STUDIES ON PREGNANCY-ASSOCIATED PLASMA PROTEIN A

by

CHRISTINE HELEN GORE

B.Sc.(Edin.)

Ph.D.

Department of Genetics

University of Glasgow

November 1982

This Thesis is dedicated to my father and mother, and all my friends and family at St. George's Tron and Glasgow University Christian Union, with grateful thanks for all their loving support and prayers over the last three years.

DECLARATION

I declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work is my own except where specifically acknowledged.

ACKNOWLEDGEMENTS

It is with gratitude and thanks that I acknowledge the help of my supervisor Dr. Roger Sutcliffe, who encouraged, guided and enthused me throughout.

I wish to thank Dr. Graham Kemp, of the department of Biochemistry, University of St. Andrews, and the staff of the labour suite at the Queen Mother's Hospital, Glasgow, for their time, assistance and gifts of material. I am also grateful to Martin Davies, Jack Hunter, Isobel Edwards and Salwa Khalef for their advice and help on technical and other matters. I gratefully acknowledge the provision of a postgraduate studentship by the MRC and thank Professor David Sherratt for allowing me to pursue my studies in the Institute of Genetics, University of Glasgow.

Finally, I would like to express my sincere thanks and gratitude to all my colleagues in the Institute who have been more than friends to me over the last three years, particularly to David Summers for proof reading my thesis, and to Paul and Simon for their special support.

CONTENTS

SUMMARY

Various pregnancy associated proteins are detected in maternal serum during pregnancy, some of which are also present at low levels in non-pregnancy serum. A review of the literature has been made and the following conclusions have been drawn. Pregnancy associated proteins are synthesised by a variety of maternal, fetal and placental tissues. Both steroid and proteinaceous hormones are produced, together with enzymes and other proteins. The functions of many of these proteins have still to be determined. In vitro investigations have suggested that some of these proteins may be involved in haemostasis and in immunosuppression. However, their precise roles in the complex and sensitive immunobiological equilibrium that exists between maternal and fetal tissues during pregnancy, has not been established.

Some placental proteins have been used, to varying degrees of success, to monitor pregnancy and fetal well-being. Others have proved invaluable for monitoring malignancies, metastasis and the recurrence of tumours after treatment. A better understanding of the function of pregnancy specific proteins would significantly increase their clinical and practical usefulness.

Pregnancy-associated plasma protein A (PAPP-A) one of the more recently discovered placental proteins, has similar physicochemical properties to the serum protease inhibitor alpha-2-macroglobulin (α_2M). It has been suggested that these two proteins may be homologues (or analogues) as has been observed for other pregnancy associated proteins. A functional homology has also been suggested for these two proteins.

Histological staining studies by various workers have localised PAPP-A to the syncytiotrophoblast, the villous cytotrophoblast, the

utero-placental fibrinoid and in reticular fibres around uterine decidual cells. This distribution is similar to that observed for the high molecular weight glycoprotein fibronectin. Tissue from the utero-placental and chorionic plates synthesised both ^{35}S-labelled PAPP-A and fibronectin after one week of culturing.

The purpose of this investigation was to develop a purification protocol for the production of physiologically active PAPP-A, and to further compare α_2M and PAPP-A both physicochemically and functionally. The following results were obtained.

The neutral carbohydrate content of PAPP-A, purified by positive affinity chromatography, was determined. PAPP-A contained a mean neutral carbohydrate content of 12.9%, the value obtained for α_2M was 3.9%. Only 4.8% of the protein in a solution of purified PAPP-A was precipitable in 12.5% trichloroacetic acid, compared with 53.9% precipitation for a solution of α_2M. However, PAPP-A was insoluble in 5% phosphotungstic acid in 2M HCl. These results are consistent with the relatively high total carbohydrate content observed for PAPP-A (19.2%).

PAPP-A was obtained in an enriched state under mild purification conditions, involving Cibacron blue dye-ligand chromatography, negative antibody affinity chromatography and gel filtration. The product was a mixture of PAPP-A and α_2M (the PAPP-A enriched preparation or PEP). The ability of these proteins to bind ^{125}I-trypsin and ^{125}I-plasmin was studied. In contrast to previous studies, there was no evidence that PAPP-A bound proteases, and on incubation with an equimolar (approx) concentration of trypsin ^{125}I-PAPP-A degradation was observed. The α_2M in these PAPP-A enriched preparations did however, bind to ^{125}I-trypsin

and ^{125}I-plasmin. Complement-induced lysis of sensitised sheep red cells was not inhibited by the PAPP-A enriched preparation. These results are contrary to those already published.

SDS-polyacrylamide gel electrophoresis analysis of scrapings from Nitabuch's layer at the utero-placental interface, revealed two high molecular weight polypeptide bands, which stained with comparable intensity with Coomassie blue. The molecular weights of these bands corresponded to those of fibronectin (230,000) and PAPP-A (187,000). Other lower molecular weight bands were also observed, some of which had molecular weights corresponding to fibrin polypeptides. On culturing Nitabuch's layer for 24 h in the presence of ^{35}S-methionine, a polypeptide with a molecular weight of 230,000 in the culture supernatant, incorporated the most radioactivity and this was concluded to be ^{35}S-fibronectin. A band at 187,000 mol wt was not observed in the culture supernatant. A control of cultured villous placenta did not show comparable incorporation of ^{35}S into fibronectin. The PAPP-A and α_2M in the PAPP-A enriched preparations did not bind to the fibronectin in the Nitabuch's culture supernatant nor to columns of gelatin Sepharose or ^{35}S-fibronectin:gelatin Sepharose complexes.

On the basis of the results in this thesis there seems to be little reason to consider that PAPP-A is homologous or analogous to the serum protein α_2M in function or finer structure. The evidence seems to indicate that PAPP-A should no longer be considered as a protein involved in immunosuppression or fibrinolysis, but primarily as a protein contributing to the structure of the extracellular matrix in the utero-placental and chorionic plates, and in certain fibrotic areas of placental villous trunks.

ABBREVIATIONS

AACE	Antibody-antigen crossed electrophoresis
α_2M	Alpha-2-macroglobulin
BSA	Bovine serum albumin
Buffer A	10 mM potassium phosphate, pH 7.5
Buffer B	0.3M NaCl in 5 mM potassium phosphate, pH 7.5
hPL	Human placental lactogen
hCG	Human chorionic gonadotrophin
mol wt	Molecular weight
NAAC	Negative-antibody affinity chromatography
PAPP-A	Pregnancy-associated plasma protein A
PBS	Phosphate-buffered saline: 0.16 M NaCl, 5 mM potassium phosphate, pH 7.5
PEP	PAPP-A enriched preparation
PTA	Phospho-tungstic acid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SP1	Pregnancy specific beta-1-glycoprotein (Schwangerschafts protein)
TCA	Trichloroacetic acid

CHAPTER 1 : INTRODUCTION

GENERAL INTRODUCTION

In this chapter a review of the current state of knowledge of the structure and function of pregnancy associated plasma protein A (PAPP-A) will be presented, together with a comparison between PAPP-A and alpha-2-macroglobulin (α_2M), a serum protease inhibitor thought to be similar to PAPP-A. However, for a better understanding of the approaches taken and the arguments presented, it is necessary to have a background knowledge of human placentology. What makes gestation such a unique process and what parts do the pregnancy-associated proteins play in this process? What can be gained by studying these proteins, and how do these studies aid clinicians in the management and treatment of expectant mothers during normal and complicated pregnancies?

1. THE HUMAN PLACENTA

The haemochorial type of placenta found in humans arises from an intimate union of fetal and maternal tissues. It has many varied purposes including the physiological exchange of materials between the maternal and fetal circulations. It possesses a limited life span of approximately 9 months, during which it develops, grows and ages; showing alterations in structure and function adapted to the requirements of the growing embryo and fetus at successive stages of gestation.

In the following sections several aspects of placental development, structure and function are described, together with a review of the immunological aspects of pregnancy.

Figure 1:1 Implantation: (a) first day
(b) second day
(Adapted from FitzGerald, 1978)

Figure 1:2 Fifth day of implantation
(Adapted from FitzGerald, 1978)

Figure 1:3 Eighth day of implantation. AS, amniotic sac; EEM, extraembryonic mesoderm; YS, yolk sac.
(Adapted from FitzGerald, 1978)

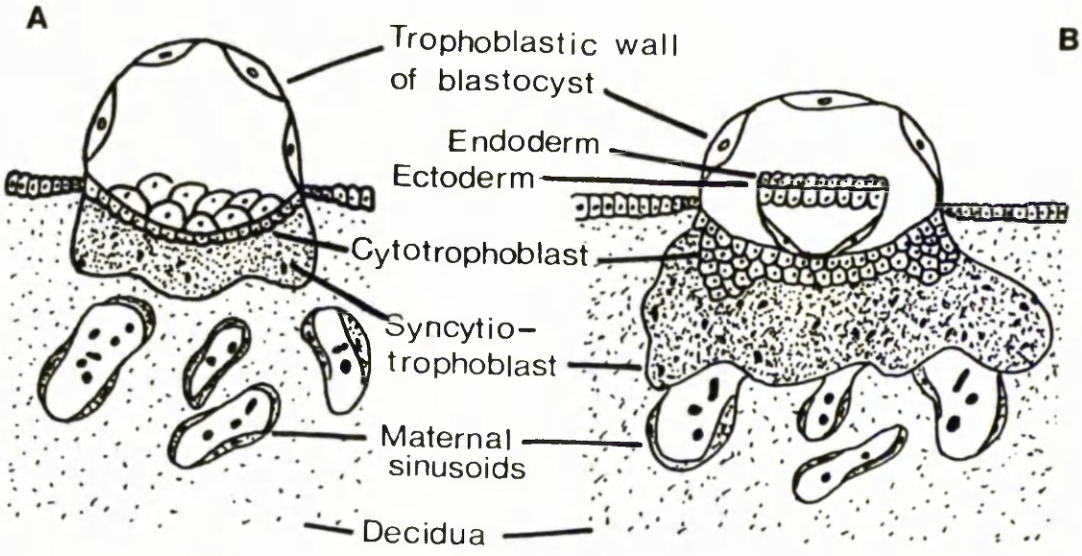


Figure 1:2

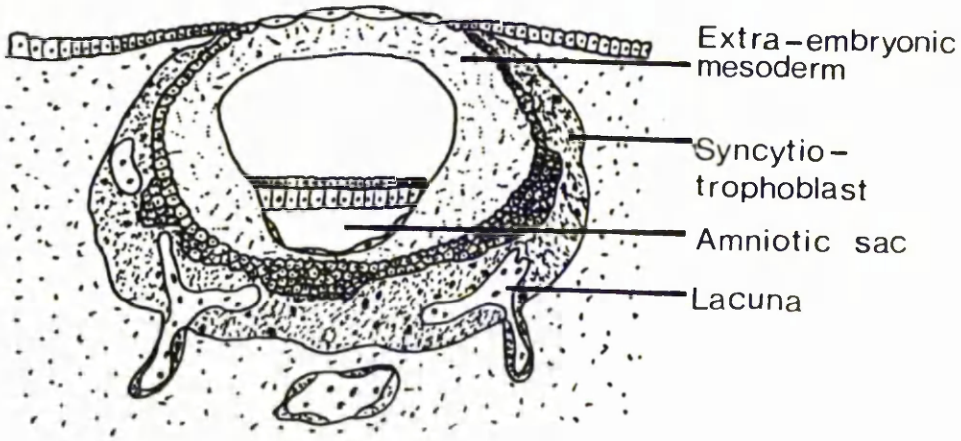
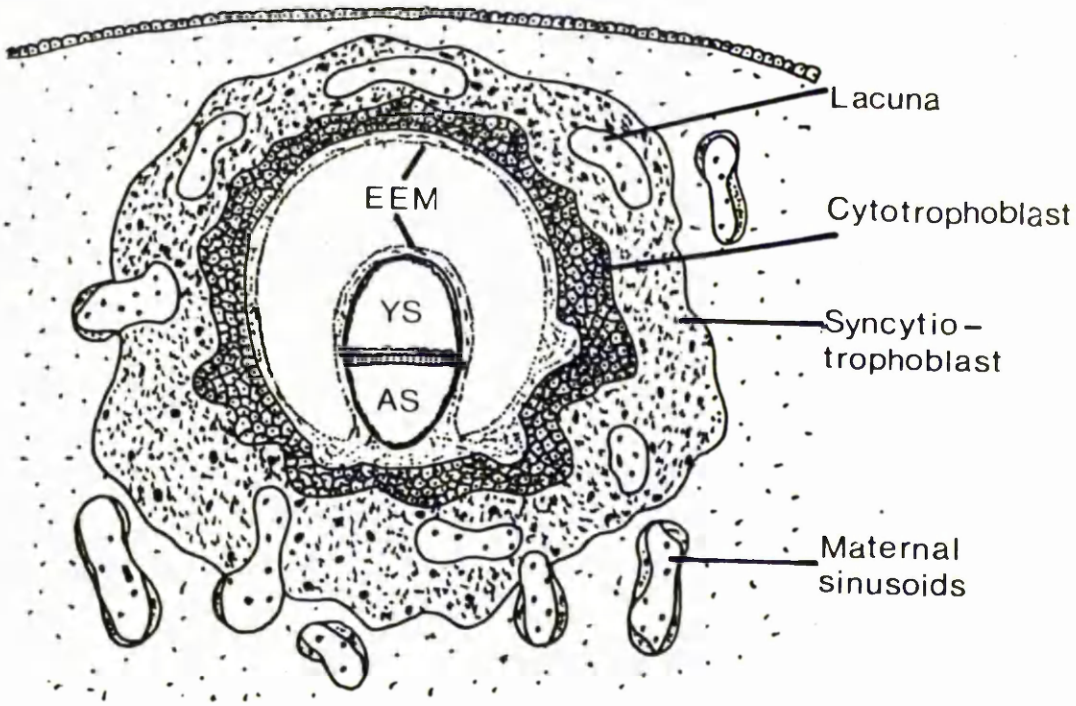


Figure 1:3



1:1 Placental development and structure

The following account has been constructed from the reviews of Aitken, Beaconsfield and Ginsburg (1979), Boyd and Hamilton (1970), FitzGerald (1978), Pijenborg *et al* (1981), Wyn (1975) and Walker and James (1976).

By the 120 cell stage the cells of the early blastocyst have differentiated into those which form the placenta (*the trophoblast*), and those which will become the fetus (*the inner cell mass*). *In vitro* the earliest time at which this has been observed is 5 days after fertilization. On the 6th or 7th day after fertilization the blastocyst implants in the wall of the uterus (*the endometrium*). The inner cell mass orientates towards the endometrial epithelium which is then penetrated by the cells of the trophoblast (Figure 1:1a). By the 12th day the blastocyst comes to lie completely embedded in the uterine endometrium (Figure 1:3). The degree of endometrial invasion by the trophoblast appears to be strictly defined and limited. Whether the controlling factors in this process are uterine or fetal (or both) in origin is not known. At the beginning of implantation, the endometrium undergoes the *decidual reaction* which begins at the site of implantation and spreads throughout the endometrium in a few days. Strictly defined, the decidua is the gestational endometrium that is shed at parturition (Wyn, 1975). A decidual cell refers specifically to the transformed stromal cells of the pregnant endometrium, and these are enlarged cells filled with glycogen and lipid. The possible decidual functions are: nutrition of the embryo; formation of a cleavage zone at parturition; limitation of trophoblastic invasion; endocrine secretion and an immunological role (Aitken, Beconsfield and Ginsburg, 1979).

After implantation the trophoblast begins to differentiate. At the point of contact with the endometrium the flat trophoblastic cells become cuboidal, then multiply to form an outer syncytial layer, the syncytiotrophoblast, which invades the endometrium. The inner cuboidal cells constitute the cytotrophoblast (Figure 1:1a). Mitotic figures are abundant in the cytotrophoblast; disintegration of its plasma membranes can be observed with the electron microscope as new syncytium is formed. The syncytium then undergoes vacuolation to form a system of lacunar spaces (lacunae), which connect with the maternal sinusoids and fill with maternal blood (Figures 1:1b and 1:2). In this way the placenta is supplied with maternal arterial blood directly from the uterine vessels. The proliferating cytotrophoblast enters the syncytial processes, forming cell columns within them. Thus the primary villi of the placenta are formed (day 15 approx). These villi are not truly villous in form, however, as they interconnect freely. Active proliferation of the cytotrophoblast carries the tips of the primary villi through the syncytial layer and into the decidualised endometrial stroma. A proportion of these villi fuse with the decidua and remain avascular. Their function is to anchor the placenta to the uterine wall. At the same time the cytotrophoblast cell columns spread laterally in the peripheral syncytium and endometrium, and join together to form a shell around the conceptus; the cytotrophoblastic shell (Figures 1:2 and 1:3). Completion of the shell limits the area of trophoblast invasion. The shell is pierced by maternal sinusoids at their points of rupture into the lacunae, which freely intercommunicate, giving rise to the labyrinthine intervillous space (Figure 1:4).

Figure 1:4 Implantation, tenth day.
AS, amniotic sac.
(Adapted from FitzGerald, 1978)

Figure 1:5 The placental barrier
(Adapted from FitzGerald, 1978)

Figure 1:4

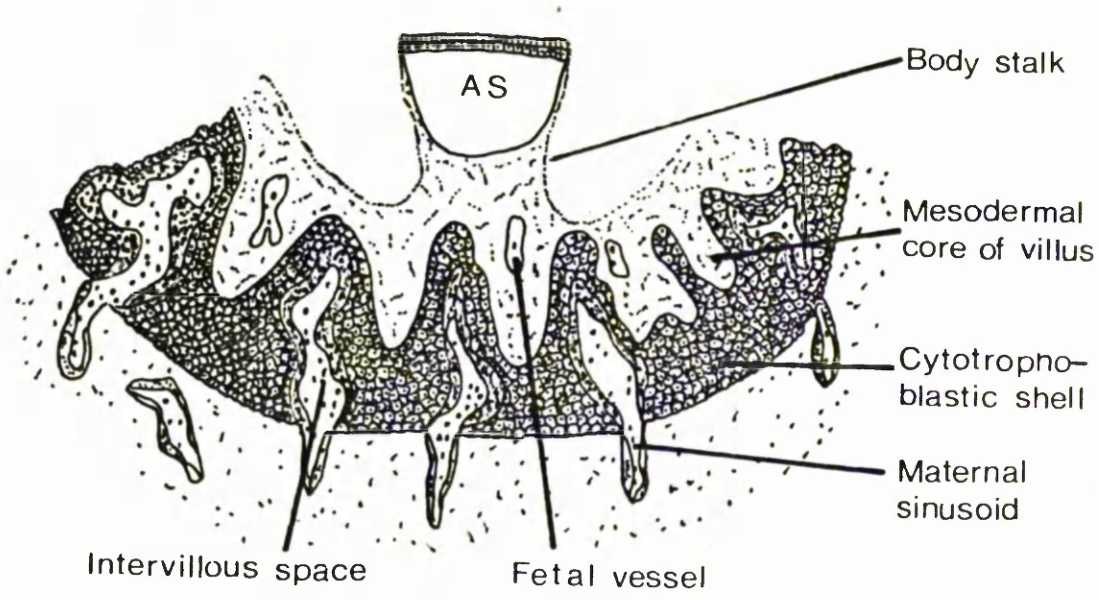
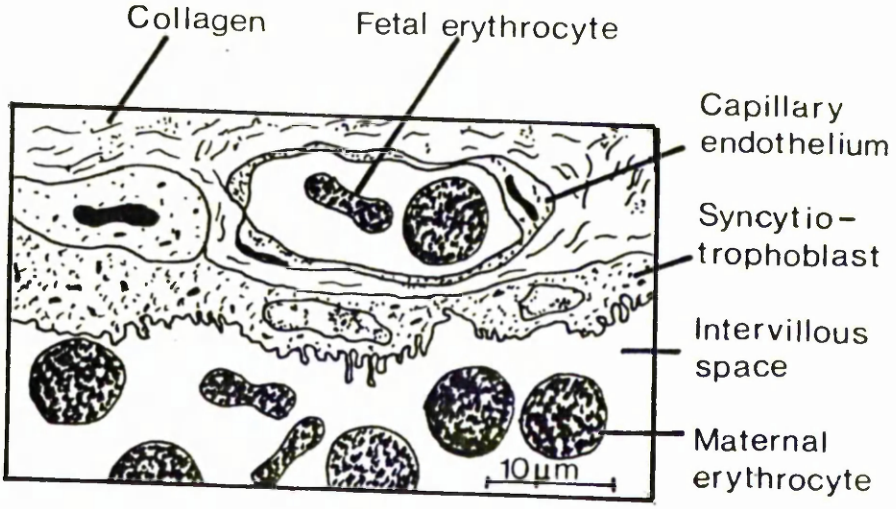


Figure 1:5



The extra embryonic mesoderm enters the villi, transforming them into secondary villi. It does not penetrate as far as the cytotrophoblastic shell. Capillaries develop in situ from this mesoderm. Villi containing these blood vessels are called tertiary villi. These villi increase in complexity by means of side branches. Up to 200 tree-like colonies of villi are formed (fetal cotyledons), each fed by one fetal artery and drained by one fetal vein. Fetal and maternal blood are separated by the syncytium (which may possess a brush border and frequently shows anucleate stretches at such levels), the trophoblastic basement membrane, the stromal connective tissue, the vascular basement membrane, the capillary endothelium and the cytotrophoblast, in those regions where it persists (Figure 1:5; Boyd and Hamilton, 1970).

A sluggish circulation of maternal blood takes place through the early lacunae but the circulation becomes more brisk as the intervillous space enlarges. The uterine arteries undergo major structural alterations during the formation of the placenta. Trophoblastic cells migrate into the maternal spiral arteries (but no deeper than the medial terminal arteries beyond the myometrium; Pijenborg et al, 1981), supplying the intervillous space (Robertson, Brosens and Dixon, 1975). The intra-arterial invasion starts with the plugging of the distal tips of these arteries by trophoblastic tissue, continuous either with the cytotrophoblastic shell or with the proliferating tips of the anchoring villi (Pijenborg et al, 1981). Trophoblastic cells also become incorporated in the arterial wall , which loses its normal histological characteristics to become a tube of fibrinoid material with a wider diameter than the original vessel. These structural changes disrupt the autonomic supply to the vessels, with the result that the

Figure 1:6 Diagrams to illustrate the enlargement of the chorionic vesicle and progressive obliteration of the uterine cavity.
a) 6 weeks after fertilization
b) 16 weeks after fertilization
(Adapted from FitzGerald, 1978)

Figure 1:7 Component parts of the placenta
(Adapted from FitzGerald, 1978)

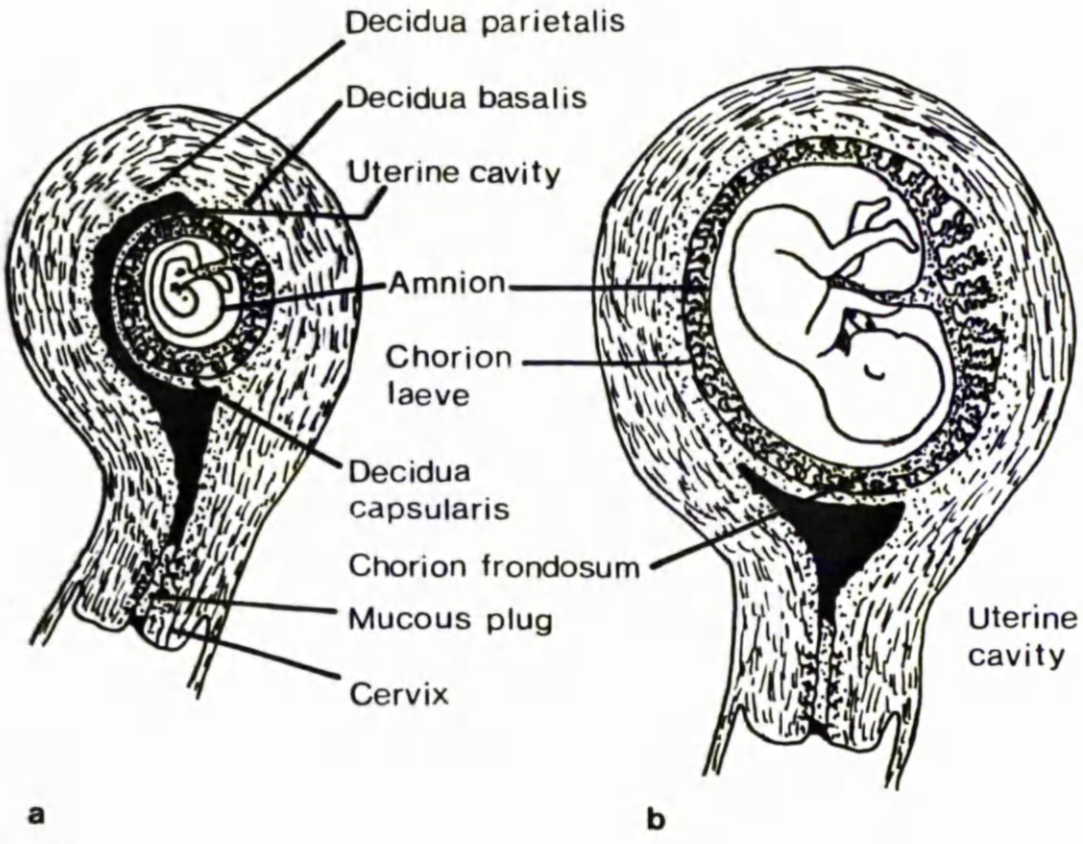
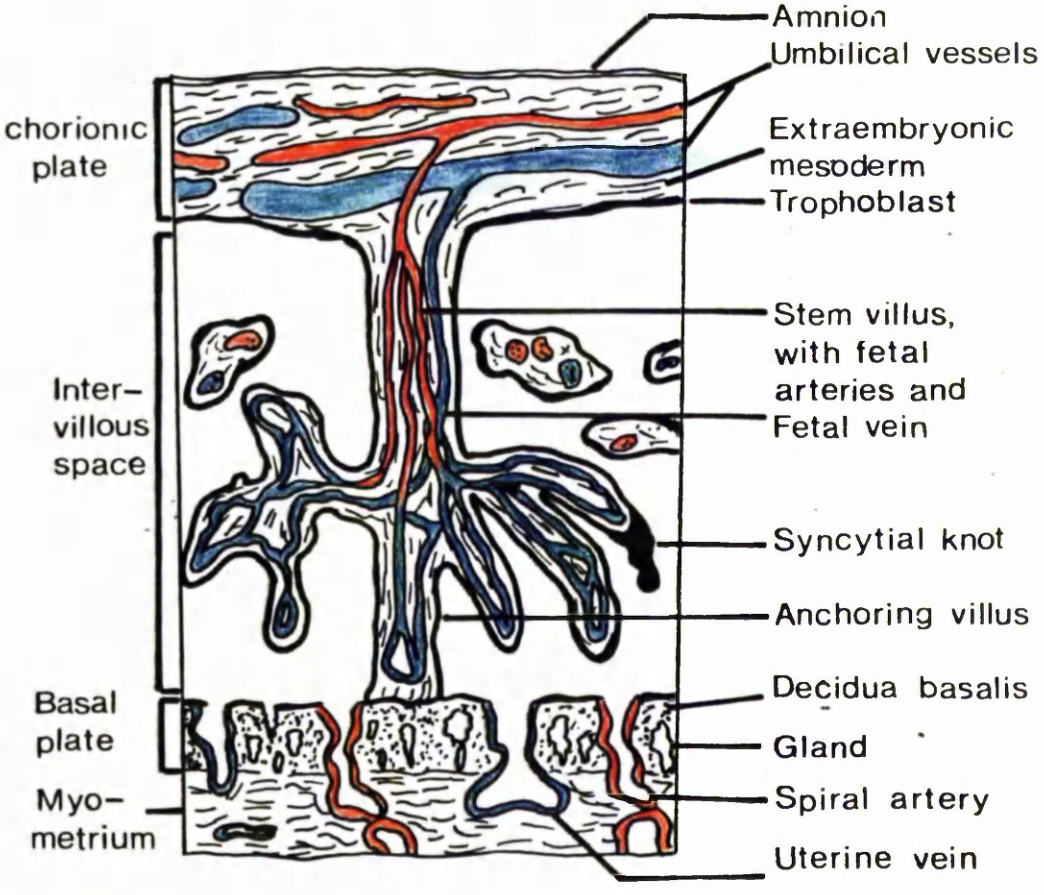

Decidua parietalis
Decidua basalis
Uterine cavity
Amnion
Chorion laeve
Decidua capsularis
Chorion frondosum
Mucous plug
Cervix
Uterine cavity
a
b

Figure 1:7


Amnion
Umbilical vessels
chorionic plate
Extraembryonic mesoderm
Trophoblast
Stem villus, with fetal arteries and
Fetal vein
Inter-villous space
Syncytial knot
Anchoring villus
Decidua basalis
Basal plate
Gland
Myo-metrium
Spiral artery
Uterine vein

placental vascular bed does not actively participate in reflex homeostatic adjustments of the maternal system. The placental vasculature forms a low resistance system, supplied directly from the systemic circulation so that flow through the placenta depends on the prevailing systemic pressure. Hence, despite the absence of autonomic vasomotor supply, changes in placental bed haemodynamics occur as a passive, secondary response to changes in the systemic pressure, and/or to chemical stimuli.

