-macroglobulin as an inhibitor of active endopeptidases is already known (see Barrett and Starkey, 1973). The possibility that PAPP-A shows physicochemical homology with $alpha_2$-macroglobulin is currently being investigated in my supervisor's laboratory.

A comparison of the levels of PAPP-A in third trimester pregnancy blood of normal women and those affected with insulin-dependent diabetes, gestational diabetes, fetal intrauterine growth retardation and a variety of other disorders was reported in Chapter 7. It revealed one group of women with a significantly reduced mean PAPP-A concentration. This group was the insulin-dependent diabetics. Lin et al. (1977; see Chapter 1, Section 4.1) have previously reported no significant difference between PAPP-A levels of insulin-dependent diabetics and controls during the last month of pregnancy. It is not known why the present findings differ from those of Lin et al. (1977) but the difference may be due to a variety of factors connected with the type and management of the patients included in both studies. The present findings suggest that the levels of PAPP-A in maternal circulation may be influenced by some aspects of maternal carbohydrate metabolism. Unfortunately, detailed obstetric data were not available on the donors of the blood samples used in the present study. Further investigations to establish the factor, or factors, associated with some insulin-dependent diabetic pregnancies which can effect the levels of PAPP-A would be desirable. These may well uncover a specific area where PAPP-A measurements will be of diagnostic value. The present study was the first study of the plasma levels PAPP-A in gestational diabetics. Although the mean concentration for this group was not found to be significantly different from that of the control normal women, a few very high values were observed. Further investigations to establish the factors responsible for these occasional high values may also be of value.

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APPENDIX: PUBLISHED PAPERS

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