During the third week after fertilization the growing chorionic vesicle enlarges into the cavity of the uterus. The decidua outside the vesicle is now called the decidua capsularis and that on the maternal aspect of the vesicle is the decidua basalis (Figure 1:6a). The decidua parietalis lines the remainder of the body of the uterus. After some 12 weeks of development the decidua capsularis comes into contact with, and begins to fuse with the decidua parietalis opposite (Figure 1:6b), gradually obliterating the uterine cavity. Although the early chorionic villi develop all around the blastocyst, the later (tertiary) villi become progressively restricted to the decidua basalis, where they enlarge to form the chorion frondosum. The chorion laeve, in contact with the decidua capsularis undergoes atrophy.

During the middle trimester of pregnancy the placenta continues to grow (Figure 1:10a), but the original relationship between fetal and maternal components is maintained. The tertiary villi continue their arborescent growth right up to term; their growing tips displaying knoblike thickenings of syncytiotrophoblast which contain many nuclei; these are known as syncytial knots (Figure 1:7). At 12 weeks the villi are large (up to 0.3 mm in diameter), with 2 well-defined layers of trophoblast. Capillaries are small and located

Figure 1:8 Diagram of a portion of the mature placenta. Only one villous tree is represented. Note: The intervillous space is normally filled with maternal blood; the arrows indicate its direction of flow.
(Adapted from FitzGerald, 1978)

Figure 1:9 Illustration of the mechanism by which blocking antibodies prevent the attack of sensitized lymphocytes (immunological enhancement).
(Adapted from Cooper, 1980).

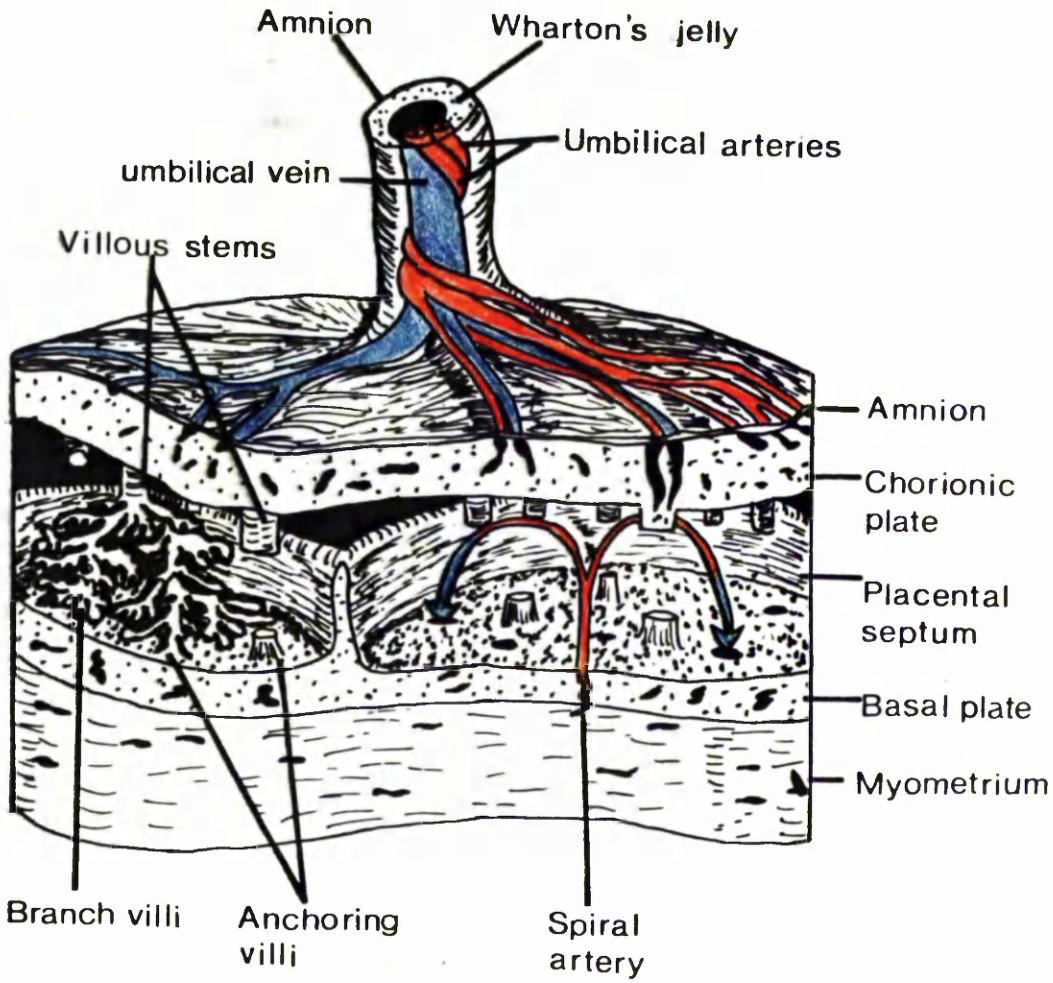
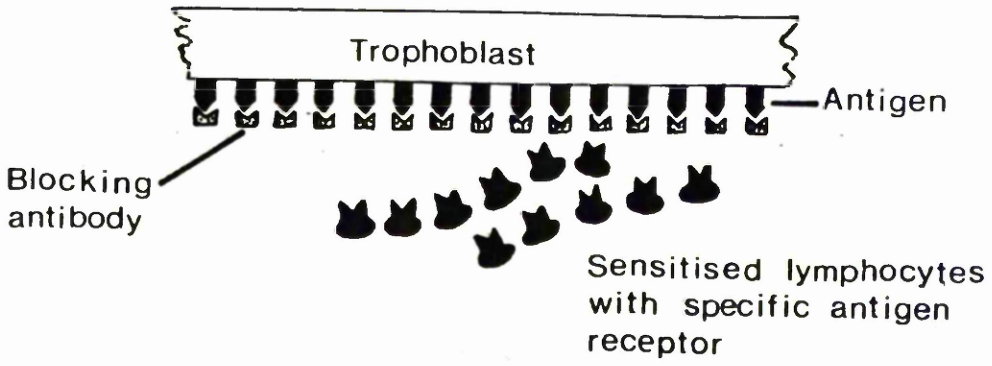

Amnion
Wharton's jelly
Umbilical arteries
umbilical vein
Villous stems
Amnion
Chorionic plate
Placental septum
Basal plate
Myometrium
Branch villi
Anchoring villi
Spiral artery

Figure 1:9


Trophoblast
Antigen
Blocking antibody
Sensitised lymphocytes with specific antigen receptor

centrally in the connective tissue core. At term the villi are much smaller (0.05-0.1 mm in diameter), the capillaries are nearer the surface, the syncytiotrophoblast thins and cytotrophoblastic cells are few. Fibrinoid deposits are commonly seen on the surface of the villi. Fresh syncytium is continually recruited from the Langhans cytotrophoblast, which diminishes in amount as pregnancy advances. During the 1st trimester of pregnancy the cytotrophoblast invades the decidua and inner myometrium, and fusion of these mononuclear cells creates the syncytiotrophoblastic multinuclear giant cells. Together with the basal decidua and inner myometrium these make up the placental bed. The transformation of cytotrophoblast into syncytiotrophoblast throughout the 2nd and 3rd trimesters results in interstial giant cells being the predominant trophoblastic elements seen in the placental bed at term (Pijenborg et al, 1981). Some of these giant cells in the uterine tissue migrate into the decidual spiral arteries. Progressive erosion of maternal vessels accompanies enlargement of the intervillous space. Placental septae are formed which are narrow ridges of interconnected decidua that project into the intervillous space, giving rise to more or less cup-shaped structures (Figure 1:8), the maternal cotyledons.

The placenta at term is disc-shaped, about 20 cm in diameter, 2.5 cm thick in the middle and tapering towards the edges, and weighs approximately 700 g(Figure 1:10a). The fetal surface is covered by the amnion beneath which lies the chorion and fetal vessels. The maternal surface is lobulated in appearance. The maternal cotyledons correspond to one, or more usually, several fetal cotyledons, the grooves between them are created by placental septae. A spiral artery enters each maternal cotyledon and several peripheral veins return the blood to the maternal circulation (Figure 1:8). The umbilical cord

contains two arteries and one vein in a jelly-like substance (Wharton's jelly), covered with the amnion. It is usually inserted near the centre of the placenta and its length normally lies between 50-100 cm.

Dissection of the placenta shows the chorionic plate attached to which are the fetal cotyledons. These project towards the decidua basalis which together with the cytotrophoblastic shell on its fetal surface make up the basal plate (Figure 1:7). The smallest fetal cotyledons form groups of villi near the chorionic plate. The largest are derived from the anchoring villi of early pregnancy; their anchorage extending from the chorionic plate to the basal plate. The side branches of these villi give rise to a profusion of terminal villi (Figure 1:8), floating in the intervillous space, some of which are united by syncytial bridges, or by deposits of fibrinoid substances on the surface of adjacent villi.

Each villus contains an independent capillary network; a wide capillary loop which follows its irregular contours (Figure 1:7). This system greatly increases the vascular bed of the branch villi, which slows the flow of blood so that the circulation time through the terminal villous loop is increased and more complete physiologic exchange with the maternal blood is effected. Some 200 spiral arteries open into the intervillous space with about 500 ml of maternal blood in total passing through per minute. Movement of intervillous blood has not been observed in humans, but in monkeys the arterial blood spurts into the intervillous space, reaching the chorionic plate before being dispersed. It returns more slowly, percolating through the villous sponge on its way to the venous exits. Gentle rhythmic contractions of the human uterus are thought to assist in emptying the intervillous space. The

anchoring villi contain smooth muscle which, by drawing the chorionic plate towards the basal plate, may prevent the formation of stagnant pools of maternal blood.

From very early on in pregnancy deposits of fibrin and the related substance fibrinoid (see Boyd and Hamilton, 1970; Sutcliffe et al, 1982a), are found in the boundary zone between maternal and fetal tissues, and in relationship to the syncytium in the villi (see Chapter 4:6). Fetal tissues come into direct contact with maternal blood, lymph and interstial tissue fluids, all of which contain fibrinogen. It is therefore not surprising that fibrin comes to be laid down in developing membranes and placenta. What is surprising is the comparative lack of coagulation observed in the intervillous space. Perhaps, like the endothelium in the maternal blood vessels, fetal syncytium possesses qualities which inhibit clotting, or augment fibrinolytic activity, and so keeps coagulation at a minimum. This alteration in coagulation status had been attributed to steroid production by the fetus and placenta (Alexander et al, 1956). However, recent reports have suggested that a protein produced specifically by the syncytiotrophoblast (placental protein 5, PP5) may be involved in the maintenance of intervillous blood flow, by locally influencing the coagulation system (Grudzinskas et al, 1979). PP5 interacts with both heparin and thrombin in vitro and it has been proposed (Salem et al, 1980), that it may represent a placental analogue of antithrombin III.

1:2 Placental Physiology

In addition to providing an anatomical barrier between the fetal and maternal circulation, the placenta has 4 functions namely: for respiratory exchange; to provide nutrients, amino acids, sugars,

lipids, minerals and vitamins; to eliminate the endproducts of fetal metabolism, and to produce hormones, enzymes and other proteins necessary for the maintenance of pregnancy.

Various mechanisms are involved in transporting substances across the placenta (Bissonnette, 1982), namely: simple diffusion, facilitated diffusion, active transport and special processes such as pinocytosis, reverse pinocytosis and leakage. These mechanisms take place across the so called placental barrier, which is composed of the fetal capillary endothelium, a thin layer of collagen fibres, the basement membrane of the villus, a film of syncytiotrophoblast and fibrinoid (Figure 1:5). The placental barrier is therefore entirely fetal in origin. In addition to the exchange that occurs between the mother and fetus via the placenta, it is possible that some fluid and electrolytes (and even large molecules) may pass by way of the chorio-amnion para-placenta and the vessels of the decidua parietalis.

The transfer of respiratory gases O_2 and CO_2, occurs by simple diffusion, the rate of which is dependent on the relative flow rates of maternal and fetal bloods, difference in concentration on the 2 sides of the membrane, the surface area of the membrane (11 sq m approx) and the thickness of the membrane. The greater O_2 affinity of fetal haemoglobin is of assistance, although not essential to normal fetal development. The placenta itself requires an oxygen supply equivalent to some 25% of that used by the fetus.

Molecules required for the nutrition of the fetus are transferred by active transport and facilitated diffusion. These include substances such as glucose (the principal energy source of the fetus), vitamins and amino acids (where the L isomer is selectively transferred). In

general, transfer of nutrient materials across the placenta is a fairly rapid process, perhaps occupying only a few minutes for substances transported by simple and facilitated diffusion. In the case of active transport, duration of 30 min to 1 h, or less, is likely to be involved (Dewhurst, 1981).

The synthetic activity of the placenta is a very important function and it produces a variety of steroid and protein molecules (some of which are not detected in normal non-pregnancy serum). The role of the steroid hormones is well documented (see Dewhurst, 1981). However, the precise roles of specific protein hormones and other placental proteins are incompletely understood. Currently known placental-specific proteins can be divided into 3 groups:

(1) Hormones - human chorionic gonadotrophin (hCG), human placental lactogen (hPL), human chorionic thyrotrophin (hCT), uterotrophin and others.

(2) Enzymes - histamines, placental alkaline phosphatase, oxytocinase, steroid dehydrogenase and transglutaminase.

(3) Other Proteins - glycoproteins that are secreted almost entirely into the maternal circulation. Most of these proteins have been identified by immunological techniques and most are of unknown biological function. There are at least 6 placental proteins that fit into this category (see Klopper, 1980; Tatarinov, 1980), namely Schwangerschafts protein 1 (β, glycoprotein or SP1), placental protein 5 (PP5), pregnancy-associated plasma proteins A and B (PAPP-A and PAPP-B) and placental-specific α microglobulins (PAMG-1 and PAMG-2).

The evidence is strong that the endocrinologically active tissue of the human placenta is the syncytiotrophoblast (Wyn, 1975), with possible small contributions from other well-differentiated and

specialized forms of trophoblast. All the ultrastructural 'machinery' required for synthesis such as endoplasmic reticulum, Golgi bodies and mitochondria, have been identified in the syncytiotrophoblast (Wyn, 1975). The cytotrophoblast (Langhan's cells), on the other hand, have been shown to contain few such organelles (Wyn, 1975) and are concerned with cellular growth and differentiation rather than synthesis of endocrine or exocrine products. It is thought that most of the placental-specific proteins mentioned above are also products of the syncytiotrophoblast, but evidence is mainly for localization rather than synthesis (Bohn and Winckler, 1977; Horne et al, 197; Lin and Halbert, 1976).

How effective is the placental barrier? At one time it was thought that the placenta protected the fetus from a number of noxious substances in the maternal circulation such as infectious diseases or drugs. But we now know that this view is too simplistic. Certain common infectious diseases such as rubella and chicken pox can affect the fetus in utero. Some drugs, even when administered in late pregnancy, may pass to the fetus with harmful effects. For example, fetal goitre can be caused by potassium iodide or thiouracil treatment, and thalidomide, administered in early pregnancy, caused tragic deformaties in the affected fetuses. Selective transport of antibodies from the maternal circulation is an important immunological function of the human placenta as it confers passive immunity upon the fetus and newborn infant. In vitro studies (Wild, 1973) of transport across the perfused human placenta have shown that immunoglobulins of the antibody class IgG are the only ones to pass through, those of classes IgA and IgM do not. Implicit, therefore, is a requirement for a

selective molecular recognition of IgG by the placenta. The Fc region of the IgG molecule has been identified as the functional element in the recognition process. Specific cell surface receptors on the placental membrane are thought to become attached to the Fcγ region (see the review of Johnson and Brown, 1981). Selective transport of the IgG across the syncytiotrophoblast occurs via receptor-mediated endocytosis in coated vesicles (see Wild, 1979; Bissonnette, 1982). Fcγ receptors may also be present on other placental cell types, and serve in a quite different function, that of protecting the fetus from immune complexes formed with maternal IgG antibodies *in situ* in the placenta (see section 1:3 of this Chapter).

The placenta, or specifically its trophoblastic component, has come to be looked upon more as a filter than the barrier it was once thought to be. However, there is no *direct* communication between the fetal and maternal blood streams, except perhaps at parturition, when fetal red cells are known to appear in maternal blood (though there is some small-scale exchange of all the formed blood elements during gestation; Beer and Billingham, 1976). There is some evidence that maternal lymphoid cells can gain access to the fetus (as seen in graft-versus-host disease; see Beer and Billingham, 1976). But generally, with respect to cellular traffic, the placenta may well present an impenetrable barrier.

1:3 Immunological aspects of pregnancy

Throughout gestation the fetus is surrounded by a layer of trophoblast at the materno-fetal interface. This layer extends from the placenta out and around the extra embryonic membranes (see section

1:1 of this chapter). If this trophoblastic layer carries alloantigens then it should present to the mother an antigenic challenge capable of initiating an immune response leading to embryonic tissue rejection. However, the conceptus is not usually rejected, which would seem to suggest that it either does not carry paternal antigens or that there has been a modifying or failure at some level of the afferent and/or efferent arm of the maternal immune response (i.e. non-recognition, non-response or a modified response).

The antigens that are recognised as foreign on a normal allograft are the histocompatibility antigens. Those which provoke the fastest removal of tissue are a group of closely linked loci known as the major histocompatibility antigens (MHC), namely HLA-A, B, C and D. Antigens which provoke weaker responses are controlled by gene loci collectively referred to as the minor histocompatibility loci (MIH), which include the ABO and Rhesus (Rh) blood groups. Trophoblastic localization studies of HLA antigens have lead to conflicting reports (Loke, Joysey and Borland, 1971; Faulk and Johnson, 1977; McIntyre and Faulk, 1979). However, the current opinion is that HLA-D antigens are absent from all trophoblastic tissue, whereas HLA-A, B and C are present on non-villous trophoblast, but absent from villous trophoblast (Dr. C.A. Sunderland, personal communication). A subpopulation of extravillous cytotrophoblast in the placental bed is also thought to carry the HLA-A, B and C antigens (Sunderland, Redman and Stirrat, 1981; Sunderland *et al*, 1981). These antigens are also present on the cells of the villous stroma (Faulk, Sanderson and Temple, 1977). ABO blood group antigens and Rh-antigens are also thought to be absent from trophoblastic tissue (Szulman, 1973; McCormick *et al*, 1971). The

fetus, unlike the trophoblast, expresses all MHC antigens, even in the early stages of development, since tissues of the inner cell mass of mouse embryos have been shown to cross-react with MHC antisera (Heyner, 1973). The conceptus therefore, would appear to be "semi-allogeneic" in nature (Johnson, 1982).

The antigenic status of the conceptus may be masked by the presence of an immunological barrier lying between the fetal and maternal tissues, thus preventing the mother from recognising the foreign conceptus. Substances such as peritrophoblastic, non-cellular fibrinoid and sialic-rich mucoproteins (Kirby et al, 1964), and transferrin (Galbraith, Galbraith and Faulk, 1980), have been put forward as candidates for potential masking agents. Often only incomplete layers of peritrophoblastic fibrinoid are observed and in some species there is a complete lack (Boyd and Hamilton, 1970). Kirby et al (1964) proposed that mucine fibrinoid has a protective effect against the maternal immune response, as it is a highly negatively charged mucoprotein. Currie and Bagshawe (1967), reported that removal of sialic acid from mouse trophoblast cells by neuraminidase treatment rendered them immunogenic. However, subsequent attempts to repeat this experiment have been unable to reproduce this observation (Simmons et al, 1971). The fibrinoid layer at the utero-placental interface (Nitabuch's layer), has been found to be an insoluble material and it may therefore, act as a barrier either against haemorrhage of maternal placental blood or to limit the migration of trophoblast, maternal lymphocytes, or other cellular elements (Sutcliffe et al, 1982a). It has been speculated (Galbraith, Galbraith and Faulk, 1980), that trophoblastic transferrin receptors might, by their ability to bind maternal transferrin, prevent

maternal immune recognition of the trophoblast. Although these agents may, to a certain degree, form a "barrier" between the mother and the fetus, it seems unlikely they are solely responsible for the non-rejection of the fetus. A further theory put forward to explain the apparent non-recognition of the conceptus is that of the uterus as an immunologically privileged site. The non-pregnant uterus is normal with regards to antigen recognition and immune effector mechanisms (Beer and Billingham, 1974). It seems likely, therefore, that the decidual response (in some, as yet undefined, way) is important in maintaining the foreign conceptus. The well documented occurrence of ectopic pregnancies in extra-uterine sites which elicit decidual reactions, indicates that the presence of the trophoblast and the evoked decidual reaction at the site of implantation play a key role in the feto-maternal co-existence.

Cells from the conceptus can gain access to the maternal circulation. Trophoblastic fragments (emboli) exfoliate and are carried away in the maternal blood at a rate of about 100,000-200,000 per day. These emboli are eventually broken down in the maternal lungs. A small-scale exchange of all the formed fetal blood elements, including platelets, is also common (Beer and Billingham, 1976), and it is evident that the mother is immunologically cogniscent of her conceptus as some of these antigens stimulate a maternal response. Perhaps the most well known of these examples is haemolytic disease of the new born, where a Rh-negative mother makes antibodies to her Rh-positive baby, which can ultimately cause fetal erythroblastosis.

Allograft survival can be prolonged if the recipient is given immunosuppressive treatment and there have been many claims that

immunosuppressive substances play a part in pregnancy. Whether such immunosuppression is systemic or local, specific for challenging paternal markers or non-specific, is a current area of investigation. Possible pregnancy specific agents which may mediate immunosuppression are summarised in Table 1:1. However, there is controversy about the immunosuppressive activity of many of these substances *in vivo* and therefore caution should be taken when interpreting these findings (Cooper, 1980). It seems unlikely that all of the agents which have been put forward as possible pregnancy immunosuppressants can have such an effect, otherwise the immune capacity of the mother would be greatly reduced (which would be a strong evolutionary disadvantage). Pregnant women are not usually more susceptible to infectious diseases (Llewellyn-Jones, 1969), though some viral diseases are more common in pregnancy (Menser, 1976). Why is the fetus not rejected therefore, by the apparently immunologically competent mother? First, as previously stated, the trophoblast envelope separates the fetus from the mother and consequently excludes most cells. Also IgM, the major complement fixing antibody (which is therefore involved in cytotoxicity and graft rejection) is not bound or transported by the placenta. Using this information, and the knowledge from tumour and transplantation immunology, a tentative general scheme can be drawn up (Voisin, 1979). Enhancing antibodies made by the mother toward paternal antigens on the conceptus can act according to 2 mechanisms (peripheral and central). One is 'peripheral' and protects the target by masking the paternal antigens on accessible cells of the conceptus. These are also known as 'blocking' antibodies (see Figure 1:9), and act by preventing the sensitised maternal lymphocytes and other cellular components from binding to their

TABLE 1:1 Proposed agents of immunosuppression during pregnancy

a) PROTEINS

Alpha-fetoprotein (AFP)

Chorionic gonadotrophin (hCG)

Pregnancy-associated α_2-glycoprotein (α_2PAG)

Early pregnancy factor (EPF)

Pregnancy-specific α_1-glycoprotein (SP1)

Human placental lactogen (hPL)

Placental transcortin

b) STEROIDS

Cortisol

Progesterone and other sex steroids

c) FLUIDS WITH SEVERAL OR MANY COMPONENTS

Pregnancy serum (blocking factors)

Placental eluates (containing maternal and fetal immunoglobulins)

*Reproduced from Cooper (1980)

target cells either by steric hindrance or by direct binding of the blocking antibodies to the appropriate receptors. The major anti-paternal alloantibody produced in murine pregnancy is the non-complement fixing IgG1, and is therefore a candidate for an enhancing antibody (Bell and Billington, 1980), especially since Fcγ receptors exist on the placenta (see section 1:2). Also, a maternal serum IgG blocking factor has been described (Rocklin et al, 1976) that inhibits lymphokine production by maternal lymphocytes responding to antigens present on paternal lymphocytes and on trophoblast cell membranes. The other 'central' mechanism acts by inhibiting the maternal lymphocytes presumably through immune complexes. Wyn (1975) has suggested that the fetal and trophoblastic cells in the maternal circulation may help to saturate the maternal antibody producing system, thus 'diverting' the mother's immune attack from the conceptus (immunodeviation). Suppressor T cells in the maternal circulation which show antigen-specific and general suppression, could inactivate primed maternal lymphocytes for paternal antigens on the conceptus (Voisin, 1979). These suppressor cells may be stimulated by the enhancing antibodies complexed to their corresponding antigens (Voisin, 1979), or by placental proteins themselves.

The placenta also acts as an immunoadsorbent, filtering out at least some of the maternal components which might otherwise attack the fetus. Cells with the morphological characteristics of mononuclear phagocytes (Hofbauer cells; Wood, 1980), are present in the chorionic villi at all stages of gestation. These cells possess Fc and C3 receptors and are therefore thought to be able to remove immune complexes entering the villus core. Johnson, Brown and Faulk (1980), have suggested that the placenta acts as a 'sink', by sequestering the

soluble immune complexes formed from the maternal antibodies and paternal antigens. However, some maternal antibodies do reach the fetus, as illustrated by the fetal acquisition of passive immunity. Fcγ receptors on the surface of the placenta (Johnson and Brown, 1981) bind maternal IgG which is then transported across the placenta to the fetus. Some of the cellular components of the maternal immune system can also cross the placenta as shown by the graft-versus-host (GVH) type reactions of the fetus to maternal T-lymphocytes (Beer and Billingham, 1976). It is interesting to note that alpha fetoprotein (AFP) a pregnancy associated protein of fetal origin, has been shown in both human and murine in vitro systems to selectively suppress cytotoxic T-lymphocytes, particularly T-helper cells (Peck, Murgita and Wigzell, 1978), and it seems likely, therefore, that AFP may help to suppress GVH reactions in the fetus.

Recent evidence suggests (see Johnson, 1982) that successful maternal acceptance of the semi-allogenic conceptus is aided by a certain degree of histoincompatability between the mother and fetus. Couples in which the woman has habitual spontaneous abortions are thought to express a greater degree of sharing of HLA loci than would be expected by reference to couples with no such clinical history (Beer et al, 1981), and a high incidence of anti-sperm antibodies are also found in these women (Jones, 1976). Further, other women who undergo recurrent consecutive abortions of unknown aetiology have been shown to lack maternal IgG blocking factors (Rocklin et al, 1976). It is interesting to note that Taylor and Faulk (1980) on the basis of the theory of the selective advantages of allogenic stimulation for a successful pregnancy, used repeated pooled leucocyte infusions to successfully treat 4 women with a history of recurrent spontaneous abortion.

However, there are risks involved in such forms of treatment such as anaphylactic hypersensitivity, infection and also cytotoxic responses that would prejudice the individual should clinical transplantations ever be required.

In summary, it seems possible to conclude that no single explanation can suffice for the survival of the conceptus as an intra-uterine "allograft". There is clearly a complex and dynamic equilibrium between the maternal and fetal systems, which probably involves the constant interplay of many factors, including at least some of those described above.

2) PRACTICAL APPLICATIONS FOR STUDIES OF PREGNANCY ASSOCIATED PROTEINS

In this section the clinical applications of placental and other pregnancy associated proteins (including steroid hormones) will be briefly considered. These fall into 3 main areas of study; namely obstetrics, oncology and immunology.

2:1 Obstetric considerations

The diagnosis of pregnancy relies on detecting substances produced by the conceptus or the uterus bearing a conceptus, found in maternal blood or urine. The 2 proteins which have proved the most useful for diagnosis of pregnancy are human chorionic gonadotrophin (hCG, the β subunit in particular) and pregnancy-specific β_1 glycoprotein (SP1), which are produced by the embryo during the early stages of implantation (Van Leusden, 1976; Grudzinskas *et al*, 1977).

Measurement of pregnancy-associated proteins can be used as diagnostic makers of fetal well-being in both early and late pregnancy

(Chard, 1976). Although hCG is a sensitive diagnostic test for pregnancy, the marked decrease in the maternal levels of this protein after the 1st trimester render it of little value in the diagnosis of pregnancy complications. However, it is of prognostic value in early threatened abortions (low hCG values), and in the detection of trophoblastic disease (high hCG values, since there is no fetal consumption of the hormone; Van Leusden, 1976). In severe rhesus iso-immunization urinary and serum hCG are increased, presumably due to the increased production of the large and hydropic placenta. However, maternal levels of placental lactogen (hPL) and SP1 rise sharply throughout pregnancy (see Figure 1:10b), and the relationship between the serum concentrations of these proteins and the status of the feto-placental unit have been extensively investigated (see reviews of Letchworth, 1976; Klopper, 1980). In about 50% of all cases with intrauterine growth retardation the serum concentrations of both of these proteins are below the normal range during the last 2 weeks prior to delivery (Bellman et al, 1980), and consistently low values of hPL in 3rd trimester maternal plasma are claimed to be indicative of fetal distress in labour (Letchworth and Chard, 1972). Low serial determinations of hPL concentrations between 10-14 weeks of gestation (and later), are useful predictors of threatened abortion. Low levels of hPL have also been associated with fetal death (lower falling levels), diabetes mellitus and in pregnancies complicated by pre-eclampsia (both mild and severe) and hypertension (Letchworth, 1976). Preliminary data suggests that SP1 may be a better indicator of fetal risk in certain pregnancy complications, particularly in assays of fetal growth retardation and pre-eclamptic toxaemia (Towler et al, 1977). Low SP1 values have also been reported to predict spontaneous abortion with a high

degree of accuracy (greater than 89%), if serial determinations are obtained (Schultz-Larsen and Hertz, 1978). Amniotic fluid levels of SP1 are elevated in cases of diabetes (a 42% increase), rhesus incompatibility (a 63% increase), and intrauterine death (a 12-fold increase; Tatra, 1979). Hence measurement of SP1 concentrations in amniotic fluid may be a valuable clinical index of high-risk fetuses. Both SP1 and hPL levels are raised in multiple pregnancies and therefore provide a useful corroborative test for the presence of twins (Towler et al, 1976).

Assays of oestrogen levels (or oestriol; a steroid hormone synthesised in the feto-placental unit), in maternal blood and urine is considered to be a reliable index of fetal well-being in late pregnancy. Reduced oestriol output is associated with an increased incidence of fetal growth retardation, fetal distress in labour and fetal death in utero (Beischer et al, 1968). Also in pregnancies with anencephalic fetuses having defective pituitaries and secondary hypoplastic adrenals, urinary levels of oestrogens (particularly oestriol) are observed at about one-tenth of those found in normal pregnancies (Thau and Lanman, 1975).

Pregnancy zone protein (PZP) and alpha feto-protein (AFP) are 2 pregnancy associated proteins not produced by the placenta, which have been used to assess fetal status. Women who show no detectable PZP in their serum tend to show a higher than normal incidence of spontaneous abortion (Beckman et al, 1974). Neural tube deformities and anencephaly can be successfully detected in 88% and 79% of cases respectively, by monitoring serum AFP levels (Brock and Sutcliffe, 1972).

In spite of these findings there are some reservations which

apply to these tests of placental function and fetal well-being. Due to assay and biological variation, overlap is present between the normal and abnormal populations. For example, a normal, but small baby will have similar hPL levels to a much larger baby in whom placental function is decreased. There is a percentage of false positive and negative results, and to reduce this problem large populations need to be surveyed to define the limits. Other clinical biochemical or biophysical tests may be better predictors of fetal well-being. Prediction of fetal risk using ultrasonography or fetoscopy may prove superior to biochemical assays since the fetus is directly assessed, whereas protein concentration, for example, is an indirect test of the feto-placental unit (Gordon and Grudzinskas, 1978). Other important criteria of such tests are that small day to day, and subject to subject variations are observed. Plasma oestrogen assays for example, can be highly variable, whereas hPL and SP1 show much less variability (Klopper, Smith and Davidson, 1979). Lack of data on the biological activities of these proteins, particularly the more recently discovered ones such as PAPP-A, PAPP-B, has hampered the proper evaluation of the clinical significance of individual circulating proteins. However, until these data are available the ease and accuracy of measurement is probably the most important criterion in judging which placental protein to use in clinical practice (Chard, 1976).

2:2 Oncological considerations

Sensitive assay systems have revealed that a wide range of trophoblastic and non-trophoblastic tumours produce specific placental proteins such as hCG, placental aklaline phosphatase and SP1, and pregnancy associated proteins such as AFP. Gordon and Chard (1979),

have stated that since findings of this type are now so common it would seem likely that all tumours can produce all placental proteins. Specific tumour markers may aid in their detection and localization as well as the detection of recurring tumours. It is current clinical practice to measure hCG in the plasma or urine of patients known or suspected to have trophoblastic tumours and in the subsequent diagnosis, management and follow-up of such patients. In about 1.5% of cases (Romney, Wharton and Fletcher, 1975), choriocarcinoma (a highly malignant, invasive derivative of fetal trophoblastic epithelium), appears after a hydatiform mole (which occurs in 1 out of 2,000 pregnancies; Romney, Wharton and Fletcher, 1975). Early diagnosis can be made by monitoring the persistence of β hCG in blood and urine (Bagshawe, 1974). Choriocarcinomas metastasize very readily via the veins, involving many tissues and organs, especially the brain and lungs in addition to the uterus and other pelvic organs. This malignant neoplasm is curable even after metastatic dissemination has occurred. The progress of metastasis and the eradication of the malignant cells with cytotoxic drugs can readily be estimated by monitoring patient's plasma, urine and cerebral spinal fluid with a radioimmunoassay for hCG.

The ectopic production of trophoblast-specific proteins by non-trophoblastic tumours is now a well recognized phenomenon. In breast cancer, 76% of patients have detectable SP1 in the cytoplasm of tumour cells, and women with SP1 negative cancers have significantly longer survival times than those who are positive (Horne, Reid and Milne, 1976). SP1 has also been detected in the sera of some patients with malignant tumours of the lung, gastro-intestinal tract and ovary (Tatarinov and Sokolov, 1977). Monitoring, serum levels of pregnancy associated α_2 glycoprotein is said to be a reliable method of predicting

metastatic disease, the levels increasing prior to clinical recognition of metastases and decreasing following successful treatment (Stimson, 1975). AFP has been detected in the serum of patients with primary hepatoma (in some areas of Africa 80% of all patients with hepatomas give a positive test, whereas in Western countries rates as low as 30% have been reported), and in primitive gonadal tumour, teratoblastoma (Alexander, 1972). Ectopic production of placental alkaline phosphatase has also been found to be associated with a wide range of cancers (Fishman, 1973).

The reason for the production of placental and fetal antigens by tumours has been the subject of much speculation, but as yet, no conclusions have been drawn. Principal theories include the reversion of normal cells to an embryonic state in association with the process of neoplasia. Goodfellow (1982), suggests that cancer results from the abnormal expression of one of a number of normal differentiation genes which has repercussions on the metabolism and physiology of the cell. New antigens on transformed cells will fall into 3 categories. First, the direct gene products of the 'abnormal' fetal differentiation antigens. Second, gene products induced or elevated by the 'phenotype shift', for example transferrin receptors, placental specific antigens etc. Third, changes in carbohydrate antigens resulting from the altered cellular physiology causing changes in the sizes of the carbohydrate pools used to make glycoproteins and glycolipids.

Certain aspects of tumour biology are comparable to the biology of the placenta (Aitken, Beaconsfield and Ginsburg, 1979), including their invasive properties, autonomous cell division, immune tolerance in the 'host' and production of proteins. However, the placenta has 'brakes' imposed on its growth, if the nature of these brakes (a

placental protein?) could be determined the repercussions on oncology would be very great.

2:3 Immunological considerations

Pregnancy-associated proteins may affect the maternal immune response during gestation and, though somewhat controversially, immunosuppressive roles have been postulated for a number of them (see Table 1:1 and section 1:3 of this chapter). The site of action of these proteins could be in the maternal systemic circulation or, more probably, locally at the utero-placental interface. Studying these proteins and their proposed roles in the maintenance of the conceptus during pregnancy may well aid our understanding of the contrasting immunological problems of graft rejection and the body's apparent tolerance of malignant tumours. If indeed these proteins do contribute to immunoprotection of the conceptus they could be used clinically in the following ways: to promote acceptance of tissue and organ transplants, to treat (or even prevent) autoimmune disorders and conditions of pregnancy with immunological aetiologies, such as pre-eclampsia, and to provoke immune rejection of malignant tumours.

Recurrent abortions and pre-eclamptic toxaemia (PET) are two conditions thought to have immunological aetiologies. The immunological theories of recurrent abortion have already been considered in section 1:3 of this chapter, to which the reader is referred. However PET will now be considered. This disease occurs to varying degrees in about 5-7% of all pregnancies (Wingate *et al*, 1975) and it is one of the 3 major causes of maternal death. The clinical symptoms of PET are hypertension, oedema, proteinuria and occasionally convulsions (developing in the 3rd trimester of pregnancy), which can result in both

fetal and maternal mortality. PET is more common in young, often submaximally nourished, primigravida who are confronted with a large placenta, due to a greater than normal genetic disparity between the mother and fetus (Beer, 1975). The dosage of fetal antigenic determinants confronting the mother may swamp her immune response so that insufficient blocking antibody is produced. This ultimately results in the production of specific cytotoxic antibodies to the trophoblast. This could lead to tissue necrosis at the choriodecidual junction of the placenta, increased fibrin deposition and damage to maternal and fetal blood vessels. Theoretically, this local tissue injury could result in the release of uterine renin, prostaglandins as well as other agents which might disturb the renin angiotensin system, evoking hypertension, oedema or premature labour. Trophoblast specific antibodies can cross-react with human kidney and brain (Curzen, 1968), which may explain why these areas are also attacked in PET. A second pregnancy by the same father may have the added advantage of blocking antibodies to paternal antigens persisting from the first pregnancy. This could explain the reduction of PET observed in subsequent pregnancies. The levels of certain pregnancy-associated proteins such as PAPP-A (Hughes et al, 1980; see section 3:4 of this chapter), and SP1 (Towler et al, 1977), are said to be affected by PET. Hence monitoring the levels of these proteins may enable the onset of PET to be predicted and suitable clinical measures to be taken to prevent its genesis, such as maternal immunization with paternal antigens or administration of blocking antibodies (specific for the paternal antigens).

Immunization against placental antigens is of special interest with regard to sterility, fertility and contraception. Antibodies to

placental antigens can arise spontaneously in women during pregnancy (see section 1:3 of this chapter). Experiments with animals (see Hearn, 1980 for recent reviews), have been carried out to monitor the effects of immunization against placental antigens on fertility and pregnancy, and the induction of abortions and reduced fertility have been observed using these techniques. Studies in this field have extended over the last few years as purified placenta-specific proteins have become available, and it is now hoped that these antigens may provide a new approach to the regulation of fertility in humans. Bohn (1980), found that monkeys immunized with SP1 and PP5 showed a significant reduction in fertility, whereas immunization with heat-stable alkaline phosphatase appeared to be less effective. Of the placental proteins looked at so far, hCG appears to be the most promising antigen with respect to fertility control (Stevens, 1980). As passive immunization with placental proteins can cause abortions it would seem to confirm the view that these proteins play an important role in the establishing and maintenance of the conceptus.

3. PREGNANCY-ASSOCIATED PLASMA PROTEIN A

Ten years ago Gall and Halbert (1972) described 4 antigenic constituents which were found specifically in the plasma of pregnant women. These were later designated pregnancy-associated plasma proteins, PAPP-A, B, C and D (Lin, Halbert and Kiefer, 1973; Lin et al, 1974b). It was subsequently found that 2 of these proteins were known pregnancy proteins, PAPP-C being SP1 and PAPP-D being hPL (Lin et al, 1974a). The remaining 2 proteins, PAPP-A and PAPP-B, were shown to be unrelated

TABLE 1:2 Purification of PAPP-A

Consecutive purification steps

Halbert and Lin (1979)	1°DEAE cellulose, ion exchange chromatography. PAPP-A eluted between 0.175-0.3M NaCl.	2°Sephadex G200, gel filtration; PAPP-A eluted in void volume.	3°Sepharose 6B, gel filtration.	4°Ammonium sulphate (50%) used to salt out PAPP-A	5°Precipitate harvested and dialysed vs. PBS.
Bischof (1979)	1°Ammonium sulphate (60%) used to salt out PAPP-A. Precipitate redissolved.	2°DEAE Sephadex A50, ion exchange chromatography. PAPP-A eluted between 0.3-0.5M NaCl pH 6.8.	3°Concanavalin-A Sepharose. PAPP-A eluted with 0.1M Met-α-mannopyranoside pH 6.8.	4°Sepharose CL4B gel filtration.	5°NAAC on anti-human serum Sepharose. PAPP-A eluted with unbound proteins.
Sutcliffe et al (1979 and 1980b)	1°Ammonium sulphate (2M) used to salt out PAPP-A. Precipitate redissolved in PBS.	2°Affinity chromatography on sheep anti-PAPP-A serum Sepharose. The column was washed with 1M NaCl and PBS. PAPP-A was eluted with 1.5M KI pH 7.5.		3°DEAE Sepharose CL6B, ion exchange chromatography. PAPP-A eluted at 0.25M NaCl pH 6.5.	4°Sepharose CL6B gel filtration.
Folkersen et al (1981a)	1°Affinity chromatography on rabbit anti-PAPP-A serum Sepharose. Column was washed with 0.1M Barbital-acetate-HCl buffer and 1M NaCl. PAPP-A was eluted with 0.3 M $MgCl_2$, 0.6 M NaCl pH 4.8.			2°NAAC on anti-human serum Sepharose. PAPP-A eluted with unbound proteins. Fractions pooled and concentrated.	

to other plasma proteins currently known to be increased in concentration during pregnancy (see Halbert and Lin, 1979).

Similar proteins are found in primates (Lin and Halbert, 1978), rats and mice (Lin, Halbert and Kiefer, 1974). It is likely, therefore, that they occur widely in mammals, which argues that they perform some essential functions in pregnancy (Klopper, 1980).

This section summarises the present state of knowledge of pregnancy-associated plasma protein A (PAPP-A), also known as pregnancy-specific protein 4 (SP4; Folkersen et al, 1979).

3:1 Purification of PAPP-A

PAPP-A has been purified using a variety of chromatographic techniques, as summarised in Table 1:2, and most have produced highly purified preparations of this protein. However, 3 of the purification procedures contained steps requiring ammonium sulphate precipitation (Table 1:2; step 4, Lin et al, 1979; steps 1, Bischof, 1979; and Sutcliffe et al, 1980b), and those employing affinity chromatography (Sutcliffe et al, 1980b; Folkersen et al, 1981a) required buffers of high salt concentration for desorbing the PAPP-A (Table 1:2). Such agents may have disrupted the conformation of the purified PAPP-A (see Chapter 3). These preparations were used to investigate the biochemical and physiological properties of PAPP-A reported in this section.

3:2 Biochemistry of PAPP-A

PAPP-A is a high molecular weight serum glycoprotein. It has an estimated molecular weight of 750,000 (740,000-820,000; Lin, Halbert and Kiefer, 1973; Bischof, 1979; Sutcliffe et al, 1980b), and is

thought to be a homotetramer, a dimer of dimers, with each monomer having a molecular weight of about 187,000 (Sutcliffe et al, 1980b). As neuraminidase treatment alters its electrophoretic mobility (Lin et al, 1974a), PAPP-A is thought to contain sialic acid (2%, approx; Sutcliffe et al, 1980b). No galactosamine could be detected in PAPP-A though a 3.1% content of glucosamine was found by Bischof (1979). On immunoelectrophoresis, PAPP-A migrates with an alpha-2 electrophoretic mobility (Lin, Halbert and Kiefer, 1973). Isoelectric focusing studies in ampholine/sucrose and ampholine/urea systems located the isoelectric point of PAPP-A at pI 4.3 and 4.42 respectively (Lin, Halbert and Kiefer, 1973; Sutcliffe et al, 1980b). PAPP-A elutes from ion exchange chromatography columns at 0.25 M NaCl (range 0.175-0.5 M NaCl; see Table 1:2 for references and details of chromatography media). Non-immunoreactive PAPP-A is obtained after treatment with 1.5 M KCNS, 2M KI (Sutcliffe et al, 1979), and proteases (Lin et al, 1974b). PAPP-A is precipitated from pregnancy plasma by 50% saturated ammonium sulphate, but unlike PAPP-B and SP1, it is soluble in 30% saturated ammonium sulphate (Halbert and Lin, 1979). A specific interaction between PAPP-A and heparin has been demonstrated, using heparin affinity crossed immunoelectrophoresis applied to late pregnancy serum (Sinosich et al, 1981).

3:3 Synthesis and localization studies

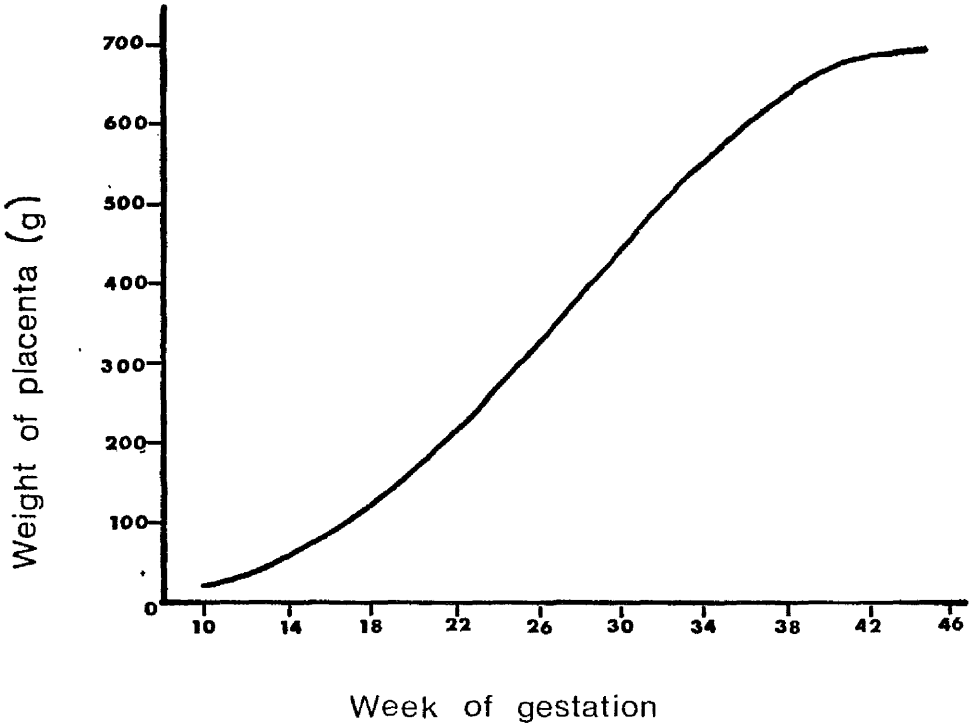
There has been some controversy recently over the nature of PAPP-A, as to whether it should be regarded as a pregnancy-specific or pregnancy-associated protein. With the development of sensitive radio-immunoassays (Bischof et al, 1981; Sinosich et al, 1982), it has been possible to detect very small amounts of PAPP-A (3-14 ng/ml of plasma).

Figure 1:10 (a) Weight increase of the placenta during gestation
(Adapted from Klopper, 1969)

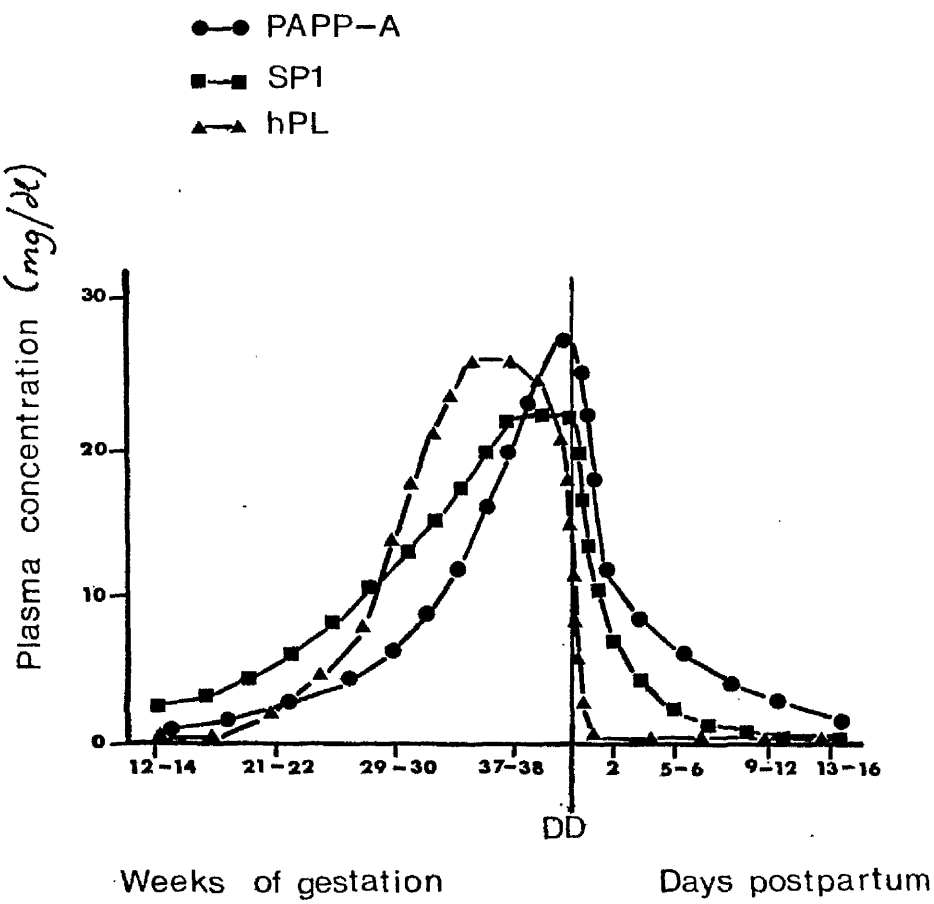
(b) Mean concentration of PAPP-A, SP1 and hPL in random plasma samples during gestation and after delivery. DD, day of delivery.
(Adapted from Lin et al, 1976)

Figure 1:10

a



b



Bischof et al (1981) claimed to have detected PAPP-A in the plasma of non-pregnant women (at 0.1 μg/ml), whereas Sinosich et al (1982) did not detect PAPP-A in the 100 non-pregnant controls they looked at. This controversy has still to be resolved and the following account of the synthesis and localization of PAPP-A will be discussed, wherever possible, bearing these 2 points of view in mind.

Circulating concentrations of PAPP-A have been detected, using a radioimmunoassay, as early as 28-32 days after ovulation in singleton pregnancies and 21 days after ovulation in twin pregnancies (Sinosich et al, 1982). Using rocket immunoelectrophoresis techniques (Bischof et al, 1979), the earliest PAPP-A can be detected is the 12th week of pregnancy. The concentration of PAPP-A rises steadily during mid-pregnancy and at a steeply increasing rate from 30 weeks right up to term (Lin, Halbert and Spellacy, 1976), with a mean concentration at term of 0.1 mg/ml. This is an uncommon pattern of secretion for a placental protein. Figure 1:10 shows the secretion pattern of PAPP-A and compares it with the secretion patterns of hPL and SP1, and a standard placental weight curve. The concentration of these placental proteins in maternal blood reaches a plateau at approximately the same time as the placental weight curve, however, the concentration curve of PAPP-A does not plateau, and its concentration reaches a maximum only at delivery. A plasma half-life of 2-3 days has been calculated for PAPP-A from its rate of fall after delivery (Smith et al, 1979). It would appear, therefore, to be more slowly removed from the maternal bloodstream than other placental proteins such as hPL (half-life of 20 min), SP1 (half-life of 1-2 days), and PAPP-B (half-life of less than 1 day; Halbert and Lin, 1979). Smith, Cooper and Thomson (1980),

TABLE 1:3 Histological localization of PAPP-A in the placenta

	TECHNIQUE	LOCATION
Lin and Halbert (1975)	Indirect immunofluorescence	Syncytiotrophoblast
McIntyre et al (1981)	Immunofluorescence	Intervillous fibrin. Syncytiotrophoblast and villous fibrinoid (both showed highly variable staining).
Wahlstrom, Teisner and Folkersen (1981)	Enzyme-bridge immunoperoxidase staining	Syncytiotrophoblast, concentrated in a thin intracytoplasmic rim at the apical border of those cells
Sutcliffe, Khalef and Horne (in preparation)	Enzyme-bridge immunoperoxidase staining	Some patchy staining in the syncytiotrophoblast. Strongly staining areas:- villous cytotrophoblast; chorionic plate; cyto-trophoblasts in fibrinoid material (Nitabuch's layer) at the utero-placental interface and in reticular fibres around uterine decidual cells.

suggest that the observations on PAPP-A concentration and half-life can be explained as follows. PAPP-A levels in late pregnancy increase with increasing placental size. As the placenta ages and its growth slows down, PAPP-A continues to rise as the mechanical trauma of uterine activity (Attwood and Park, 1961) exfoliates the placenta of potential microemboli (small fragments of trophoblast). These subsequently enter the maternal circulation and eventually die in the lungs (over a period of 2-3 days), thus releasing PAPP-A. However, this theory does not account for the concentration curves of other proteins thought to be of trophoblastic origin such as SP1, PP5 and PP12. If PAPP-A is maternal in origin (as suggested by Bischof et al, 1981), then the comparison of PAPP-A synthesis to placental weight is invalid, and the long half-life observed after parturition can be explained, since synthesis will not cease on delivery of the placenta.

Using the immunoelectrophoresis method described in Bischof et al (1979), Smith et al (1979) determined the levels of PAPP-A in various maternal and fetal compartments. No PAPP-A was found in cord blood or amniotic fluid using this method, which was taken to show that the secretion of PAPP-A was directed into the maternal rather than the fetal compartments. The concentration of PAPP-A in the maternal peripheral blood was 8x greater than in the peritoneal fluid (which suggests that the passage of PAPP-A out of the blood vessels is greatly impeded). The concentrationsin the uterine and peripheral veins were very nearly the same, which indicates that the inflow from the placenta is too slow to raise the local concentration. The mean concentration of the retro-placental blood was half that of the peripheral blood. Smith et al (1979), found this unusual distribution difficult to reconcile with the direct passage of PAPP-A into the intervillous blood, and concluded

that the intervillous space was not the main point of entry of PAPP-A into the maternal circulation. Again they postulated the theory of embolism, as described above, to explain this distribution. A further possibility they put forward was that PAPP-A was released directly into the maternal tissue as a result of the invasion of the decidua by non-villous trophoblast (see Robertson, Brosens and Dixon, 1975). Dissolution of non-villous trophoblast after parturition could account for the relatively long half-life of PAPP-A.

Sinosich et al (1982) using their radioimmunoassay did not detect PAPP-A in the umbilical cord circulation but they did observe PAPP-A in amniotic fluid (less than or equal to 7.4% of PAPP-A in maternal sera at term; i.e. 2.7 μg/ml). Bischof et al (1982a) using their radioimmunoassay also found PAPP-A in amniotic fluid (3.8 μg/ml at term), with the increase in amniotic fluid concentration during gestation paralleling the increase in PAPP-A in the maternal circulation. Neither group was able to suggest how PAPP-A might enter this fetal compartment at such concentrations. Duberg et al (1982) using the radioimmunoassay of Bischof et al (1981), found that the concentration of PAPP-A in maternal decidua at term (57.0 $\pm$ 2.0 μg/g) was 3x higher than in the trophoblast (16.9 $\pm$ 5.4 μg/g) and that the concentrations in the endometrium of non-pregnant women (1.9 $\pm$ 0.6 μg/g) was 40x higher than in corresponding plasma samples (0.05 $\pm$ 0.02 μg/ml). Duberg et al (1982) suggested therefore, that PAPP-A was of decidual rather than placental origin, (though they did not exclude an additional fetal origin).

Histological staining studies have been used to localize PAPP-A on sections of placental and decidual tissues. The results of these experiments are summarised in Table 1:3. Lin and Halbert (1976),

McIntyre et al (1981) and Wahlstrom, Teisner and Folkersen (1981), localized PAPP-A staining to the syncytiotrophoblast. However, Sutcliffe, Khalef and Horne (in preparation) found PAPP-A in the villous cytotrophoblast, chorionic plate, associated with reticular fibres around uterine decidual cells, and in Nitabuch's layer at the utero-placental interface. Some patchy staining was also observed by these workers in the syncytiotrophoblast. The PAPP-A distribution that Sutcliffe, Khalef and Horne observed was very similar to that of the high molecular weight glycoprotein Fibronectin (see Chapter 4, section 6), and both of these proteins were often observed in conjunction with reticular fibres. Sites of synthesis cannot be deduced from the results of such localization studies. However, Khalef, Buultjens and Sutcliffe (in preparation), carried out tissue culturing experiments on Nitabuch's layer (see also Chapter 4:6) and chorionic plate, both of which synthesised ^{35}S labelled PAPP-A and fibronectin. These results would seem to indicate therefore, that PAPP-A is synthesised by cyto-trophoblastic cells in these areas, and the staining of reticular fibres around decidual cells was indicative of PAPP-A accumulation rather than synthesis.

3:4 Physiological studies on PAPP-A

A variety of approaches have been employed in an attempt to elucidate the part that PAPP-A might play in gestational processes and feto-maternal homeostasis. From the localization studies discussed above, it has been suggested that the sites of staining of PAPP-A in the trophoblast are equivalent to its site of synthesis; or synthesis and action; or its site of accumulation and action. These results have led to the supposition that PAPP-A is concerned with the maintenance of

the placenta as an allograft, by suppressing the immune reactions of the mother. Other possible functional roles have been investigated by monitoring PAPP-A levels during pregnancy and comparing them with various obstetric parameters. Circulating levels of PAPP-A have also been monitored in patients with various malignant tumours. Finally *in vitro* biochemical studies, using purified samples of PAPP-A, have also been carried out and its effect on the activity and binding of several enzymes and proteins assessed. The results from these approaches will now be considered.

In vitro, Biochemical studies using purified PAPP-A

Much of the work in this area has been carried out by Dr. Paul Bischof with PAPP-A produced in the manner described in Table 1:2 and in Bischof (1979). Therefore, the results obtained must be viewed as pertaining to this particular preparation of PAPP-A. The functions assigned to PAPP-A by Bischof may or may not reflect the *in vivo* functions of this protein.

Common antigenic determinants between PAPP-A and other proteins of known biological function were looked for since this might give a clue to the physiological role of PAPP-A (Bischof, 1979). No reaction was observed in immunodiffusion studies when PAPP-A was tested against antisera to human thyroid stimulating hormone, luteinizing hormone, follicle stimulating hormone, prolactin, placental lactogen, alpha fetoprotein, SP1, SP3, α_2M, antithrombin III, α_1-antitrypsin, plasminogen, prothrombin and non-pregnancy human serum proteins.

The relatively long half-life of PAPP-A after parturition (Smith *et al*, 1979), suggested to Bischof (1979), that PAPP-A might be a steroid binding protein, thus extending the short half-life of these

hormones. However, binding studies between PAPP-A and tritiated progesterone, oestrone, oestradiol, oestriol, testosterone and cortisone gave negative results (Bischof, 1979).

On incubation of PAPP-A and plasmin, 30% inhibition of the plasmin activity in a caseinolytic assay was recorded (Bischof, 1979). Also inhibition of urokinase mediated fibrinolysis was observed (Bischof, 1979) by the fibrin plate method (Astrup and Mullertz, 1952). Both of these results suggest that PAPP-A is involved in the regulation of fibrinolysis. It is well documented that the fibrinolytic activity of maternal blood decreases as pregnancy progresses and is rapidly restored after delivery of the placenta. This points to the presence of fibrinolytic inhibitors in the placenta, and such inhibitors have been shown to exist (Astedt, 1972). However, *in vitro* studies have shown that placental protein 5 (PP5) inhibits the proteolytic activity of trypsin and plasmin (Klopper, 1980) and could therefore represent the placental protease inhibitor responsible for the observed inhibition of fibrinolysis.

PAPP-A is also reported to be an inhibitor of complement-mediated haemolysis (Bischof, 1979, 1981). The inhibitory capacity of PAPP-A produced by positive affinity chromatography (Sutcliffe *et al*, 1980b) was also tested by Bischof (1981) and identical results to those observed with his own preparation of PAPP-A were obtained. However, Sutcliffe himself did not observe inhibition of complement-mediated haemolysis using the same PAPP-A (Dr. R.G. Sutcliffe, personal communication). Bischof (1981) speculated that if PAPP-A inhibited the activity of complement during pregnancy, it might participate in the immunosuppressive mechanisms thought to prevent rejection of the fetus.

Similarly, Bischof et al (1982b), have reported that PAPP-A inhibits phytohemaglutinin (PHA) induced T-lymphocyte transformation (but not pokeweek mitogen (PWM) induced B-lymphoblastogenesis). They suggested that this was brought about by PAPP-A affecting the secretory products (possibly lymphokines possessing mitogenic activities), of the transformed T-cells. McIntyre et al (1981) found neither anti-PAPP-A nor PAPP-A itself exerted a cytotoxic effect on peripheral blood lymphocytes, and neither affected lymphoblastogenesis in response to PHA, PWM or Concanavalin A or in mixed lymphocyte reactions (MLR). They therefore concluded that PAPP-A was not involved in the regulation of lymphocyte function in vitro, which is contrary to the view held by Bischof et al (1982b). Many pregnancy-associated proteins have been postulated to be "immunosuppressive" agents (see Table 1:1), and this heterogeneous collection of compounds has been shown to modify one or more of several immunological tests such as MLR, mitogen-induced lymphoblastogenesis or the rosette inhibition assay. With the exception of steroids such as cortisone, there is little direct evidence that these agents do in fact have an immunosuppressive effect in vivo. That is, they are immunosuppressive only in the restricted sense that they can modify or inhibit an immunological test in vitro. The immunosuppressive results reported for PAPP-A by Bischof et al (1982b) should be viewed in this light.

A specific and highly selective interaction has been reported to occur between PAPP-A (in late pregnancy serum) and heparin (Sinosich et al, 1981). Interaction between heparin and a wide range of tissue and circulating proteins of the non-pregnant state has been examined using heparin-Sepharose chromatography (McKay and Laurell, 1980). This report suggested that with the exception of β-lipoprotein, the proteins which bound to the immobilised heparin could be divided into 4 functionally

related groups, i.e. coagulation proteins, protease inhibitors, cell surface proteins and complement system proteins. Sinosich et al (1981) concluded therefore, in conjunction with the localization of PAPP-A to the apical membrane of the syncytiotrophoblast (Wahlstrom, Teisner and Folkersen, 1981), that PAPP-A is involved in the coagulation system during pregnancy and in particular with the maintenance of the intervillous blood flow.

The relationship between obstetric parameters and PAPP-A concentrations

By measuring levels of PAPP-A throughout gestation (which is now possible from approximately the 5th week of pregnancy right through to the postpartum period using radioimmunoassays) and comparing them with obstetric parameters in normal and complicated pregnancies, it was hoped that the function of PAPP-A might be revealed. It was also hoped that PAPP-A might be useful as a diagnostic marker of pregnancy and early placental function, and that measurement of its concentration would increase the clinician's complement of tests for fetal well-being. The results of such investigations are summarised in Table 1:4 and will now be briefly considered.

Mothers with large placentae, as in twin and primigravid pregnancies, seem to have increased serum concentrations of PAPP-A (Lin, Halbert and Spellacy, 1976). This could be explained in two possible ways; the observed high PAPP-A values may simply reflect the fact that there is more placenta present to synthesise it; or the larger placenta may evoke a larger immunological response in the mother and therefore more immunosuppressant (i.e. PAPP-A) is produced to counter this. The latter hypothesis would seem to be supported by the high values of PAPP-A observed in PET (see section 2:3 of this chapter) and

TABLE 1:4 Correlations between PAPP-A serum levels and obstetric parameters

Parameter	PAPP-A levels c.f. control at term (pre-labour)	Reference
Large fetus	High	Lin, Halbert & Spellacy (1976)
Large placenta (especially in primigravid females)	High	Lin, Halbert & Spellacy (1976)
Twin pregnancies	High	Lin, Halbert & Spellacy (1974)
Male fetuses	High	Lin, Halbert & Spellacy (1974)
Increasing maternal age	Low/ possible increase	Bischof, Hughes & Klopper (1980)/ Sutcliffe et al (1982b)
Increasing parity	Low	Lin, Halbert & Spellacy (1974); Bischof, Hughes & Klopper (1980)
Onset of spontaneous labour	High	Smith et al (1979)
Induced labour	Higher than spontaneous	Smith, Thomson & Cooper (1981)
Premature labour	High	Hughes et al (1980); Smith, Thomson & Cooper (1981)
Pre-eclamptic Toxaemia	High	Lin et al (1977); Hughes et al (1980)
Rh-negative fetuses	High	Bischof, Hughes & Klopper (1980)
High diastollic blood pressure	High	Lin, Halbert & Spellacy (1976)
Antepartum haemorrhage	High	Hughes et al (1980)
Intra uterine growth retarded fetuses	As control	Sutcliffe et al (1982b); Hughes et al (1980)
Babies with high Apgar scores at 1 min	High	Bischof, Hughes & Klopper (1980)
Gestational diabetes in mother	As control	Sutcliffe et al (1982b)
Insulin dependent diabetes in mother	low/ As control	Sutcliffe et al (1982b)/Lin et al (1977)

male fetuses (because of the antigenic determinants on the Y chromosome). The low values of PAPP-A in the serum of mothers of increasing parity would also seem to lend support to this hypothesis since "pre-immunization" to paternal antigens may confer a degree of immunity on the mother. In subsequent pregnancies less immunosuppression would therefore be required to counteract the mother's rejection response. It would be interesting to measure the PAPP-A levels of multiparous women who have had one or more changes of husband and compare them to monogomous females of equivalent parity. Should the levels of PAPP-A be higher than expected this may lend some support to the notion that it is an immunosuppressant.

Smith, Thomson and Cooper (1981) suggest that observed increases in PAPP-A concentration associated with the onset of labour, whether spontaneous, induced or premature, is the consequence of increasing uterine activity at this time and not that PAPP-A is involved with the initiation of labour.

If PAPP-A is involved in anticoagulation, a high level at term could result in a greater postpartum persistence of the protein in the maternal circulation. This could delay the restoration of normal coagulation, which might result in a greater maternal susceptibility to antepartum haemorrhage.

Sutcliffe et al (1982b) have suggested that the effect of insulin on blood levels of PAPP-A shows that the concentration of PAPP-A is capable of altering significantly in response to certain physiological changes associated with the control of carbohydrate metabolism.

Contrary to the observations made by Lin, Halbert and Spellacy (1976) that PAPP-A levels are increased with large fetuses, the reported normal levels of PAPP-A with intrauterine growth retarded fetuses (Sutcliffe et al, 1982b), suggest that PAPP-A is not a useful index of fetal size or weight.

Circulating levels of PAPP-A in association with malignant tumours

Using immunoelectrophoresis methods, Lin et al (1974a) were unable to detect PAPP-A in the sera of 102 patients with various malignancies. However, Sinosich et al (1982), using a radioimmunoassay, detected circulating PAPP-A (15.04 μg/ml) in 2 patients with hydatiform mole, but not in patients with choriocarcinoma or a patient with endodermal sinus tumour. Bischof et al (1982c), found that circulating levels of PAPP-A in patients with non-trophoblastic tumours were not significantly different from levels in matched control groups. However, circulating levels of PAPP-A were significantly elevated pre-operatively in patients with trophoblastic tumours, whereas the post-operative concentrations gradually decreased. The production of placental proteins by tumours is not an uncommon occurrence; in fact it seems likely that all tumours can produce placental proteins (Gordon and Chard, 1979). This is thought to be linked to the process of neoplasia and a general reversion of what were otherwise normal cells to an embryonic state. So studies of placental protein production in patients with tumours does little to further our understanding of the function of these proteins.

In summary, there is circumstantial evidence that PAPP-A may be involved in immunosuppression and regulation of blood coagulation during pregnancy. To the clinician, PAPP-A could prove useful for monitoring the onset of pre-eclamptic toxaemia and labour. However, whether PAPP-A is an effective index of fetal growth and vitality has yet to be demonstrated.

4. HUMAN ALPHA-2-MACROGLOBULIN : A COMPARISON WITH PAPP-A

PAPP-A has certain characteristics in common with the serum

protein alpha-2-macroglobulin (α_2M). Before the common characteristics of these two proteins are considered (section 4:2), a basic review of the biochemistry and physiology of α_2M will be presented.

4:1 Biochemistry and Physiology of α_2M

The data presented in this section has been compiled from the following reviews on α_2M, Harpel (1976), Starkey and Barrett (1977) and Van Leuven (1982) and references therein.

α_2M is a glycoprotein containing 8-11% carbohydrate including sialic acid. It is a high molecular weight tetramer (725,000), a dimer of dimers, which can be split into halves without cleavage of covalent bonds, and into quarters (181,000-185,000 mol wt), by reduction of interchain disulphide bonds. On electrophoresis α_2M is seen to have an alpha-2 mobility, and a pI between 5.0 and 5.5.

α_2M forms complexes with and inhibits nearly all endopeptidases (but not exopeptidases or non-proteolytic enzymes), as well as binding nickel and 20% of the total serum zinc content (though this does not seem to play an essential role in the physiological functions of α_2M; Starkey and Barrett, 1977). Considering the unique interaction between α_2M and proteases in a little more detail. Active endopeptidases bind irreversibly (involving covalent bond formation) to α_2M, and saturation of a molecule of α_2M with a protease prevents the subsequent binding of other proteases. The bound enzymes retain their activity towards low molecular weight substrates and inhibitors, but are prevented from binding to large substrate molecules (i.e. their natural proteinaceous substrates). The bound enzyme is also protected from other high molecular weight inhibitors and transfer of proteases from other inhibitors to α_2M has been observed *in vitro* and *in vivo*.

As the α_2M:protease complex is formed, the polypeptide chains of α_2M are proteolytically cleaved. This proteolysis is limited, and with most endopeptidases yields α_2M fragments of the same size (85,000 mol wt, on reduction). Based on these findings Barrett and Starkey (1973) proposed a 'trap' model for α_2M, in which a short region located near the middle of the α_2M subunit polypeptide chain (the 'bait' region), is proteolytically cleaved to provoke a conformation change which entraps the enzyme. This 'bait' region has been shown to be a sequence of amino acid residues which reflect the substrate specificity of virtually all endopeptidases, hence the broad specificity of α_2M for these enzymes (Van Leuven, 1982). The inhibition of the protease is due to steric hindrance which distinguishes α_2M from other inhibitors such as soya bean trypsin inhibitor and α_1-proteinase inhibitor, which bind through the catalytic site of the enzyme, thus abolishing all activity (Starkey and Barrett, 1977). Contact with high molecular weight substrates is thus prevented, although small substrates (such as esters), can still gain access. The protease inhibitory capacity of α_2M is destroyed by certain nucleophilic reagents, particularly ammonium ions, hydrazine, hydroxylamine and a series of aliphatic primary amines such as methylamine (Steinbuch et al, 1986; Van Leuvan, 1982). Reaction of α_2M with these compounds can cause irreversible conformational changes which prevent the binding of proteases. This inactivated α_2M has characteristics which are very similar to α_2M:protease complexes, such as similar electrophoretic mobility and isoelectric focusing patterns (Barrett, Brown and Sayers, 1979).

α_2M:protease complexes are rapidly removed (half-life of 6 min approx) from the circulation by reticuloendothelial cells. This does not occur for native α_2M or other enzyme-inhibitor complexes. α_2M:protease

complexes are internalised by macrophages and fibroblasts by receptor mediated endocytosis in vitro (Willingham, Maxfield and Pastan, 1979). It has been proposed (Van Leuvan et al, 1982), that the conformational change that occurs when α_2M binds enzymes, exposes a receptor-recognition site, separate from the bait region or the internal thioester groups (which form covalent bonds with the entrapped enzyme molecule). The rapid clearance of these complexes suggests that α_2M controls proteolytic activity in the circulation primarily by removing the enzyme, and that inhibition is largely incidental. It is difficult to envisage that the residual activity of the complexed enzymes during their brief time in the circulation could be of any great importance. However, the increased vascular permeability which occurs during inflammation, allows α_2M to escape into the tissue. In this situation the α_2M:enzyme complexes will not be removed as rapidly as from the circulation and any residual activity retained by the bound proteases may be important.

There are strong indications that α_2M may contribute to the body's defences against invasive pathogens and parasites, which often enter the body with the aid of proteolytic activity, as α_2M seems to be the only plasma inhibitor of several bacterial and parasitic proteases (Starkey and Barrett, 1977). It seems that inhibitors which bind tightly to the active centre of proteases may generally be highly specialised for control of the body's own serine proteases, whereas α_2M can eliminate proteases of all classes, even when they are exogenous and the body has had no opportunity to develop specific protective mechanisms.

α_2M is a major component in the circulation of vertebrates (plasma concentration of 2-4 mg/ml in humans), and it has a long evolutionary history, with for example, α_2M-like proteins being found in

the plaice (Starkey, 1981). (The 3, apparently different, genetic polymorphisms of α_2M in man (A, B and C) are of a Mendelian autosomal, co-dominant type, and are distinguishable by their differing electrophoretic mobilities.) High concentrations of α_2M are also found in extravascular fluids such as lymph, colostrum, semen, pleural fluid and synovial fluid. The cells of the lymphoreticular system (e.g. fibroblasts and lymphocytes), are thought to be responsible for the synthesis of this protein. The serum concentrations of α_2M seem to be affected by several factors (Harpel, 1976), including: age, infants possess 2.5x as much α_2M as adults; sex, with higher concentrations in females; pregnancy, α_2M levels increase significantly; and in association with oral contraceptives, women on the 'pill' have increased α_2M levels which may account for the increased tendency to thrombosis in women taking oestrogens. In most disease states, α_2M levels remain substantially normal, though increased levels have been reported in pulmonary disorders, diabetes mellitus, agamma globulinemia and in patients with liver diseases (Harpel, 1976). Decreased levels of α_2M are reported in multiple myeloma and in women with rheumatoid arthritis. In pre-eclampsia without associated proteinuria, α_2M levels fall. But when pre-eclampsia is accompanied by proteinuria, α_2M levels increase (Harpel, 1976). To date there have been no reports of individuals lacking this protein, which suggests that such a mutation is lethal. One case of an inherited α_2M deficiency has been reported (Bergqvist and Nilsson, 1979). This defect appeared to have no overt clinical symptoms and the heterozygote individuals were in good health. It has been suggested that α_2M in the serum of patients with cystic fibrosis (Roberts et al, 1982) and multiple sclerosis (Bridges, Applegarth and

Johannson, 1982), shows abnormal protease binding properties.

The protease-binding activity of α_2M may not represent its only physiological function. So large a protein could contain a variety of independent binding sites, and there are reports of binding to other molecules such as zinc and nickel as previously stated. Such interactions are presumably reversible and quite unrelated to its reactivity with proteases (Starkey and Barrett, 1977). James (1980) has speculated that α_2M-protease binding might participate in the triggering of lymphocytes and the synthesis, release and activity of immunologically important lymphoreticular cell products. However, there is no direct evidence for this occurring *in vivo*.

Using indirect immunofluorescence studies on term human placentae, Faulk and Johnson (1977) localized intense α_2M staining in areas of peri- and inter-villous fibrin/fibrinoid. Occasionally stromal components of villi exhibited small clusters of staining for α_2M including the peri-vascular regions and apical aspects of the endothelium. Human complement components C_3 and C_4 were often localized in the same areas. α_2M shares a number of properties with these proteins (Starkey, 1981; Van Leuven, 1982), and it has been suggested (Starkey, 1981) that they may have evolved from a common precursor.

In summary, α_2M is an important defense mechanism, aimed at the inhibition and removal of potentially harmful endoproteases. These may originate endogenously (e.g. from the blood clotting cascade, injured tissue, inflammation) or exogenously (e.g. from bacteria). This protective action is not restricted to the blood circulation, since α_2M is present in body fluids (including amniotic fluid), a fact that indicates a possible role in the control of proteolytic activity in the extravascular space.

TABLE 1:5 Amino acid composition of α_2M and PAPP-A

Amino acid residue	α_2M (a)	α_2M (b)	PAPP-A (c)
	mol/100 mol	g/100g of protein	residues/100 residues
Lysine	5.92	5.74	5.40
Histidine	2.55	2.77	2.30
Arginine	3.16	3.89	4.21
Aspartic acid	8.33	7.49	9.48
Threonine	7.04	5.79	6.25
Serine	8.73	5.52	7.90
Glutamic acid	12.72	12.65	10.99
Proline	6.59	3.63	5.0
Glycine	6.68	2.91	10.6
Alanine	6.64	3.63	8.23
Valine	9.23	6.82	6.25
Methionine	1.53	1.61	1.18
Isoleucine	3.66	3.13	3.95
Leucine	9.94	7.89	7.63
Tyrosine	3.90	4.92	3.42
Phenylalanine	4.27	4.83	4.01
Tryptophan	-	1.29	0
Half cystine	-	1.73	-
Cysteic acid	-	-	2.90

Sources (a) Hamberg, Stelwagen and Ervast (1973)
(b) Hall and Roberts (1978; - average of 4 values given in table 3)
(c) Bischof (1979)

4:2 α_2M and PAPP-A; a comparison

Similarities in the amino acid composition and physicochemical properties of α_2M and PAPP-A (see Tables 1:5 and 1:6), prompted Bischof (1979, 1980), and Sutcliffe et al (1980a), to independently propose that these 2 proteins were in some way related. Gordon and Chard (1979) have suggested that all placental proteins are analogues of proteins present in the normal adult, sharing some chemical immunological and biological features. Although this has not been shown for all placental proteins it is certainly true of many that have been thoroughly characterised (Gordon and Chard, 1979). For example, adrenocorticotrophic hormone (ACTH) and gonadotrophin-releasing hormone are essentially the same as the adult product. Human chorionic gonadotrophin (hCG) and human placental lactogen (hPL) are analogues of luteinizing hormone and prolactin respectively. The gene for murine α-fetoprotein (AFP), a serum protein made by the fetal liver and yolk sac, has been sequenced and shown to have extensive homology to the serum albumin gene (Law and Dugaiczyk, 1981). Although the exact function of AFP is unclear, it now seems very likely that it is a fetal counterpart of adult serum albumin.

Sutcliffe et al (1980a), having observed the apparent similarities in size and electrophoretic mobility of α_2M and PAPP-A, carried out comparative studies into the structure and antigenicity of these two proteins. Peptide mapping was used to look for structural homology. ^{125}I-labelled protein was digested with V8 protease from Staphylococcus aureus, which cleaves polypeptides at the COOH-terminal side of aspartic and glutamic acid residues. Digestion products were analysed by SDS polyacrylamide gel electrophoresis. Substantial differences between

TABLE 1:6 A comparison of the physicochemical properties of α_2M and PAPP-A*

Property	α_2M	PAPP-A
Type of protein	serum glycoprotein (macroglobulin)	serum glycoprotein (macroglobulin)
Molecular weight (approx)	725,000	750,000
Structure	Homotetramer (dimer of dimers)	Homotetramer (dimer of dimers)
Molecular weight of monomer (approx)	181,000	187,000
Molecular weight of major tryptic fragment (approx)	85,000	82,500
Electrophoretic mobility	α_2	α_2
pI in reducing conditions	4.34	4.34
Total carbohydrate	8.6%	19.2%
Hexosamine	3.24%	(a) 3.1%
Sialic acid	1.14%	2% approx
Dissociating agents/ AACE precipitin arc-sensitivity	(b) 1.5 M KCNS & 2.0 M KI	(b) 1.5 M KCNS & 2.0 M KI

*All data from Sutcliffe et al (1980b) and Hall and Roberts (1978); except (a) Bischof(1979) and (b) Sutcliffe et al (1979).

the digest patterns were observed. Sutcliffe et al (1980a) concluded that this apparent lack of correspondence in the digest patterns did not rule out polypeptide homology, since proteins may be homologous in some amino acid sequences which are not bounded by aspartic or glutamic acid residues which can be cleaved by V8 (PAPP-A contains more aspartic acid residues than α_2M and fewer glutamic acid residues; see Table 1:5). However, subsequent cyanogen bromide peptide mapping of these 2 proteins also showed no detectable stretches of homology (Gore and Sutcliffe; in press).

Sheep anti-PAPP-A serum raised by Sutcliffe et al (1980a), was found to contain some anti-α_2M specificities. Crossed-immunoelectrophoresis experiments were carried out to determine whether PAPP-A and α_2M antibodies were raised against contaminating protein in the original immunization. No reactions of partial or complete identity were observed between α_2M and PAPP-A. In a more sensitive radioimmunoassay, α_2M was unable to compete with purified PAPP-A for binding sites on immobilised anti-PAPP-A. However, in the reciprocal assay, purified PAPP-A was able to displace α_2M from immobilised anti-α_2M. Sutcliffe et al (1980a), suggested that the significance of this experiment depended on the level of α_2M contamination in the purified PAPP-A, which was not considered to be very great as α_2M could not be detected on gel electrophoresis or immunoelectrophoresis.

Bischof (1979, 1980), looked for possible similarities in biological function between α_2M and PAPP-A. As previously stated (section 4:1 of this chapter), α_2M is a potent inhibitor of endoproteases and therefore is directly and indirectly involved in the regulation of the haemostatic, inflammatory and complement pathways.

PAPP-A has been reported to inhibit plasmin, urokinase (α_2M was thought to inhibit urokinase, Ogston et al, 1973; but there have been several subsequent reports contradicting this; see Starkey and Barrett, 1977), and the haemolytic activity of complement, in vitro (Bischof, 1979, 1981). Therefore, these preliminary results tend to suggest that PAPP-A and α_2M show functional similarities. In pre-eclamptic toxaemia (when associated with proteinuria), both PAPP-A and α_2M levels are raised (Hughes et al, 1980; Harpel, 1976), which may also be indicative of functional similarities.

Using indirect immunofluorescence studies on full term placentae, McIntyre et al (1981), and Faulk and Johnson (1977), have located α_2M and PAPP-A staining in intervillous fibrin/fibrinoid together with complement component C4, plasminogen and the blood clotting factor, factor VIII. These observations are again compatible with the notion that α_2M and PAPP-A are both involved in the complement and fibrinolytic pathways.

There is no genetic information available on PAPP-A at present. However, Sutcliffe et al (1980b), have speculated on the possible origins of PAPP-A. They suggest that PAPP-A is structurally related to α_2M and is expressed in the placenta from a structural gene distinct from that of α_2M. They also suggest that it is possible that PAPP-A is a post-transcriptionally modified form of the placental α_2M gene product, which is modified in a way different from that occurring when α_2M is synthesized in other tissues. Another possibility indicated, is that serum α_2M is converted to PAPP-A in the placenta. The observed lack of serological cross-reactivity between these proteins does not seem to support the theory of a common genetic origin for α_2M and PAPP-A. However, when

the antigenic determinants of these two proteins have been determined this apparent discrepancy may be resolved.

5. THE USE OF BINDING ASSAYS AS A MEANS OF DETECTING ENZYME INHIBITION

The interaction between proteases and their inhibitors is characterised by the formation of highly associated complexes, in which the active site of the enzyme is covered or occupied by the inhibitor (Feeney, 1971; Heimburger, 1975). In the case of α_2M, complex formation with proteases results in structural changes in the α_2M molecule (see section 4:1), which lead to an irreversible complex being formed. This has been particularly well demonstrated by Saunders *et al* (1971), who were unable to displace ^{125}I-trypsin from α_2M complexes on the addition of cold trypsin (and *vice versa*). In the case of α_2-plasmin inhibitor (α_2 PI; the primary inhibitor of plasmin in human plasma), binding to plasmin is also rapid and irreversible (Mullertz and Clemmenson, 1976). Such irreversibility may not be absolute for all inhibitors. For example, there is a slow transfer of trypsin from complexes with soya bean trypsin inhibitor and, also α_1-antitrypsin (α_1 protease inhibitor) to α_2M (see Starkey and Barrett, 1977). Therefore, enzymes and inhibitors should perhaps, in most cases, be considered to form tightly associated complexes rather than irreversible ones.

Activity assays have been routinely used to assess enzyme inhibition (for examples see Table 1 in Starkey and Barrett, 1977), such as the caseinoloysis assay of Crawford, Ogston and Douglas (1976) used to investigate the plasmin inhibitory capacity of PAPP-A (Bischof, 1979).

A disadvantage of such an activity assay is that the purity of the enzyme and inhibitor has to be of a very high standard, otherwise a precise identification of the protein species which is actually interacting with the enzyme cannot be made. However, it is not always possible to obtain such pure protein preparations, especially when purifying under physiological conditions. Therefore, other methods for assessing enzyme inhibition have had to be employed. Binding assays involving the radio-labelling of one of the protein components (usually the enzyme) and the resolution of the resulting components (chromatographically, electrophoretically and immunologically), have been most useful when working with partially purified solutions, and as an alternative to an activity assay when using highly purified solutions. Harpel (1977) separated incubation mixtures of α_2M, α_2PI and ^{125}I-plasmin (after the addition of a SDS-urea solution and boiling), by SDS gel electrophoresis. The electrophoretic mobility of the complexes and free protein species were sufficiently different to allow the precise identification of the various components to be made, and their degree of ^{125}I-plasmin binding to be quantified. Hence the distribution of radiolabelled plasmin between the two protease inhibitors could be compared, and Harpel (1977) was able to conclude that α_2 PI rather than α_2M was the major circulating plasmin inhibitor. Using an activity assay in conjunction with the binding assay, Harpel (1977) was able to show that ^{125}I-plasmin bound as readily to these inhibitors as did the native enzyme. Barrett and Starkey (1973) have shown that α_2M also binds radiolabelled trypsin, chymotrypsin, papain and cathepsin B1. They separated free enzyme from enzyme:α_2M complexes by gel filtering the partially purified α_2M-enzyme incubation mixtures.

Samples of the chromatographed proteins or enzyme-plasma mixtures were run on immunoelectrophoresis gels against anti-whole human serum and anti-α_2M, and the plates stained for activity. From these experiments, Barrett and Starkey (1973) were able to show that α_2M could bind to essentially all proteases. The stability of α_1 antitrypsin:trypsin complexes on chromatographic and electrophoretic separation was demonstrated by Jeppsson and Laurell (1975), and it would appear, therefore, that these two methods are generally useful for identifying enzyme:inhibitor complexes.

An "immunocapture" technique was independently developed by both Harpel and Hayes (1981) and Sutcliffe (Dr. R.G. Sutcliffe, personal communication). Harpel and Hayes (1981) used this technique to specifically isolate α_2M:^{131}I-βtrypsin complexes from plasma, and from purified and iodinated α_2M, trypsin mixtures. Protein solutions were incubated with anti-α_2M immobilised on an agarose gel matrix. The antibody-bound α_2M:trypsin complexes were remarkably stable since they remained associated with the agarose gel matrix even through repeated washing. This assay was a highly sensitive and specific assay. Other immobilised antibodies bound only insignificant amounts of α_2M, and ^{131}I-βtrypsin itself did not bind to the immobilised anti-α_2M. Trypsin activity (amidolysis) was observed and used as a specific measure of α_2M:trypsin complexes. Harpel and Hayes (1981), concluded that this technique was able to facilitate i) the rapid isolation and concentration of α_2M:protease complexes, and ii) the measurement of their catalytic capacity in biological fluid in various physiologic and pathologic states.

In summary, activity assays as a means of identifying enzyme inhibitors, are only valid if highly purified preparations of proteins are involved. When using whole plasma or partially purified preparations

of potential protease inhibitors, identification of the interacting protein species can be made by using an enzyme binding assay. This can be carried out with great precision if techniques, such as the specific and sensitive "immunocapture" assay, are employed.

6. EXPERIMENTAL OBJECTIVES

> "To date the experimental approach to elucidating biological function (of placental proteins), has concentrated on assessing purified proteins which may have been denatured during purification, and in future, approaches may well be directed to the study of combinations of molecules"
>
> Y.B. Gordon and T. Chard (1979)

> "The isolation of the new placental proteins opens new vistas. Although attention has been fixed on assays of these proteins as a measure of placental function, it is likely that their importance stretches well beyond this. Their true significance cannot be assessed until we know more about their function..... We have too easily fallen for the temptation to seek immediate clinical applications for the assay of placental proteins. We should now isolate enough pure material to test hypotheses about their function. The future lies there"
>
> A. Klopper (1980)

The experimental objectives of this thesis were two fold: i) to develop a purification protocol for the production of physiologically active PAPP-A; and ii) to further compare α_2M and PAPP-A, both physico-chemically and physiologically.

6:1 The purification of physiologically active PAPP-A

The relevance of any *in vitro* functional investigations to *in vivo* systems depends on a number of factors, an important one of which is that the purified protein is in sufficient of a native state to permit observations on its possible physiological role. The methods

of PAPP-A purification used to date have produced highly purified samples of PAPP-A, which were useful for studying protein composition and subunit structure. However, Bischof (1979), Halbert and Lin (1979) and Sutcliffe et al (1980b), used ammonium sulphate fractions of maternal serum as a source of PAPP-A for chromatography. This may be significant as ammonium salts can cause irreversible conformational changes in α_2M which prevent the binding and inhibition of proteases (see section 4:1 of this chapter). In addition, some purification procedures for PAPP-A contained steps requiring buffers of high salt concentration (see Table 1:2; 1M NaCl and 1M KI, Sutcliffe et al, 1980b; 1M NaCl, Folkersen et al, 1981a). Such agents may disrupt the conformation of the molecules enough to interfere with any functional properties PAPP-A might have.

The first objective, therefore, was to develop a method of purifying PAPP-A which did not involve ammonium sulphate precipitation or high salt concentrations, in order to avoid such conformational or other changes.

6:2 Homologies, analogies or anomalies?

To what extent can the comparison between α_2M and PAPP-A be taken? Are these proteins structurally and functionally homologous or analogous, or can the observed similarities be explained in other terms? The second objective of this thesis was to further compare α_2M both physicochemically and physiologically with PAPP-A purified under mild conditions, and to determine if this PAPP-A was indeed an inhibitor of plasmin and the complement system as stated by Bischof (1979, 1981).

CHAPTER 2 : CARBOHYDRATE ANALYSIS OF PAPP-A PRODUCED BY ANTIBODY AFFINITY CHROMATOGRAPHY

1. INTRODUCTION

The physicochemical properties of a protein have an important influence on the elucidation of its structure and ultimately its biological function. PAPP-A and α_2M are both glycoproteins (Lin et al, 1974a; Starkey and Barrett, 1977), and because of the similarities in their overall molecular weight, electrophoretic mobility and amino acid composition, it has been suggested that these two proteins may be structurally related (Bischof, 1979 and 1980; Sutcliffe et al, 1980b). Isoelectric focusing studies on PAPP-A and α_2M (Sutcliffe et al, 1980b), showed that their isoelectric points were indistinguishable under both reducing and non-reducing conditions. Estimations of the total carbohydrate content have shown that the value for PAPP-A exceeds that of α_2M by 10.6% (Sutcliffe et al, 1980b). Bischof (1979) estimated the glucosamine content of PAPP-A to be 3.1%, compared with a mean value of 3.2% for the hexosamine content of α_2M (Hall and Roberts, 1978). Neuraminidase treatment decreases the apparent size of both proteins by approximately the same amount (2%; Sutcliffe et al, 1980b), which indicates that their sialic acid contents are also comparable.

As these proteins have co-incident isoelectric points and since their amino acid compositions are similar, it would suggest that the contribution of their carbohydrate residues to their net molecular charges is also similar. It is possible, therefore, that PAPP-A is equivalent to a molecule of α_2M with additional, attached carbohydrate. If so, this 'extra' carbohydrate must be composed of neutral sugars or equal numbers of positively and negatively charged residues (or both), thereby maintaining the overall molecular charges the same. To investigate this hypothesis, the neutral carbohydrate content of PAPP-A and α_2M were determined.

2. MATERIALS AND METHODS

MATERIALS

α_2M and PAPP-A were prepared according to the method of Sutcliffe et al (1980b). Bovine serum albumin, ovalbumin and phosphotungstic acid were obtained from Sigma Chemical Company. Galactose, trichloroacetic acid (Anal-R) and phenol (chromatographic grade), were obtained from BDH. H_2SO_4 and acetone (both Anal-R), were obtained from Hopkins and Williams.

METHODS

Protein precipitation

Trichloroacetic acid (TCA; 12.5%, w/v at 4°C) was added to an equal volume of protein solution and mixed. After standing on ice for 15 min the solutions were centrifuged (2,500 g for 15 min), and the precipitates recovered. TCA soluble proteins were precipitated with phosphotungstic acid (PTA) as follows. The protein solutions (1 ml) containing 6.25% TCA (w/v final concentration), were mixed with 0.2 ml of 5% (w/v) PTA in 2M HCl at 4°C. The precipitates were recovered as described above, after standing on ice for 10 min.

For acetone precipitation, 5 volumes of acetone were added to 1 volume of protein solution (1 ml). After 15 min, at room temperature, the precipitate was recovered by centrifugation (2,500 g for 15 min).

Determination of neutral carbohydrate

Neutral carbohydrate was assayed by the phenol/H_2SO_4 method of Dubois et al (1956), using a galactose standard. The protein for analysis (400-600 µg/ml) was either in PBS or had been resuspended in water after precipitation in TCA, TCA plus PTA or acetone.

TABLE 2:1 Precipitation of α_2M and PAPP-A

	Method of precipitation	(a) Total protein (µg)	(b) Protein precipitated (µg)	% precipitated	Mean
α_2M	TCA	910	440	48.4)	
		910	540	59.3)	53.9
	TCA + PTA	960	340	35.4	35.4
	Acetone	330	60	18.2)	
		440	73	16.6)	20.3
		960	250	26.0)	
PAPP-A	TCA	420	30	7.1)	
		420	20	4.8)	4.8
		500	10	2.4)	
	TCA + PTA	280	140	50.0	50.0
	Acetone	280	150	53.6)	
		280	160	57.1)	55.4

(a) Determined by the Folin method (Lowry, 1951)

Figure 2:1 The effect of TCA on carbohydrate and protein estimation assays:-

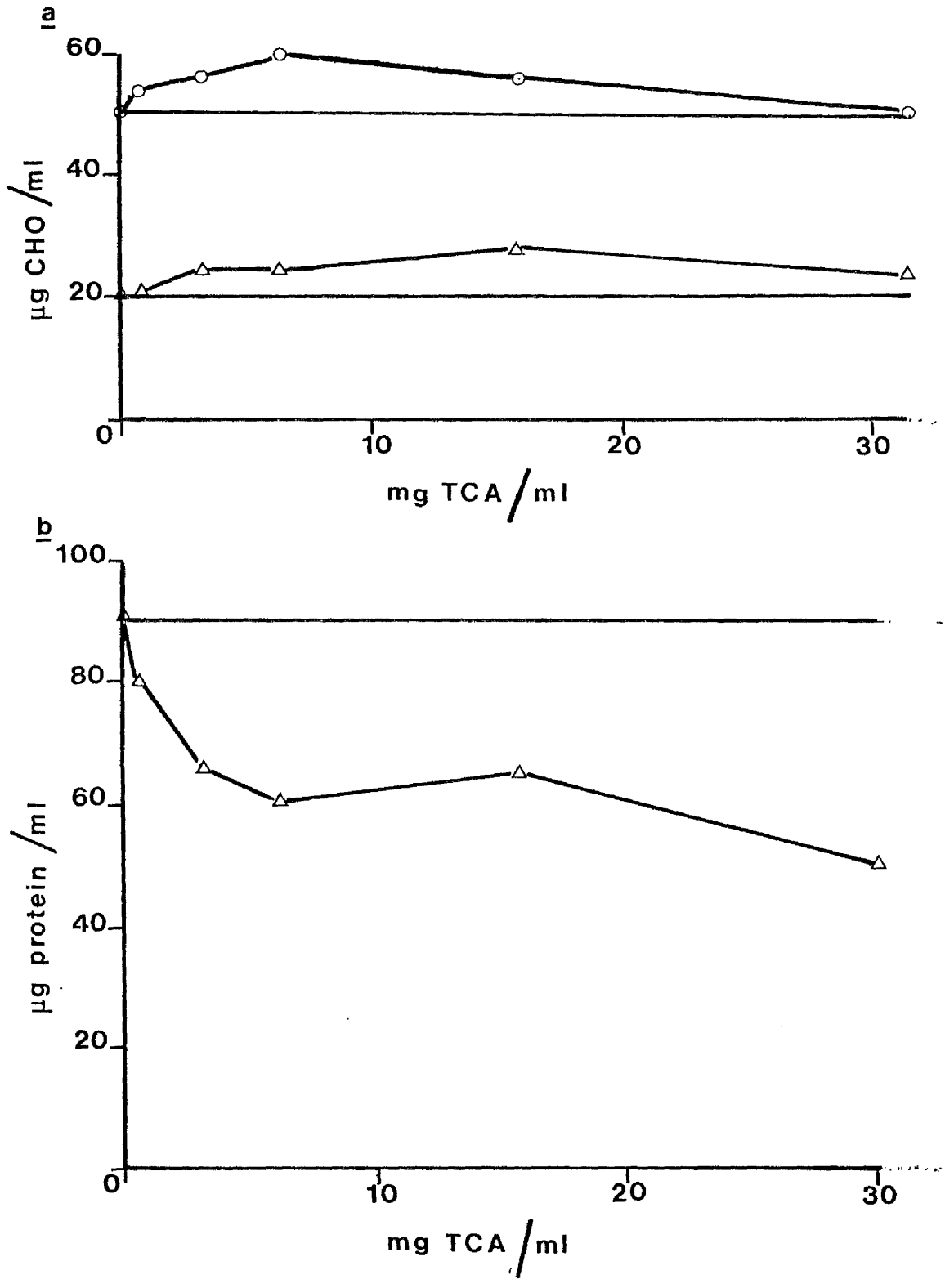
a) The effect of TCA on the phenol/H_2SO_4 assay:
(———) expected values of standards
(O—O) galactose standard (50 µg/ml)
(Δ—Δ) ovalbumin standard (20 µg of carbohydrate/ml)

b) The effect of TCA on the Folin assay:
(———) expected value of standard
(Δ—Δ) ovalbumin standard (90 µg/ml)

All points plotted are the average of triplicate determinations.

CHO = carbohydrate.

Figure 2:1


a
60
40
20
0
µg CHO /ml
10
20
30
mg TCA /ml
b
100
80
60
40
20
0
µg protein /ml
10
20
30
mg TCA /ml

Determination of protein

Protein was assayed by the Folin method (Lowry et al, 1951), with bovine serum albumin (BSA), as standard. Acid-precipitated samples were first neutralised.

The effect of TCA and PTA on the determination of carbohydrate and protein

The effects of TCA and PTA on the phenol/H_2SO_4 and Folin assays were determined. Increasing amounts of TCA (0-32.5 mg/ml) or PTA (0-5 mg/ml) were added to 100 µl of galactose (50 µg/ml) and 100 µl of ovalbumin (90-650 µg/ml). These solutions were then analysed for their neutral carbohydrate and protein content.

3. RESULTS

Precipitation of protein

Unlike α_2M, it was found that PAPP-A precipitated to only a small extent (4.8%; Table 2:1) in 6.25% TCA (w/v). Precipitation was increased to 50% on the addition of 5% PTA (w/v) to the TCA/PAPP-A solutions, which was comparable with the value obtained on acetone precipitation (Table 2:1). A reduction in precipitated α_2M was observed with TCA plus PTA (35.4%; Table 2:1), and acetone (20.3%), compared with TCA alone (53.9%).

The effect of TCA and PTA on the determination of neutral carbohydrate and protein content

There was no detectable effect on the estimation of neutral carbohydrate content as the amount of TCA or PTA added to the phenol/H_2SO_4 assay was increased (Figures 2:1a and 2:2a). Observed values

TABLE 2:2 Carbohydrate content of PAPP-A and α_2M

	Method of precipitation	Mean carbohydrate content (g/100 g protein) PAPP-A	α_2M	PAPP-A-α_2M
Neutral carbohydrate (phenol/H_2SO_4 assay; Dubois, 1956)	a) TCA	10.0	4.7	5.3
	b) TCA + PTA	12.9	6.2	6.7
	c) Acetone	15.9	5.6	10.3
	Mean of (a)-(c)	12.9	5.5	7.4
	d) no precipitation	12.9 (4) [9-15.7]	3.9 (3) [3.8-4.0]	9.0
Total carbohydrate (Anthrone assay; Koehler, 1952)	e) no precipitation+	19.3 (3) [17.7-21.5]	8.6 (3) [7.3-9.6]	10.6

Each value was obtained as a mean of at least 2 determinations. Values in parentheses refer to number of independent determinations (each in duplicate), and values in square brackets represent the range of values observed for non-precipitated proteins.

Abbreviations: TCA, trichloroacetic acid; PTA, phosphotungstic acid.

+ data from Sutcliffe et al (1980b).

Figure 2:2 The effect of PTA on carbohydrate and protein estimation assays.

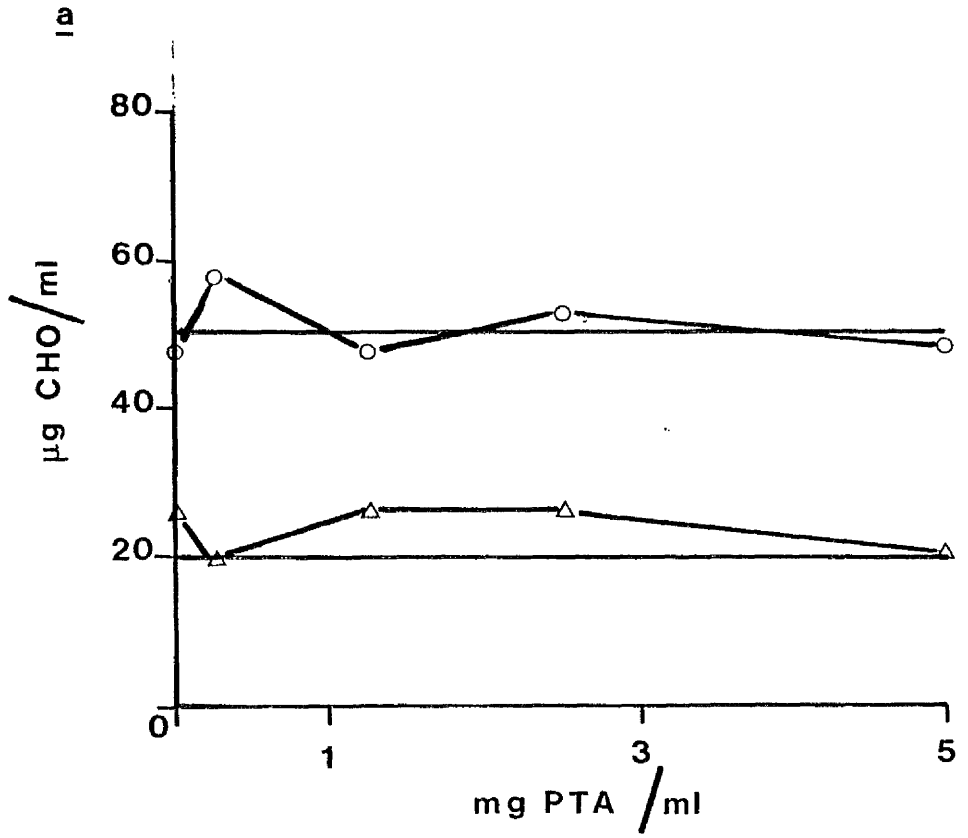
a) The effect of PTA on the phenol/H_2SO_4 assay:
(———) expected values of standards
(O—O) galactose standard (50 μg/ml)
(Δ—Δ) ovalbumin standard (20 μg carbohydrate per ml)

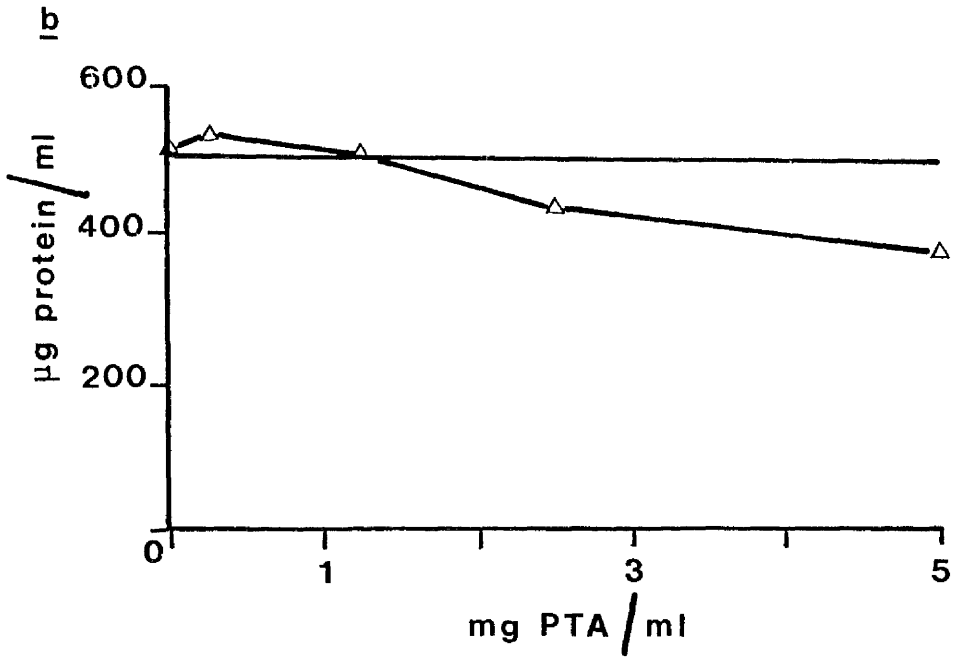
b) The effect of PTA on the Folin assay:
(———) expected value of standard
(Δ—Δ) ovalbumin (500 μg/ml)

All points plotted are the average of triplicate determinations.

CHO = carbohydrate.

Figure 2:2


a
80
60
40
20
0
µg CHO/ml
1
3
5
mg PTA /ml


b
600
400
200
0
µg protein/ml
1
3
5
mg PTA /ml

varied little from the expected, even at the highest concentrations of acid (TCA: 32.5 mg/ml; PTA: 5 mg/ml). However, as the amount of acid added to the Folin assay was increased a corresponding decrease in the estimated protein concentration was detected (Figures 2:1b and 2:2b). For example, the amount of protein observed at the highest concentrations of TCA and PTA were 55.6% and 74% respectively of the expected values.

Neutral carbohydrate content of PAPP-A and α_2M

The neutral sugar content was investigated by the phenol/H_2SO_4 method of Dubois et al (1956), using a galactose standard (see Table 2:2). Proteins were precipitated prior to analysis to separate the protein-bound carbohydrate from any free carbohydrate in the samples, acquired during the preparation of the proteins from the chromatography media. The mean neutral carbohydrate content of PAPP-A was 12.9%. This value was observed for both precipitated and non-precipitated samples. However, the amount of neutral carbohydrate was found to be greater in precipitated α_2M (mean of 5.5%), than in the non-precipitated samples (mean of 3.9%).

4. DISCUSSION

PAPP-A and α_2M prepared in the manner described by Sutcliffe et al (1980b) were analysed for their neutral carbohydrate content (Table 2:2). A value of 12.9% was obtained for the neutral carbohydrate content of PAPP-A, with and without prior precipitation. The mean value of neutral carbohydrate in α_2M, without prior precipitation, was 3.9%, which compares closely with the published values of 3.08-4.5% (see Hall and Roberts, 1978). However, an increase in the neutral carbohydrate content was observed after precipitation, when a value of

5.5% was obtained. The cause of this increase was unclear, especially as no such effect was observed with precipitated PAPP-A. It is not due to the effect of residual acid on the carbohydrate determination as TCA and PTA were shown to have no effect on the phenol/H_2SO_4 assay (Figures 2:1a and 2:2a). The Folin assay was shown to be sensitive to the presence of acid (Figures 2:1b and 2:2b), so resuspended pellets were neutralised before they were assayed. This neutralisation may have been an unnecessary precaution as it was estimated that a residue of 10 µl (approx.) of acidified solution would remain in the precipitates leading, after resuspension, to a concentration of PTA and TCA of 0.08 mg/ml and 0.6 mg/ml respectively. The effect on the Folin assay of such concentrations was found to be minimal (Figures 2:1b and 2:2b).

α_2M and PAPP-A exhibited different precipitation properties in TCA, PTA and acetone (Table 2:1). PAPP-A precipitated to only a small extent in TCA, unlike α_2M, but precipitation could be increased on the addition of PTA or by using acetone instead. TCA solubility has been observed for a range of human serum glycoproteins (Putnam, 1965) and for bovine fetuin which has a large carbohydrate content (5.5% hexosamines and 8.3% hexoses; Spiro, 1960). PAPP-A, like fetuin, has a relatively high proportion of carbohydrate, being composed of 19.2% total carbohydrate (Sutcliffe et al, 1980b), and 12.9% neutral carbohydrate (see Table 2:2). Similarly, both proteins precipitate in 5% PTA (Spiro, 1960; Table 2:1), forming a heavy precipitate of insoluble protein-tungstate salts (Rendina, 1971). Presumably their high carbohydrate content increases their TCA solubility through the relatively high surface density of hydroxyl groups.

The mean total carbohydrate content of PAPP-A was found to be

19.2% compared with a mean value of 8.6% for α_2M (Sutcliffe et al, 1980b). These determinations were carried out on un-precipitated samples, therefore it is possible that they may be overestimates due to the presence of free carbohydrate acquired during the purification of these proteins. However, the observed TCA solubility confirms that the carbohydrate content of PAPP-A must be relatively high and it was apparent from the neutral carbohydrate assay that precipitation had no effect on the estimated values. Also the estimated total carbohydrate content of α_2M is in good agreement with those already published (see Hall and Roberts, 1978), which indicates that contaminating carbohydrate is not having a significant effect on this estimation.

The observed difference in neutral carbohydrate content of PAPP-A and α_2M (7.4-9.0%) accounts for 69.8-84.9% of the difference in total carbohydrate content. It is interesting to note that if the amounts of neutral carbohydrate (12.9%; see Table 2:2), glucosamine (3.1%; Bischof, 1979) and sialic acid (2%; Sutcliffe et al, 1980b) in PAPP-A are totalled, then the figure obtained for total carbohydrate is 18%. Using this value, the contribution of neutral carbohydrate to the difference in total carbohydrate observed between α_2M and PAPP-A increases from 69.8-84.9% to 78.7-95.7%. These results would appear to support the hypothesis that a high proportion of the additional carbohydrate present in PAPP-A compared with α_2M is made up of neutral sugars, which have little or no effect on the overall molecular charge. The molecular weight of the PAPP-A polypeptide, as estimated from polyacrylamide gels (Sutcliffe et al, 1980b), exceeds that of α_2M by about 6,000 or 3.3%. Therefore, the notion that PAPP-A is equivalent to α_2M plus additional carbohydrate is too simplistic, as the molecular weight analysis suggests that the PAPP-A polypeptide is smaller than that of α_2M.

CHAPTER 3 : PURIFICATION OF PAPP-A UNDER MILD CONDITIONS

1. INTRODUCTION

PAPP-A has been isolated from maternal serum by several different methods (see Table 1:2), some of which have produced highly purified samples of this protein. These samples were useful for studying its composition and subunit structure (Bischof, 1979; Sutcliffe et al, 1980b). However, these preparations of PAPP-A may have been denatured during their purification. Some purification procedures contained steps requiring buffers of high salt concentration (1M NaCl and 1.5M KI, Sutcliffe et al, 1980b; 1M NaCl, Folkersen et al, 1981a). Such agents may disrupt the conformation of PAPP-A sufficiently to interfere with any functional properties it might have. Similarly, ammonium sulphate precipitation, as used by Bischof (1979), Halbert and Lin (1979) and Sutcliffe et al (1980b), may also interfere with the functioning of PAPP-A, since ammonium salts can cause conformational changes in α_2M (see chapter 1:6).

In order to avoid such conformational and other changes occurring, a method of purification of PAPP-A was developed which did not involve ammonium sulphate precipitation or high salt concentration. This method was expected to produce PAPP-A in sufficient of a native state to permit observations on its possible physiological role.

2. MATERIALS AND METHODS

MATERIALS

Samples of maternal blood (25-175 ml) were collected per vagina from normal patients at delivery. Sodium citrate was added to the blood to give a final concentration of 0.5% (w/v), though some unavoidable clotting occurred before treatment with the anti-coagulent.

The individual blood samples were pooled at 4°C, centrifuged at 9,000g for 15 min and the plasma stored at 20°C.

Sephadex G200 and Sepharoses 4B and CL6B were obtained from Pharmacia (GB) Ltd. Cibacron blue was obtained from Ciba Geigy Ltd. and was coupled to Sepharose CL6B by the method of Travis et al (1976). Anti-adult male serum was coupled to Sepharose using the method of Porath, Axen and Ernback (1967).

Sheep anti-adult male serum and sheep anti-PAPP-A were raised as described by Sutcliffe et al (1978, 1979), rabbit anti-α_2M and anti-human albumin were obtained from Behring Diagnostics. SDS and Tris were obtained from the Sigma Chemical Company, all other chemicals and materials were obtained from BDH.

METHODS

All chromatographic procedures were carried out at 4°C. The supernatants obtained after centrifugation of the blood samples were filtered as previously described by Sutcliffe et al (1980b), to prevent the possible accumulation of any clot-like material on top of the columns.

2:1 Approach Experiment: Negative antibody affinity chromatography; a possible single step purification procedure

Aliquots of filtered supernatant (100 ml and 200 ml) were applied to a column of Sepharose 4B (9 x 16.5 cm) on which had been immobilised 1.5g of immunoglobulin from sheep antisera to adult male serum (Sutcliffe et al, 1978). The antibody column had previously been equilibrated with 0.3M NaCl, 5 mM potassium phosphate pH 7.5 (buffer B). Samples were applied and eluted at 400 ml/h. The eluate from the column was passed through a Uvicord II analyser

(LKB Instruments), and the A_{280} recorded. The proteins which did not bind to the column were collected and antibody-antigen crossed electrophoresis (AACE) analysis used to detect their points of elution.

For regeneration and re-use, the column was later treated with 300 ml of 0.5M acetic acid followed by 50 mM potassium phosphate pH 7.5. When the pH of the eluate had reached neutrality the column was re-equilibrated with buffer B.

2:2 Purification protocol: A 3 step procedure

Depletion of albumin from plasma by dye ligand chromatography

A sample of filtered supernatant (6 g of protein in 100 ml), which had previously been dialysed against 10 mM potassium phosphate, pH 7.5 (buffer A), was applied to a column (2.5 x 90 cm) of Sepharose CL6B to which Cibacron blue dye had been conjugated. The column was equilibrated with buffer A, at a flow rate of 5 ml/h. The eluate from the column was passed through a Uvicord II analyser (LKB Instruments), and the A_{280} recorded. When the absorbance began to fall, the column was washed with 1M NaCl in buffer A, to remove the bound protein. The column was re-equilibrated with buffer A at 50 ml/h. Peak PAPP-A fractions were pooled (100 ml approx.) and stored at -20°C.

Negative antibody affinity chromatography (NAAC)

The salt concentration of the pooled PAPP-A fractions from the Cibacron blue column was adjusted to 0.3M NaCl and applied to the NAAC column. Chromatography was carried out as described in the section headed 'Approach Experiment'.

Gel filtration

The PAPP-A pool from the negative antibody affinity step was

concentrated to 10 ml by ultrafiltration, using a 7.6 cm diameter XM100A membrane (Amicon). The PAPP-A concentrate was applied to a Sephadex G200 gel filtration column equilibrated with buffer B. The sample was eluted at 4 ml/h and the PAPP-A enriched fractions stored at -20°C.

2:3 Electrophoresis

Antibody-antigen crossed electrophoresis (AACE)

Fractions containing PAPP-A, α_2M and albumin were identified by AACE (Laurell, 1966), with the modification that 100 mM Tris was present in the tank and gel buffers (Sutcliffe et al, 1980b).

Polyacrylamide gel electrophoresis (PAGE)

SDS/Tris polyacrylamide gel electrophoresis (method 2 in Sutcliffe et al, 1980b), was used to ascertain the purity of the eluate collected during the isolation of the PAPP-A enriched preparation. The molecular weight standard used on these gels was made up of bovine serum albumin (68,000 mol wt), ovalbumin (43,000 mol wt), carbonic anhydrase (29,182 mol wt) and β-lactoglobulin (18,363 mol wt). PAPP-A standards were prepared by positive affinity chromatography (Sutcliffe et al, 1980b), and α_2M standards by gel filtration of human serum on Sepharose CL6B. Gels were stained with Coomassie brilliant blue R.

3. RESULTS

Approach experiment: Negative antibody affinity chromatography

The results from the fractionation of 100 ml and 200 ml of filtered supernatant are summarised in Figure 3:1 and Table 3:1. Considering the 200 ml fractionation in detail; of the PAPP-A loaded onto the column (16 mg), 13 mg (81.25%) were recovered between 0.52 l

TABLE 3:1 Protein recovery from NAAC of filtered maternal blood

Sample applied	Protein applied (g)	Protein recovered unadsorbed (g)	Recovery (%)	Elution range (l)
100 ml of filtered maternal blood	2.7	0.92	34.1	0.14-1.54
200 ml of filtered maternal blood	5.4	2.11	39.1	0.14-1.46
PAPP-A applied in 200 ml sample	0.016	0.013 (0.6%)	81.25	(a) 0.52-1.302
Albumin applied in 200 ml sample	5.0	1.75 (83%)	(b) 35.0	0.49->1.302

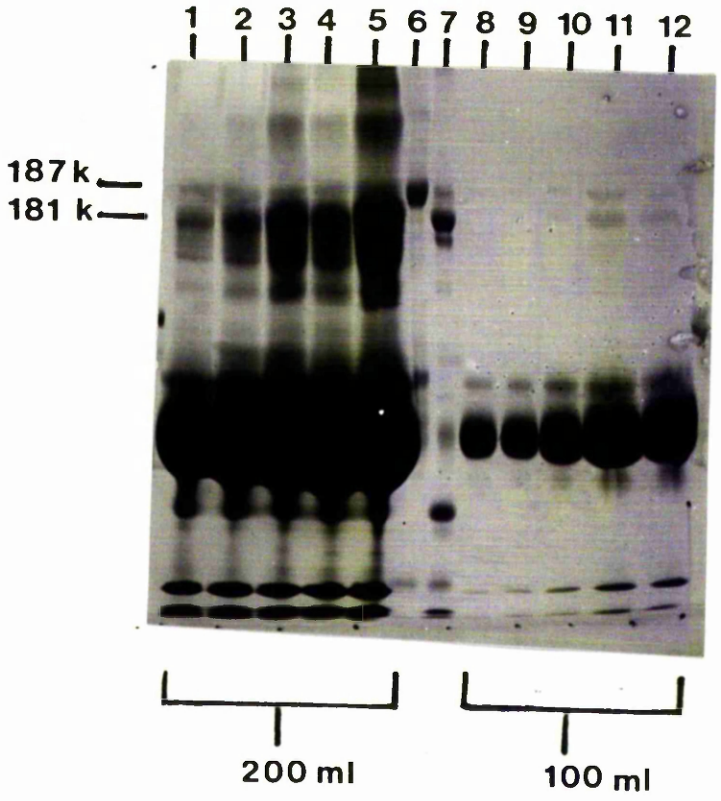
Figures in parentheses are percent of total unadsorbed protein.

(a) maximum PAPP-A and α_2M elution points at 0.96 l and 1.06 l respectively.

(b) Albumin recovered in PAPP-A elution range. Ratio of PAPP-A: Albumin, 1:134.6.

Figure 3:1 Analysis of the fractions eluted from a NAAC column. Tracks 1-5, fractionation of 200 ml of filtered maternal blood; unbound protein eluting between 0.934-0.99 l. Tracks 8-12, fractionation of 100 ml of filtered maternal blood; unbound protein eluting between 0.846-0.902 l. Track 6, PAPP-A standard. Track 7, α_2M standard.

Figure 3:1



and 1.302 l eluted (Table 3:1). This represented 0.6% of the total unadsorbed protein recovered from the column. The albumin eluted in the PAPP-A range (1.75 g; Table 3:1), amounted to 35% of that loaded and 83% of the total unadsorbed protein. α_2M was also detected in the PAPP-A range, however, its recovery was not calculated. The fractions of maximum PAPP-A concentration (0.85-0.9 l and 0.93-0.98 l eluted for 100 ml and 200 ml of filtered supernatant respectively), were analysed on SDS polyacrylamide gels (4½ - 12½%; Figure 3:1). All fractions contained proteins other than PAPP-A. When 100 ml of filtered supernatant were fractionated, 5 polypeptide bands were visible at 187,000, 181,000, 86,000, 68,000 and 36,000 mol wt. The polypeptides at 187,000, 181,000 and 68,000 were judged to be PAPP-A, α_2M and albumin respectively. The amount of albumin in each fraction greatly exceeded any of the other proteins. On fractionation of 200 ml of filtered supernatant these polypeptides were again observed, as were at least 6 others, at approximately 47,000, 92,000, 108,000, 140,000, 250,000 and 300,000 mol wt. The identity of these polypeptides was not determined. As before albumin was the main contaminant and a ratio of 1:135 for PAPP-A to albumin was observed in the PAPP-A range.

Purification of PAPP-A

Albumin depleted plasma was prepared by passing filtered supernatant of maternal blood through a column of Cibacron blue Sepharose. The results from a typical fractionation are shown in Figures 3:2, 3:3 and Table 3:2. The protein eluted between 120-220 ml (Figure 3:2) contained 77% of the PAPP-A originally applied to the column (Table 3:2), and compared with the starting material there was a 248-fold reduction in albumin content in this pool. The progress

TABLE 3:2 Dye Ligand Chromatograph of Filtered maternal blood on Cibacron Blue Sepharose*

	Volume (ml)	Total protein (mg)	Albumin (mg)	α_2M (mg)	PAPP-A (mg)	PAPP-A/α_2M x 100%
Filtered maternal blood loaded	100	6100	3800	169	7	4.1
Protein in pooled fractions (unbound protein)	100	965 (16%)	15.4 (0.4%)	129 (76%)	5.4 (77%)	4.2

* Figures in parentheses are % recovery.

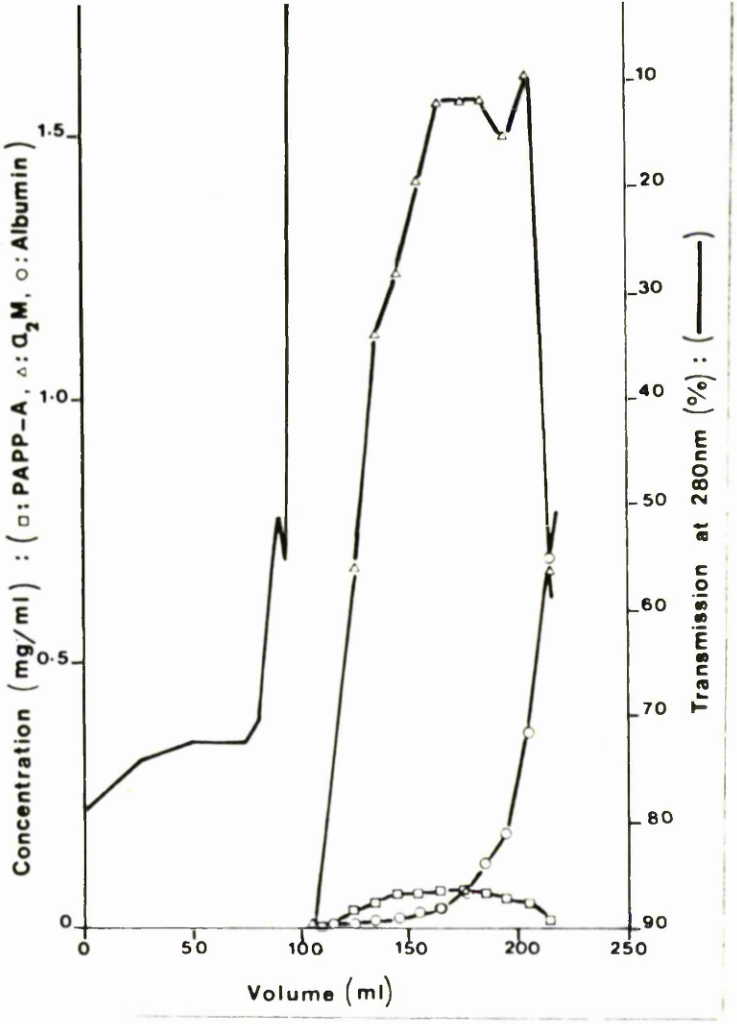
Figure 3:2

Transmission at 280 nm (———) of the effluent from the Cibacron blue column. Filtered maternal blood (100 ml) was applied at zero volume. The concentrations of PAPP-A (□—□), α_2M (Δ—Δ) and albumin (○—○) were measured by AACE.

Figure 3:3

Analysis of the fractions eluted from the Cibacron blue column. Track 1, α_2M; track 2, molecular weight standard; track 3, PAPP-A standard; tracks 4 and 5, 50 and 25 µl of 1/20 diluted filtered maternal blood. Tracks 6-12, aliquots of fractions from Figure 3:2 containing unbound protein; tracks 3-16, aliquots of fractions subsequently eluted with the salt wash.

Figure 3:2



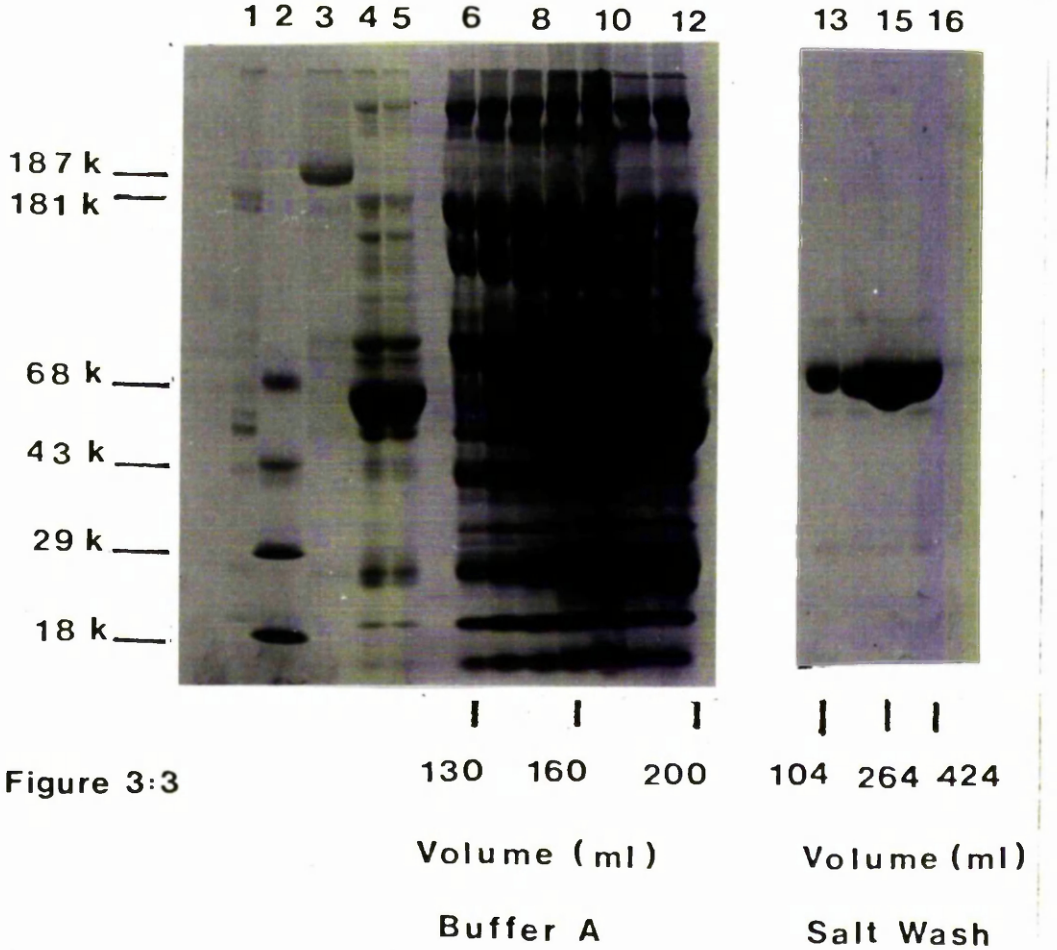


Figure 3:3

TABLE 3:3 Negative antibody affinity chromatography of pooled fractions containing PAPP-A from the column of Cibacron blue Sepharose*

	Volume (ml)	Total protein (mg)	Albumin (mg)	α_2M (mg)	PAPP-A (mg)	PAPP-A/α_2M x 100%
Protein loaded onto column	180	1512	23	84	6.2	7.4
Protein in pooled fractions (unbound protein)	684	47.6 (3%)	$<$0.02 ($<$0.1%)	22.5 (26.8%)	5 (81%)	22.2

*Figures in parentheses are % recovery.

Figure 3:4 The PAPP-A pool from the Cibacron blue column was adjusted to 0.3 M NaCl and applied to the NAAC column. The unbound protein was eluted with buffer B and monitored via transmission at 280 nm (———). .. The concentrations of PAPP-A (□—□) and α_2M (△—△) were measured by AACE. The fractions indicated were pooled and concentrated by ultra filtration.

Figure 3:5 Analysis of the unbound protein eluted from the NAAC column. Track 1, molecular weight standards; track 2, α_2M; track 3, PAPP-A. Tracks 4-15, 500 µl of the various fractions were dialysed against buffer A, lyophilised and resuspended in 50 µl of H_2O, before running on the gel. Track 16; 10 µl of the material applied to the NAAC column.

Figure 3:4

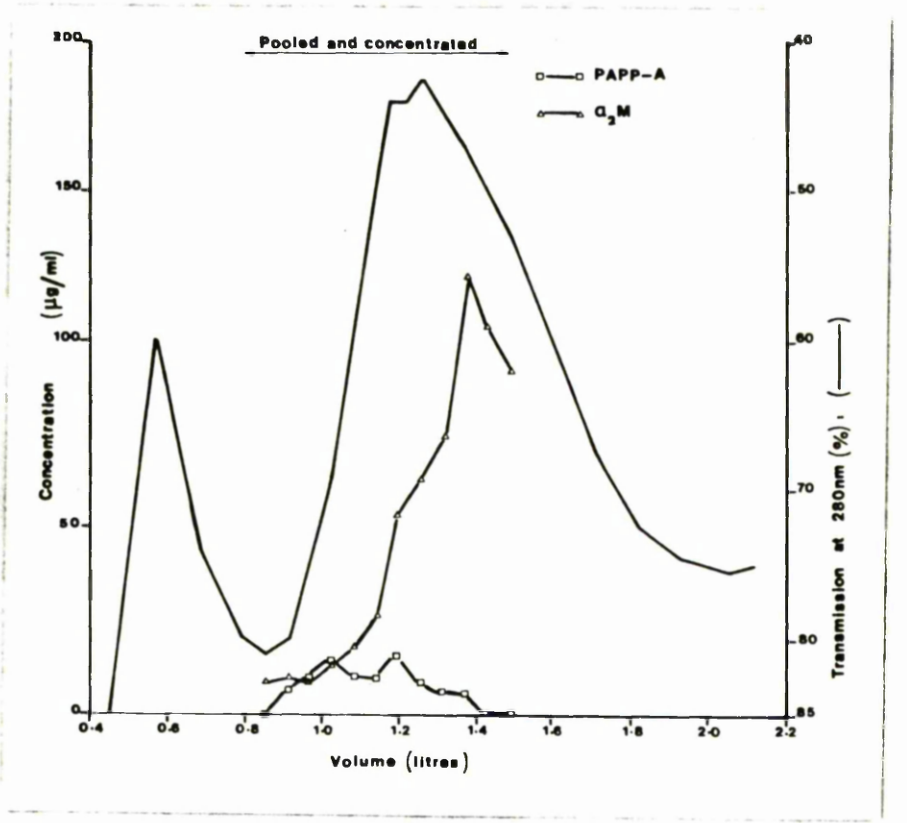


Figure 3:5

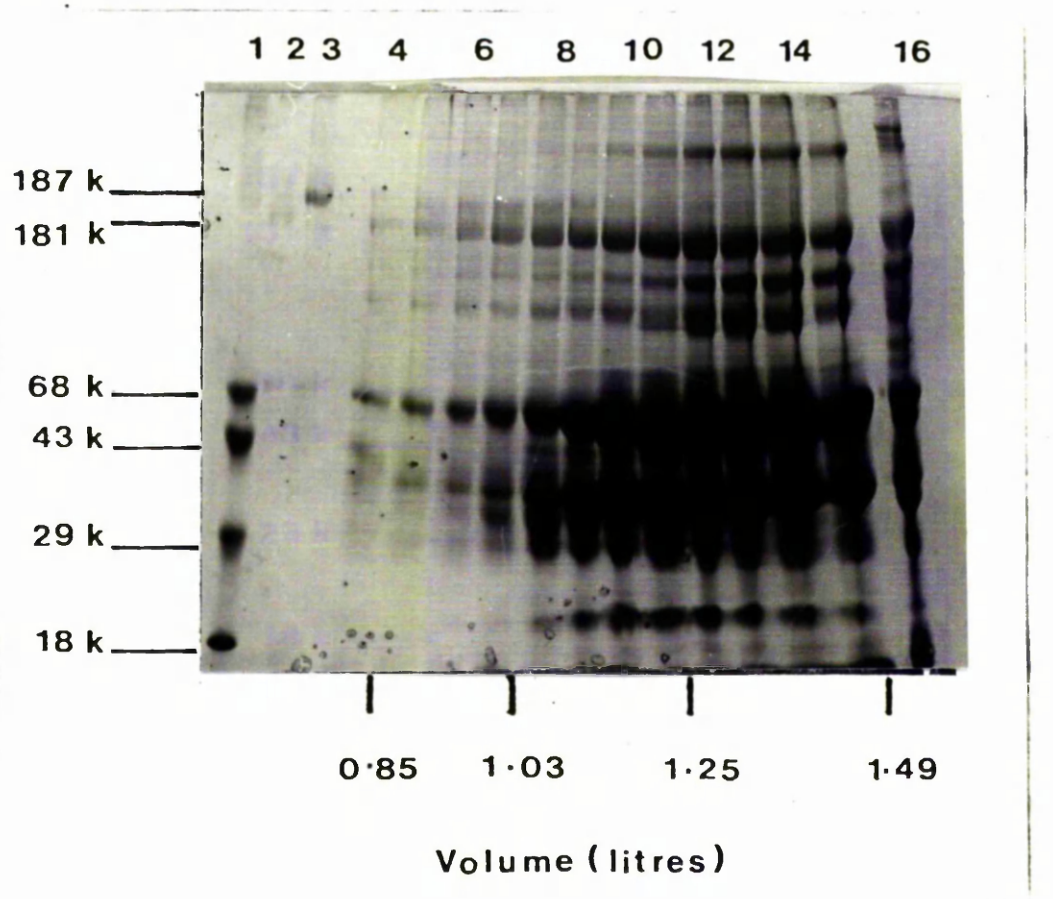


TABLE 3:4 Gel filtration of the pooled and concentrated fractions containing PAPP-A from the NAAC column*

	Volume (ml)	Total protein (mg)	Albumin (mg)	α_2M (mg)	PAPP-A (mg)	PAPP-A/α_2M x 100%
Protein loaded	8	42.4	nd	4	1.2	30
Protein in pooled fractions	30	nd	nd	3.6 (90%)	1.0 (83%)	27.8

*Figures in parentheses are % recovery

nd; values not determined.

Figure 3:6 Transmission at 280 nm (———) of the effluent from Sephadex G200 gel filtration column. The concentrations of PAPP-A (□—□) and α_2M (△—△) were measured by AACE.

Figure 3:7 Analysis of the fractions eluted from the gel filtration column. Track 1, α_2M; track 2, molecular weight standards; track 3, PAPP-A. Tracks 4-16, aliquots of alternate fractions containing PAPP-A and α_2M as ascertained by AACE.

Figure 3:6

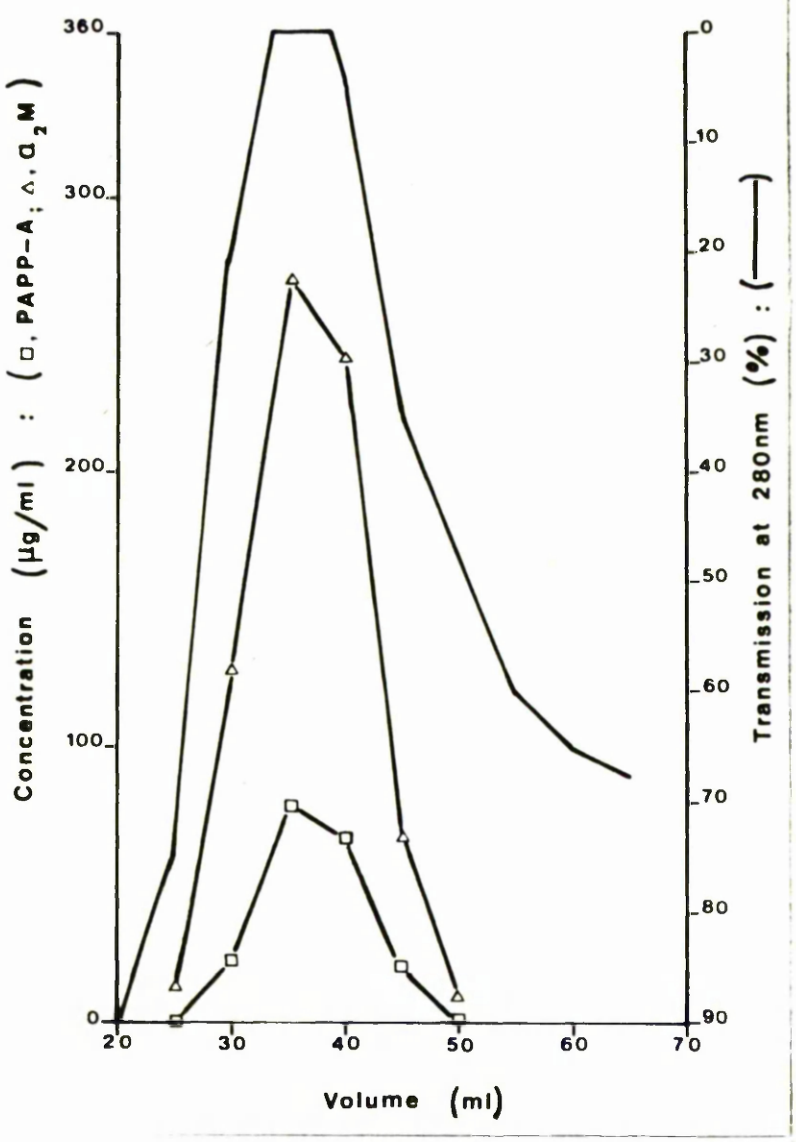
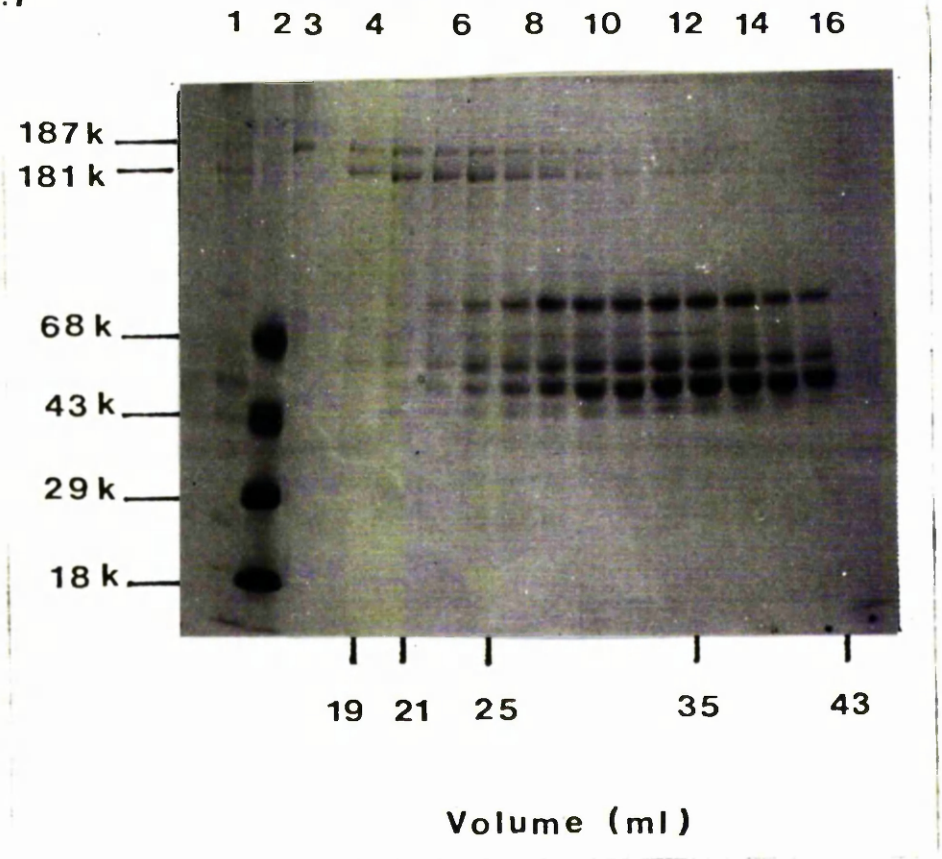


Figure 3:7



of fractionation is shown in Figure 3:3. Compared to the proteins in the starting material (Figure 3:3, tracks 4 and 5), the protein retained and then eluted from the column (tracks 13-16), contained predominantly serum albumin (mol wt 68,000). In addition, certain polypeptides increased in concentration from tracks 6 through to 12, this includes albumin (see also Figure 3:2), but also polypeptides of 120,000, 78,000, 48,000 and 24,000 mol wt. A heavy staining band of molecular weight similar to α_2M was also present in the fractions (tracks 6-12), only a faint band was seen of size corresponding to PAPP-A.

The PAPP-A pool from the column of Cibacron blue Sepharose was further purified by immunoadsorption of the contaminating proteins onto an antibody affinity column of sheep anti-adult male serum proteins. PAPP-A eluted before the peak of α_2M (Figure 3:4). Similarly on SDS gels (Figure 3:5) a polypeptide of molecular weight corresponding to pure PAPP-A (see Figure 3:5, track 3), eluted earlier from the column (tracks 4-10), than the polypeptide corresponding to α_2M (tracks 4-15). Compared with the starting material (Table 3:3), the pooled protein which failed to bind to the column contained no detectable albumin. In this step, the content of α_2M had been reduced 3-fold and the recovery of PAPP-A was 81%. Sephadex G200 filtration (Figure 3:6), was accompanied by an 83% recovery of PAPP-A (Table 3:4) and separated away many low molecular weight contaminants (Figure 3:7). The α_2M to PAPP-A ratio was not affected by this step as these proteins co-elute on gel filtration (Figure 3:6).

4. DISCUSSION

Approach experiment

As an approach towards purifying PAPP-A under mild purification

conditions, maternal blood was fractionated using negative antibody affinity chromatography (NAAC). All of the fractions containing PAPP-A were found to be contaminated with other serum proteins (Figure 3:1). The degree of contamination was dependent on the volume of filtered supernatant applied to the column, as many more polypeptides were observed on PAGE when 200 ml of maternal blood (Fig 3:1, tracks 1-5), were fractionated, compared with 100 ml (tracks 8-12). The major contaminant in these fractions was serum albumin, which made up 83% of the unadsorbed protein recovered after the 200 ml fractionation (Table 3:1). A high recovery of PAPP-A was observed, equivalent to 81.25% (Table 3:1) of the amount loaded, but only 0.6% of the total unadsorbed protein. This indicated that under these conditions the capacity of the NAAC column for immuno-adsorption of adult non-pregnancy specific proteins had been saturated, and it was evident, therefore, that if a more pure preparation of PAPP-A was to be isolated, this protocol would have to be modified. On consideration of these results, it was decided that there were three possible ways to approach the problem, namely; by reducing the volume of filtered supernatant loaded onto the column; by increasing the gel filtering capacity of the NAAC column or by incorporating a further chromatographic step to selectively remove the major contaminants. A reduction in the amount of filtered supernatant loaded onto the column could result in a reduction in the PAPP-A recovery. A lower limit of 100 ml of filtered supernatant was therefore decided upon. On fractionation of this volume (Figure 3:1. tracks 8-12) only 4 significant contaminating polypeptides were observed in the PAPP-A fractions. Separation of these lower molecular weight contaminants from the PAPP-A could possibly be achieved if the gel filtering capacity of the NAAC column

were increased (by changing its shape from short and wide to longer and narrower), though it seems unlikely that the problem of albumin contamination would be fully resolved by this step. As albumin is the main contaminant in the NAAC-PAPP-A fractions, and because of the problems considered with the other approaches, it was decided to incorporate a step to selectively remove albumin before the negative antibody affinity chromatography step. Travis et al (1976) showed that a column of Sepharose conjugated to the dye Cibacron blue could be used as an affinity column for serum albumin. Albumin represents at least 50%, by weight, of the proteins in plasma, therefore this step could remove over one half of protein with only very minor mechanical losses of other proteins. Thus much smaller quantities of protein have to be processed in subsequent steps. The efficiency of binding of Cibacron blue Sepharose was reported to be very high (Travis et al, 1976), with approximately 98% of the albumin being removed on passage of whole human plasma through the column. Moreover, this was said by Travis et al to be a mild procedure and that denaturation of the unretarded protein was minimized. Virca et al (1978) and Travis et al (1976) reported that Cibacron blue Sepharose also retarded lipoproteins and gamma globulins, but not α_2M. Hence a column of Cibacron blue Sepharose was used as an initial step in the purification protocol to selectively remove albumin while effecting the molecular sieving of the remainder of the plasma proteins.

Isolation of PAPP-A enriched preparations

PAPP-A was isolated from maternal blood by a series of 3 chromatographic steps using mild purification conditions. Albumin depleted plasma was produced by dye ligand chromatography on Cibacron blue Sepharose as an alternative to an ammonium sulphate precipitation

step as used previously (Bischof, 1979; Halbert and Lin, 1979; Sutcliffe et al, 1980b). Compared with the starting material, a 248-fold reduction in albumin concentration was observed in the pooled fractions, whilst α_2M and PAPP-A were obtained with high recovery values (Table 3:2). Analysis of the salt wash (Figure 3:3, tracks 13-16), showed that serum albumin was the predominant protein binding to the Cibacron blue. Some albumin eluted with the later fractions of unbound protein (Figure 3:2), since the column had become saturated with albumin. However, this was sufficiently delayed to allow a relatively albumin-free PAPP-A pool to be collected (Figure 3:2). As the fractionation progressed (Figure 3:3, tracks 6-12), there was an increase in certain polypeptides which did not bind appreciably to the matrix (tracks 13-16). Gel filtration is the most likely explanation for this, and probably contributed to the purification process at work in this step.

Immunoadsorption of the adult, non-pregnancy specific proteins from this albumin-depleted pool, produced fractions containing PAPP-A, α_2M and other low molecular weight contaminants. Most of the PAPP-A eluted before the peak of the α_2M (Figures 3:4 and 3:5), though some α_2M was observed in all the PAPP-A containing fractions (Figure 3:5). These proteins would normally co-elute from a column of Sepharose 4B, the disparity between their maximum elution points in this step is due to the saturation of anti-α_2M antibodies on the Sepharose 4B. A 3-fold reduction in α_2M content was achieved in the pooled PAPP-A fractions compared with the starting material and 81% of the PAPP-A applied to the column was recovered in the pool (Table 3:3). Both proteins were obtained with high recovery from the subsequent gel filtration step (Table 3:4).

The PAPP-A produced by this method was not as pure as that obtained after positive affinity chromatography (Sutcliffe et al, 1980b; Folkersen et al, 1981a). However, this was largely an operational factor, since the purity of the PAPP-A could be markedly increased by reducing the volumes loaded onto the columns and restricting the pool size to those fractions containing the maximum concentration of PAPP-A. Inevitably this stringency would lead to a reduction in the recovery of PAPP-A. The problems of the contaminating α_2M can be resolved as these two proteins can be separated by ion exchange chromatography (see Chapter 4:3). However, to avoid further processing of the PAPP-A, this step was not employed until after investigating its functional properties (see Chapter 4). It is not possible to be certain that the present purification scheme has maintained PAPP-A in an essentially native and potentially active state, but it does avoid the more severe conditions used in previous methods.

CHAPTER 4 : FUNCTIONAL STUDIES ON PAPP-A ENRICHED PREPARATIONS

1. INTRODUCTION

This chapter contains the experimental details and results of the functional studies carried out on the PAPP-A purified in the manner described in Chapter 3. The following functional aspects of the PAPP-A enriched preparation (PEP) were investigated:-

i) The trypsin binding properties

ii) The plasmin binding properties

iii) The complement inhibitory properties

iv) The fibronectin and gelatin binding properties.

α_2M is a potent protease inhibitor (see Starkey and Barrett, 1977). Most of the research on its inhibitory properties has been carried out using trypsin inhibition as a model. α_2M also inhibits plasmin (Harpel, 1977; Starkey and Barrett, 1977) and it has been reported (Bischof, 1979) that PAPP-A inhibits this enzyme too. Hence the trypsin and plasmin binding properties of the PAPP-A enriched preparation (PEP) were investigated. The interaction between proteases and their inhibitors is characterized by the formation of highly associated complexes, in which the active site of the enzyme is covered or occupied by the inhibitor (see Chapter 1:5). Compared with activity assays, protease binding assays have the initial advantage that a precise identification can be made of the protein species which is actually interacting with the enzymes. This is important in the present work where the PEP is contaminated with α_2M.

PAPP-A has also been reported to inhibit complement mediated red cell lysis (Bischof, 1981), therefore the complement inhibitory properties of PEP were determined. Finally, the fibronectin and gelatin binding properties were also studied since immunoperoxidase

staining of placental tissues has located these proteins in similar areas (Sutcliffe, Khalef and Horne, in preparation).

2. GENERAL MATERIALS AND METHODS

MATERIALS

PAPP-A enriched preparation (PEP) was purified as described in Chapter 3. For those experiments involving column chromatography, aliquots of PEP (1 ml) were dialysed overnight at $4^{o}C$, against 2 l of the column buffer.

Sepharoses of types 4B, CL6B and DEAE CL6B were obtained from Pharmacia (GB) Ltd. Column buffers were either 10 mM potassium phosphate pH 7.5 (buffer A) or 0.3 M NaCl 5 mM potassium phosphate pH 7.5 (buffer B).

Sheep anti-PAPP-A was raised as described by Sutcliffe et al (1979), rabbit anti-α_2M was obtained from Behring Diagnostics (Hoescht Pharmaceuticals) or Seward Laboratories. All protein samples were coupled to Sepharose 4B (15 mg protein/g Sepharose 4B, approx), using the method of Porath, Axen and Ernback (1967).

Tris, SDS, agarose, trypsin (type III, from bovine pancreas) and all other proteins were obtained from the Sigma Chemical Company. Chemicals and other materials were obtained from BDH unless otherwise stated. Enhance was purchased from New England Nuclear.

Carrier-free ^{125}I was obtained from Amersham with a specific activity of 100 µCi/ml.

METHODS

Ion exchange chromatography

Protein solutions were applied to a column of Sepharose

DEAE CL6B (0.8 x 0.9 cm) equilibrated at 4°C with buffer A. The column was washed with 10-15 ml of the same buffer at 10 ml/h . An 18 ml (10 ml in the trypsin binding assay) salt gradient (0-0.4 M NaCl in buffer A) was then applied at 5 ml/h, followed by a further 5 ml of 0.4 M NaCl in buffer A. The column was re-equilibrated with buffer A before re-use.

Precipitation studies using protein-Sepharose conjugates

The fractions obtained after ion-exchange chromatography and gel filtration were analysed using protein-Sepharose conjugates and the subsequent precipitation values recorded. Duplicate aliquots (50-100 µl) of these fractions were shaken vigorously, overnight at room temperature with 75-100 µl of the immobilised protein conjugates which had been diluted with Sepharose 4B (anti-α_2M Sepharose, 1/10 diluted; anti-PAPP-A and horse serum Sepharoses, 1/4 diluted). Anti-PAPP-A Sepharose was mixed with 100 µl of adult male serum per ml of Sepharose prior to immunoadsorption (except in the gel filtration approach experiment to trypsin binding), to block any contaminating pregnancy non-specific binding sites (see Sutcliffe et al, 1980a). The unbound counts were removed by 3 cycles of washing with PBS and centrifugation (2,500 g for 10 min). The counts in each tube before and after washing were measured using a 1275 minigamma counter (LKB Instruments).

Antibody antigen crossed electrophoresis (AACE)

AACE was used for the detection of α_2M and PAPP-A in the column eluates, using the method of Laurell (1966), with the modification that 100 mM Tris was present in the tank and gel buffers (Sutcliffe et al, 1980b). A 2% antibody solution was used in the antibody beds

for both antisera. This method was also used to estimate the concentration of α_2M and PAPP-A in the PEP, against standards of known concentration.

Polyacrylamide gel electrophoresis (PAGE)

SDS/Tris PAGE, under reducing and non-reducing conditions (see methods 1 and 2, Sutcliffe et al, 1980b) was used as described in the subsequent methods sections. The molecular weight standards used on these gels were made up of bovine serum albumin (68,000 mol wt), ovalbumin (43,000 mol wt), carbonic anhydrase (29,182 mol wt), and β-lactoglobulin (18,363 mol wt). PAPP-A standards were prepared as described by Sutcliffe et al (1980b), and α_2M standards by gel filtration of human serum on Sepharose CL6B. Gels were stained with Coomassie brilliant blue R.

Autoradiography, when required, was carried out on the dried-down gels. ^{125}I counts were detected using NS-2T film (Kodak) and ^{35}S counts were detected using X-omatS film (Kodak) on enhanced gels (1 h in enhance followed by 1 h in H_2O, before drying down). Films were exposed at room temperature unless otherwise stated.

TCA and PTA precipitation of ^{125}I-protein counts

Aliquots (100 µl) of the fractions collected during ion-exchange chromatography and gel filtration were mixed with 400 µl of BSA (RIA grade 1 mg/ml) in 50 mM potassium phosphate pH 7.5, to which was added 500 µl of 12.5% (w/v) TCA at 4°C or 100 µl of 5% (w/v) PTA in 2 M HCl. After standing on ice for 15 min the precipitates were recovered by centrifugation (2,500 g for 15 min). The counts present were recorded before and after precipitation using a 1275 minigamma counter (LKB Instruments).

Protein Iodinations

Iodinated trypsin and PEP were prepared by the chloramine T method of Hunter and Greenwood (1962), with final concentrations of chloramine T and sodium metabisulphite in the reaction mixture of 80 µg/ml and 200 µg/ml respectively. Iodinated plasminogen was prepared by the lactoperoxidase method (Bolton, 1977).

3. TRYPSIN BINDING PROPERTIES OF PEP

3:1 INTRODUCTION

In an initial experiment the ^{125}I-trypsin binding capacities of the components in a partially purified preparation of PAPP-A made by negative antibody affinity chromatography of a small volume of maternal blood (see section 3:2 of this chapter), were investigated. This preparation contained some α_2M and other contaminants such as albumin. Separation of these components was achieved by gel filtration. An immune-adsorption assay was then used to identify specific protein-trypsin complexes (for validation of this assay see Gore and Sutcliffe, in press). Subsequent binding assays were carried out using PEP as a source of PAPP-A, which was separated from contaminating α_2M by ion exchange chromatography. Again specific trypsin-protein complexes were identified by an immunoadsorption assay.

3:2 MATERIALS AND METHODS

MATERIALS

The following materials were obtained from the Sigma Chemical Company: trypsin (type III from bovine pancreas), human serum albumin, trypsin inhibitor (type II-O from egg white), Soya bean trypsin inhibitor, BSA (RIA grade) and phosphotungstic acid (PTA).

Anti-human albumin was purchased from Behring Diagnostics (Hoescht Pharmaceuticals). α_2M was prepared by gel filtration of human serum on Sepharose CL6B. BSA buffer, composed of 0.1% BSA in 50 mM potassium phosphate pH 7.5, was the column buffer in the gel filtration experiment.

METHODS

Approach Experiments

i) To determine the trypsin binding activity of a partially purified preparation of PAPP-A

The PAPP-A used in these experiments was produced on an NAAC column (2.5 x 95 cm) equilibrated with buffer B. A 25 ml sample of filtered maternal blood was applied to the column at 30 ml/h. A fraction containing 25 µg PAPP-A/ml (approx) was used in gel filtration experiments as an approach to investigating the trypsin binding properties of PAPP-A. However, the assay was initially developed using α_2M and ^{125}I-trypsin.

The elution point of ^{125}I-trypsin was determined prior to the α_2M binding assay. A 100 µl aliquot of trypsin (0.2 µg; 18.8 µCi/µg) was mixed with 200 µl of human albumin (1 mg/ml) and 700 µl of BSA buffer. This was then applied to a column of Sepharose CL6B (0.9 x 52.5cm) equilibrated with BSA buffer at 4^{o}C. The sample was eluted at 10 ml/h (for 8 h) with the same buffer. The radioactivity in the fractions was measured and the elution point of albumin determined by AACE. Protein bound ^{125}I and free ^{125}I were located by TCA precipitation.

α_2M (0.7 ml, 1 mg/ml) was incubated with 200 µl of human albumin (1 mg/ml) and 100 µl of ^{125}I-trypsin (0.2 µg, 18.8 µCi/µg) for 10 min at room temperature. This sample was fractionated as for the

^{125}I-trypsin experiment. The radioactivity in the fractions was measured and the elution points of α_2M and albumin determined by AACE. α_2M:trypsin complexes were located by immunoadsorption with immobilised anti-α_2M. A TCA precipitation was also carried out.

PAPP-A (0.9 ml; 0.32 mg) from an NAAC fraction (as described above), was incubated with 100 µl of ^{125}I-trypsin (0.2 µg; 18.8 µCi/µg) for 10 min at room temperature. Gel filtration was carried out as for the α_2M binding assay. PAPP-A and albumin elution points were detected by AACE and PAPP-A bound counts were precipitated using immobilised anti-PAPP-A. As PAPP-A is soluble in TCA (see Chapter 2), a PTA precipitation assay was used to locate protein-bound and free ^{125}I.

ii) Validation of the trypsin binding assay incorporating ion exchange chromatography

The assay was initially developed using α_2M (400 µl; 0.1 mg/ml) incubated for 1 h at 37°C with a mixture of ^{125}I labelled and unlabelled trypsin (100 µl, 0.2 µg, 19 µCi/µg and 5 µl, 0.25 µg respectively). Unbound trypsin was removed by incubation (½ h at room temperature), on a column (0.5 x 4 cm) of soya bean trypsin inhibitor (12 mg) coupled to Sepharose 4B. The column was equilibrated in buffer A and unbound protein eluted with same buffer, 1 ml fractions were collected. The fractions containing the main peak of counts were pooled and applied to the ion exchange column (see general methods, this chapter). The radioactivity and conductivity of the fractions were determined, together with the α_2M elution point. A TCA precipitation and immunoadsorption with immobilised anti-α_2M, were carried out.

Separate ion exchange experiments were performed on iodinated trypsin (0.1 µg; 19 µCi/µg) and PEP (67 µg, PAPP-A; 239 µg, α_2M) to

determine the molarity of their points of elution in the salt gradient.

Trypsin binding properties of PEP

A 1 ml sample of PEP containing approximately 50 µg of PAPP-A and 150 µg of α_2M (previously dialysed against buffer A), was incubated for 1 h at 37°C with 0.3 µg/100 µl of iodinated trypsin (19 µCi/µg) and 0.25 µg of non-iodinated trypsin (5 µl; 50 µg/ml). To remove unbound trypsin the mixture was then incubated for ½ h at room temperature on a column of Sepharose 4B (0.5 x 4 cm), to which 12 mg of soya bean trypsin inhibitor had been coupled. The column had first been equilibrated with buffer A. The unbound protein was eluted with buffer A and 1 ml fractions collected. The counts eluted were recorded using a bench top gamma counter (Mini Assay type 6-20, Mini Instruments), and the fractions containing the main peak of counts were pooled and applied to the ion exchange column (see general methods, this chapter). The radioactivity and conductivity of the fractions were measured, together with the elution points of α_2M and PAPP-A. For immune adsorptions, aliquots of the individual fractions were incubated with anti-PAPP-A, anti-α_2M and a control of non-immune (horse) serum, coupled to Sepharose 4B (see general methods, this chapter). The precipitated counts were measured.

The effect of trypsin on the PAPP-A enriched preparation

The degradation of PEP in the presence of trypsin was investigated. An iodinated aliquot (300 µl) of PEP containing 1.5 µg PAPP-A and 1.5 µg α_2M approx (10.6 µCi/µg) was incubated for 30 min with 1.5 µg of trypsin (15 µl; 0.1 mg/ml) at room temperature. Samples (15 µl) of the incubation mix were withdrawn after 0, 1, 3, 5, 15 and 30 min, and the reaction stopped by the addition of 10 µl of egg

Figure 4:1 AACE analysis of the partially purified preparation of PAPP-A after fractionation of 25 ml of filtered maternal blood on a NAAC column.

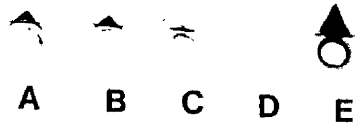
(a) antiserum, sheep anti-PAPP-A; wells A-D, 10 µl of consecutive fraction pools; Well E, 10 µl of filtered maternal blood (0.1 mg/ml PAPP-A approx).

(b) antiserum, rabbit anti-α_2M; Wells A-D, 10 µl of consecutive fraction pools; well E, 1 µl of filtered maternal blood (3 mg/ml α_2M approx).

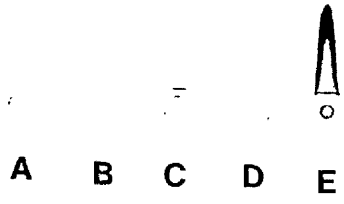
(c) antiserum, rabbit anti-albumin; Wells A-D, 1 µl of consecutive fraction pools; well E, 1 µl of 1/20 diluted filtered maternal blood (2 mg/ml albumin approx).

Figure 4:1

a



b



c

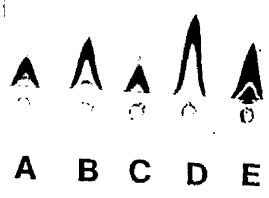
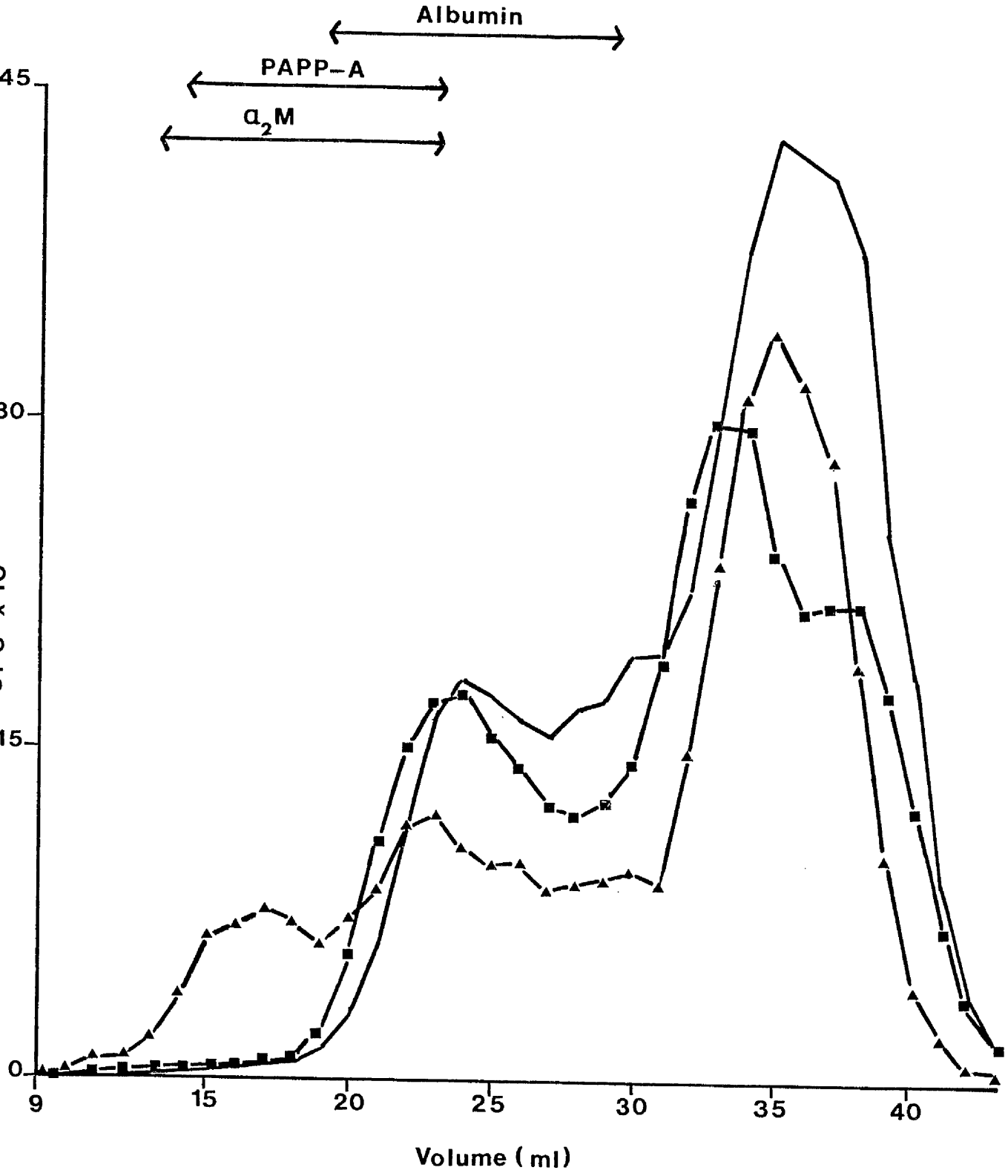


Figure 4:2 Gel filtration of partially purified preparations of α_2M (▲—▲) and PAPP-A (■—■) after incubation with ^{125}I-trypsin, plus a filtration of ^{125}I-trypsin (———) as control. Whole fraction counts were recorded.

Figure 4:2



white trypsin inhibitor (100 µg/ml). After 30 min, 200 µl of trypsin inhibitor were added to the remaining ^{125}I-PEP/trypsin mix (210 µl). This solution was then fractionated by ion exchange chromatography (see general methods, this chapter). The radioactivity and conductivity of the fractions were measured. SDS-PAGE (5-12½%) under reducing conditions was carried out on the samples collected during the ^{125}I-PEP/trypsin incubation. Similarly, the fractions eluted during the salt gradient were analysed by SDS-PAGE, under both reducing and non-reducing conditions. These gels were dried down after staining with Coomassie blue and autoradiographs set up. Integrated densitometry on the autoradiograph from the trypsin digestion of PEP, was used to determine the degree of trypsin digestion of the PAPP-A and α_2M.

3:3 RESULTS

Approach experiments

i) Determination of the trypsin binding activity of a partially purified preparation of PAPP-A

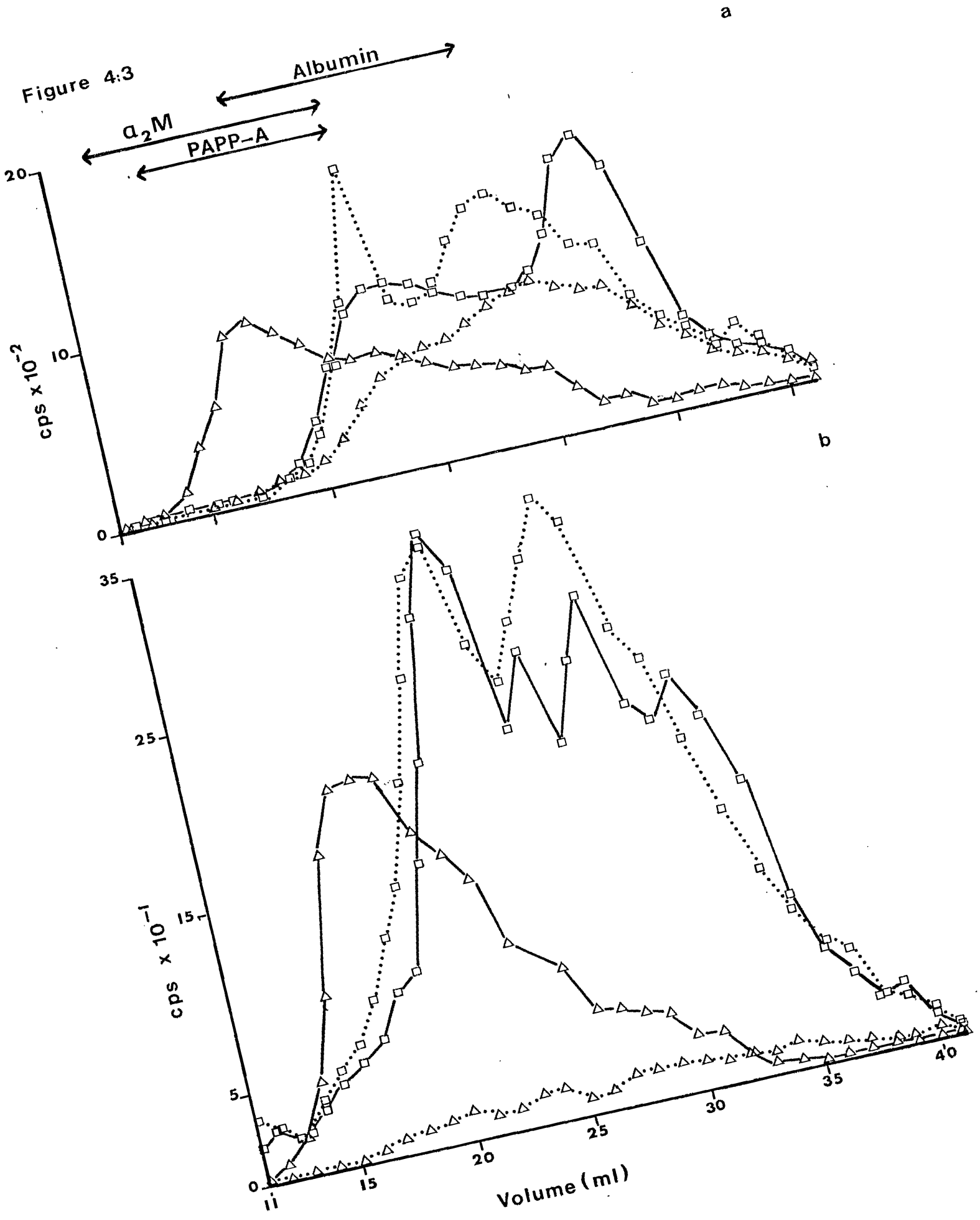
Radiolabelled trypsin was incubated with partially purified preparations of α_2M and PAPP-A (see Figure 4:1), and the resulting mixtures fractionated on Sepharose CL6B. When ^{125}I-trypsin plus human serum albumin (HSA) carrier were gel-filtered 2 peaks of radioactivity were observed (Figure 4:2). The first and smallest peak reached its maximum at 24 ml eluted, and the second at 35 ml eluted. Gel filtration of an α_2M/^{125}I-trypsin/HSA mixture gave rise to 3 peaks of radioactivity. The first and smallest peak reached its maximum at 17 ml, the second at 23 ml, and the third and largest peak at 35 ml eluted (Figure 4:2). On fractionation of a PAPP-A/^{125}I-trypsin

Figure 4:3 Precipitation of ^{125}I-trypsin counts after gel filtration assay.

(a) TCA/PTA precipitations:
α_2M/trypsin (△——△) and trypsin (△- -△) fractionations; TCA precipitable counts.
PAPP-A/trypsin (□——□) and trypsin (□- -□) fractionations; PTA precipitable counts.

(b) Immune adsorptions:
α_2M/trypsin (△——△) and trypsin (△- -△) fractionations; anti-α_2M precipitable counts. PAPP-A/trypsin (□——□) and trypsin (□- -□) fractionations; anti-PAPP-A precipitable counts.

Figure 4:3


a
Albumin
α_2M
PAPP-A
20
10
0
cps x 10^{-2}
b
35
25
15
5
0
cps x 10^{-1}
11
15
20
25
30
35
40
Volume (ml)

mixture, the elution profile for the radioactivity was similar to that obtained with ^{125}I-trypsin/HSA (Figure 4:2), with peaks of radioactivity at 24 ml and 33 ml eluted.

AACE analysis of the fractions, located the α_2M elution range to 13-23 ml eluted (max. at 18 ml); the PAPP-A range to 14-23 ml eluted (max at 17 ml) and the HSA range to 19-29 ml eluted (max at 23 ml). From the AACE and TCA/PTA precipitation results the fractions were divided up into 3 ranges, designated as follows; the α_2M/PAPP-A range (14-24 ml); the ^{125}I-trypsin range (24-34 ml) and the ^{125}I-range (34-44 ml). The percent of counts recorded after precipitation with TCA/PTA or antiserum were calculated for each range (Table 4:1). TCA precipitations on the eluate from the fractionation of ^{125}I-trypsin gave a broad precipitation curve (Figure 4:3a), which started in the α_2M range and reached its maximum at 30 ml eluted. This was judged to be the point of maximum trypsin concentration. In the trypsin range, 62.5% of the counts precipitated with TCA were recorded, compared with 17.5% and 20% in the α_2M and ^{125}I ranges respectively (Table 4:1). Similarly, after PTA precipitation, 65% of the precipitable counts were observed in the trypsin range, 18% in the PAPP-A range and 17% in the ^{125}I range. This time, however, 2 precipitation peaks were observed, the first at the point of maximum albumin concentration (23 ml eluted) and the second at the point of maximum trypsin concentration (29 ml eluted). TCA precipitations on the eluate from the α_2M/trypsin fractionation gave a broad precipitation curve (Figure 4:3a). The fraction of maximum radioactivity and α_2M concentration were co-incident (at 18 ml eluted). In this range 59% of the TCA and 72.5% of the anti-α_2M precipitable counts were recorded (Table 4:1). Very few of the control ^{125}I-trypsin counts were precipitated by the anti-α_2M in any of the ranges (Figure 4:3b).

TABLE 4:1 Precipitation values for trypsin binding assay (gel filtration method)

Precipitation(a)		FRACTION RANGE 14-24 ml α_2M/PAPP-A range	24-34 ml trypsin range	34-44 ml ^{125}I- range
TCA	(b)	59% (17.5%)	33.5% (62.5%)	7.5% (20%)
anti-α_2M	(b)	72.5% (17.0%)	24.0% (67.0%)	3.5% (16.0%)
PTA	(c)	16.0% (18.0%)	54.0% (65.0%)	30.0% (17.0%)
anti-PAPP-A	(c)	32.0% (26%)	58.0% (63%)	10.0% (11%)

(a)Values given as % of TCA, PTA or immune precipitable counts

(b)Average of 2, α_2M/trypsin gel filtration experiments

(c)Results from 1, PAPP-A/trypsin gel filtration experiment

Figures in parentheses are values obtained for trypsin/albumin gel filtration controls.

Figure 4:4 Ion exchange chromatography of PAPP-A enriched product. Proteins were eluted with an NaCl gradient (- - -) and their absorbance at 280 nm recorded (———).

Figure 4:5 Ion exchange chromatography of an α_2M preparation after incubation with ^{125}I-trypsin. Proteins were eluted with an NaCl gradient (- - -) and the counts in an 100 µl aliquot of the fractions, recorded (———). TCA (■—■) and anti-α_2M (▲——▲) precipitable counts were determined.

Figure 4:4

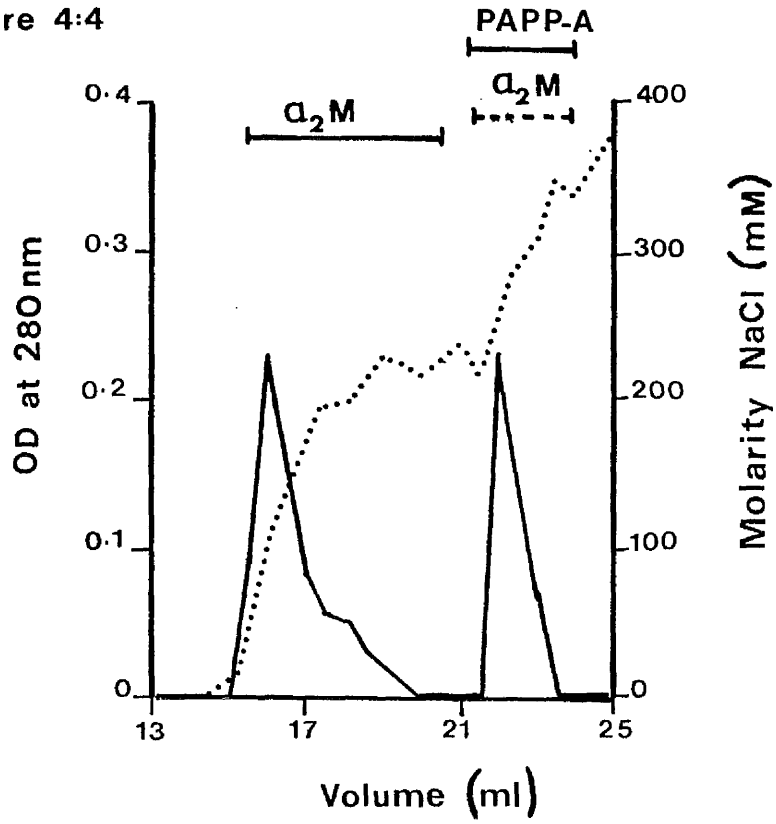
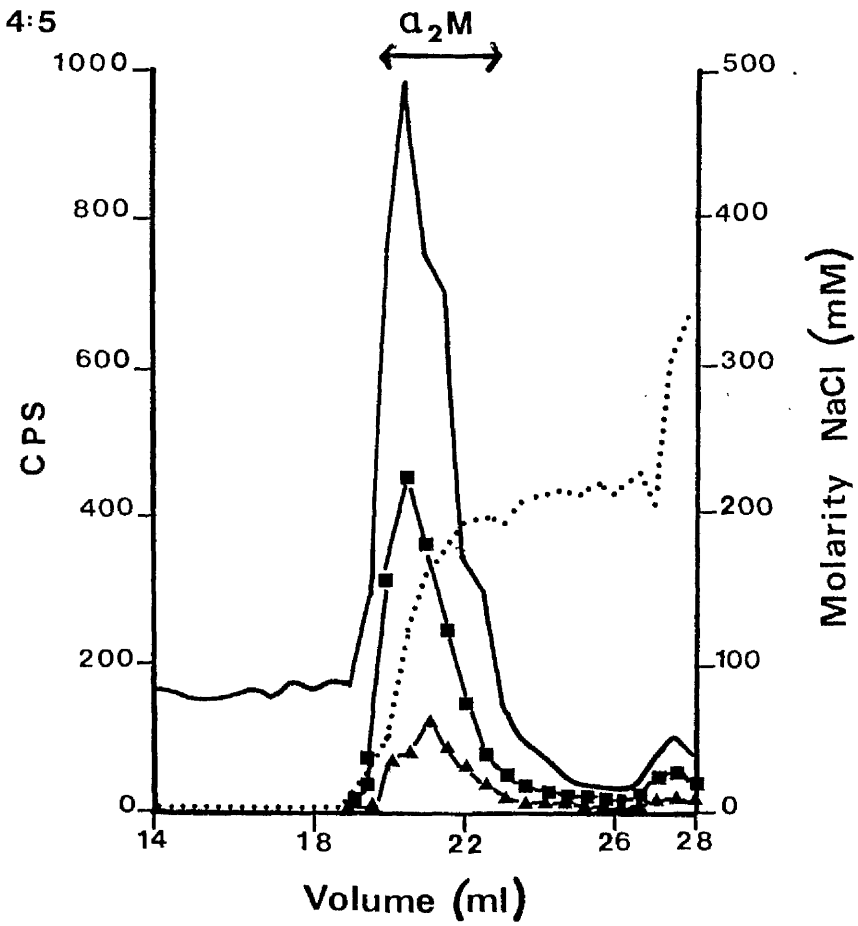


Figure 4:5



Two peaks of PTA precipitable counts were observed in the eluate from the PAPP-A/trypsin fractionation. The first occurred at 23-24 ml eluted (max. albumin concentration) and the second at 33 ml eluted. In the PAPP-A range 16% of the PTA and 32% of the anti-PAPP-A counts were precipitated. In the trypsin range the values were 54% and 58% respectively, and for the ^{125}I range 30% and 10%. Immobilised anti-PAPP-A precipitated 26%, 63% and 11% of the ^{125}I-trypsin counts in the PAPP-A, trypsin and ^{125}I ranges in the control fractionation (Table 4:1; Figure 4:3).

ii) Validation of the trypsin binding assay incorporating ion exchange chromatography

Separate ion exchange experiments on ^{125}I-trypsin and PEP were performed to ascertain their points of elution in a NaCl gradient. The ^{125}I-trypsin was found to elute maximally at 180 mM NaCl (data not shown). The elution maxima for α_2M and PAPP-A were at 120 mM and 270 mM NaCl respectively (Figure 4:4). Some α_2M was also eluted in the fractions containing PAPP-A.

The assay was developed using α_2M incubated with ^{125}I-trypsin (Figure 4:5). Two peaks of radioactivity were eluted by the salt gradient. The first and largest peak occurred at 20.5 ml eluted (120 mM NaCl) with the second peak at 27.5 ml eluted (295 mM NaCl). The α_2M concentration was maximum at 120 mM NaCl (20.5 ml eluted), which coincided with the peak of the TCA precipitable counts (45.9% precipitation). The peak of anti-α_2M precipitable counts (15.1% precipitation) was at 156 mM NaCl (21 ml eluted). TCA and anti-α_2M precipitable counts were also observed in the second peak (52.5% and 9.2% respectively), however, α_2M was not detected in this range by AACE (Figure 4:5).

Figure 4:6 Ion exchange chromatography of a PAPP-A enriched fraction after incubation with ^{125}I-trypsin. Proteins were eluted with an NaCl gradient (- - -) and whole fraction counts recorded (▲——▲).

Figure 4:7 Immunoadsorption of ^{125}I-trypsin/α_2M complexes. Lower panel: ^{125}I-trypsin counts precipitated by anti-α_2M (Δ—Δ) and horse serum (●—●). Upper panel: concentration of α_2M (Δ—Δ) determined AACE.

Figure 4:8 Immunoadsorption with immobilised anti-PAPP-A. Lower panel: ^{125}I-trypsin counts precipitated by anti-PAPP-A (□—□) and a control of non-immune serum (●—●). Upper panel: concentration of PAPP-A (□—□) determined by AACE.

Figure 4:6

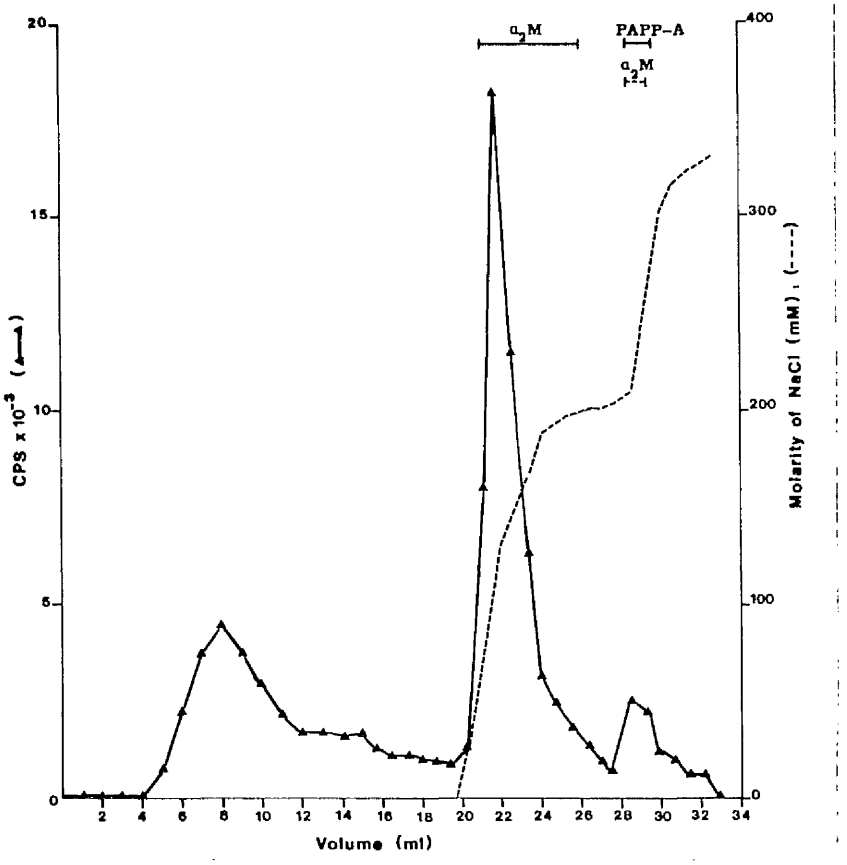


Figure 4:7

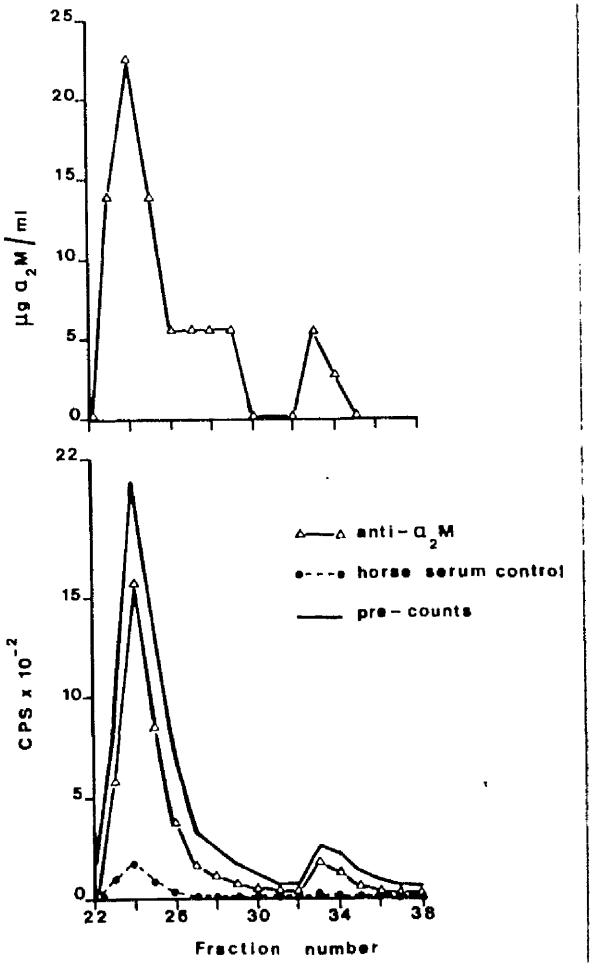
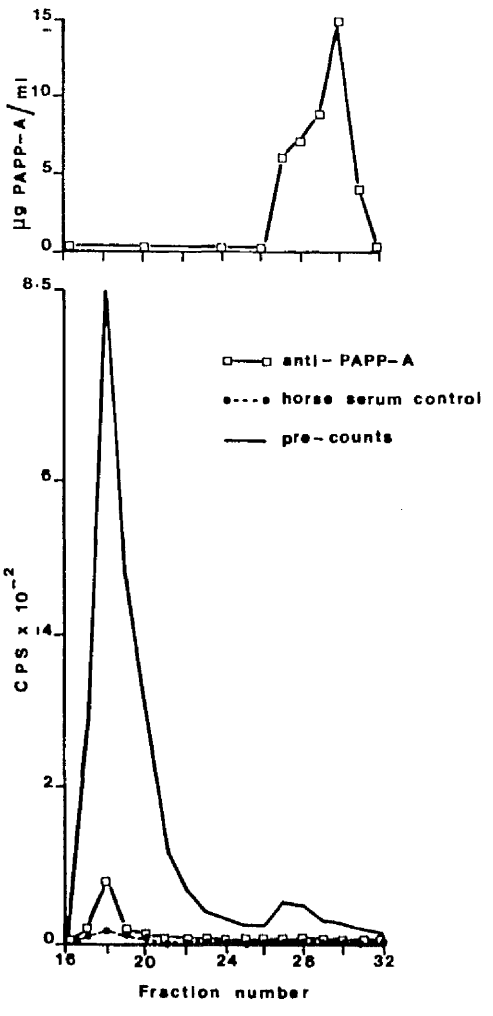


Figure 4:8



Trypsin binding properties of PEP

In this experiment ^{125}I labelled trypsin was added to the purified preparation of PAPP-A. Since this preparation contained both PAPP-A and α_2M it was essential to separate the two proteins by ion exchange chromatography in order to investigate the trypsin binding capacity of PAPP-A. This chromatographic step was carried out after the addition of labelled trypsin. The number of steps the PAPP-A was exposed to before it was tested for trypsin binding was thus reduced. The α_2M present was used as a positive control for the trypsin binding procedure. Three peaks of radioactivity were observed (Figure 4:6). The counts in the first peak, which eluted before the NaCl gradient, did not precipitate with TCA and were therefore judged to be free ^{125}I. The second peak was largest and eluted maximally at 130 mM NaCl. The third peak was smallest and began to elute at 200 mM NaCl (max. 210 mM). AACE assays showed that most α_2M was eluted in those fractions containing the largest peak of radioactive trypsin (20-26 ml eluted; Figure 4:6). PAPP-A was eluted at 28-30 ml and some α_2M was also detected there. In the fraction of maximum radioactivity (21.75 ml eluted), 75.5% of the iodine counts were precipitable by immobilised anti-α_2M (Figure 4:7), and 65% of the counts were precipitated in the peak fraction from the smallest peak (28.5 ml eluted). Results of similar immunoadsorptions with immobilised anti-PAPP-A are shown in Figure 4:8. The precipitated counts were very low and compared closely with the controls in Figure 4:7.

The effect of trypsin on the PAPP-A enriched preparation

Trypsin (0·15 µg) was incubated with an iodinated sample of PEP (1.5 µg PAPP-A, 1.5 µg α_2M), in a 3:1 molar ratio of trypsin to α_2M

TABLE 4:2 Integrated densitometry on the autoradiograph from the trypsin/^{125}I-PEP incubation[a]

Incubation time (min)	PAPP-A (mg)[b]	α_2M (mg)[b]
0	1.2	4.1
1	0.6	2.1
3	0.3	1.9
5	0.3	1.9
15	0.2	1.5
30	0.2	1.5

(a) See Figure 4:9a

(b) Densitometric peaks were cut out and weighed.

Figure 4:9 Analysis of the degradation of iodinated PAPP-A and α_2M in PEP after incubation with an equimolar (approx) ratio of trypsin.

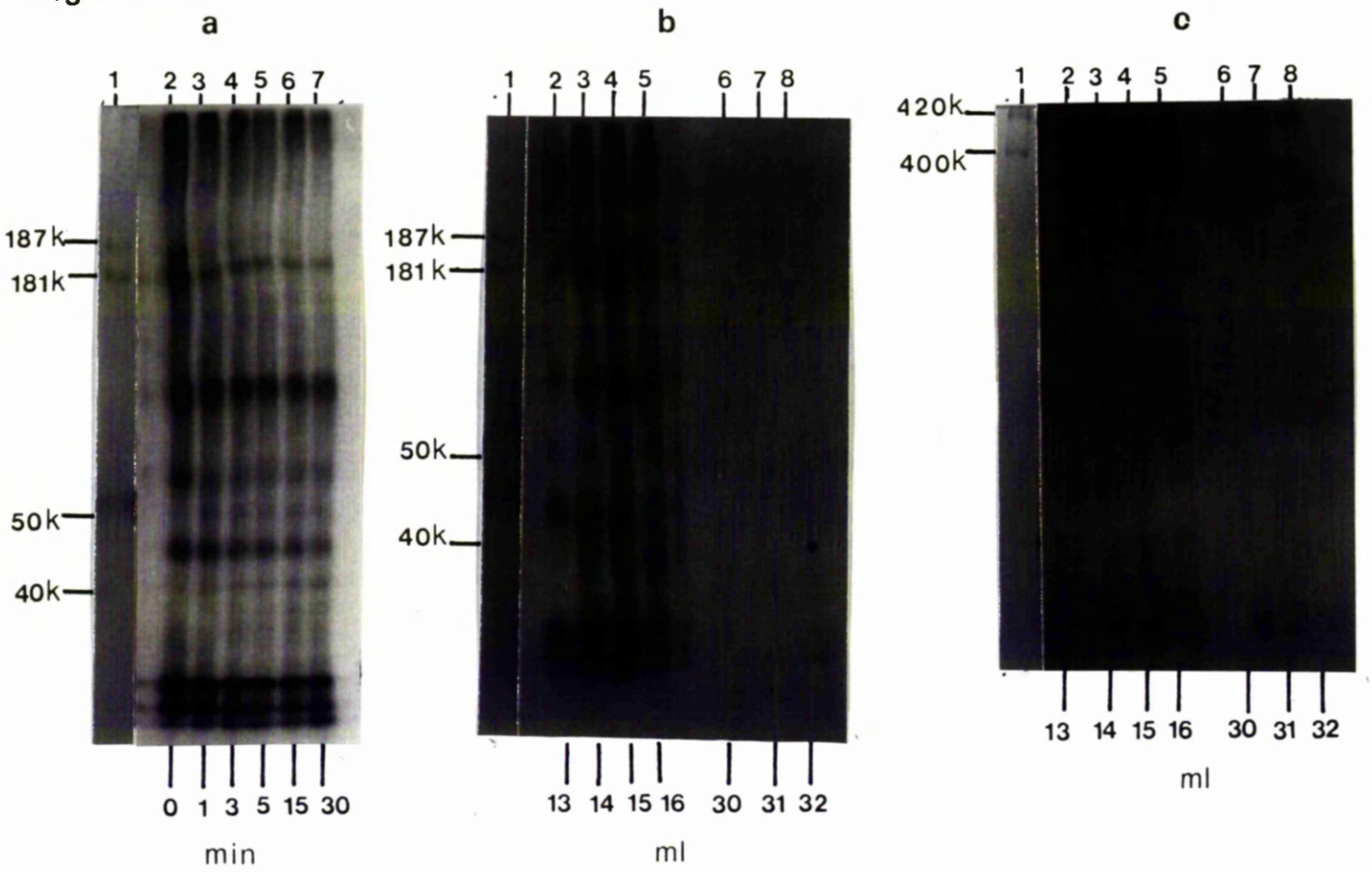
(a) SDS-PAGE under reducing conditions. Track 1, reduced α_2M (181 k) and PAPP-A (187 k) standard, stained with Coomassie blue. Tracks 2-7, autoradiogram showing the degradation of ^{125}I-PEP during a 30 min incubation with trypsin. Aliquots were withdrawn at 0, 1, 3, 5, 15 and 30 min respectively.

(b) SDS-PAGE under reducing conditions on the fractions collected after ion exchange chromatography of ^{125}I-PEP/trypsin, after a 30 min incubation. Track 1, reduced α_2M (181 k) and PAPP-A (187 k) after staining with Coomassie blue. Tracks 2-8 autoradiogram of the fractions in the α_2M peak (tracks 2-5, 13-16 ml eluted) and in the PAPP-A peak (tracks 6-8, 30-32 ml eluted) in the salt gradient.

(c) as for (b) except that the gel was run under non-reducing conditions. Track 1, non-reduced PAPP-A (420 k) and α_2M (400 k approx) after staining with Coomassie blue.

All autoradiograms were exposed for one month at -20°C.

Figure 4:9

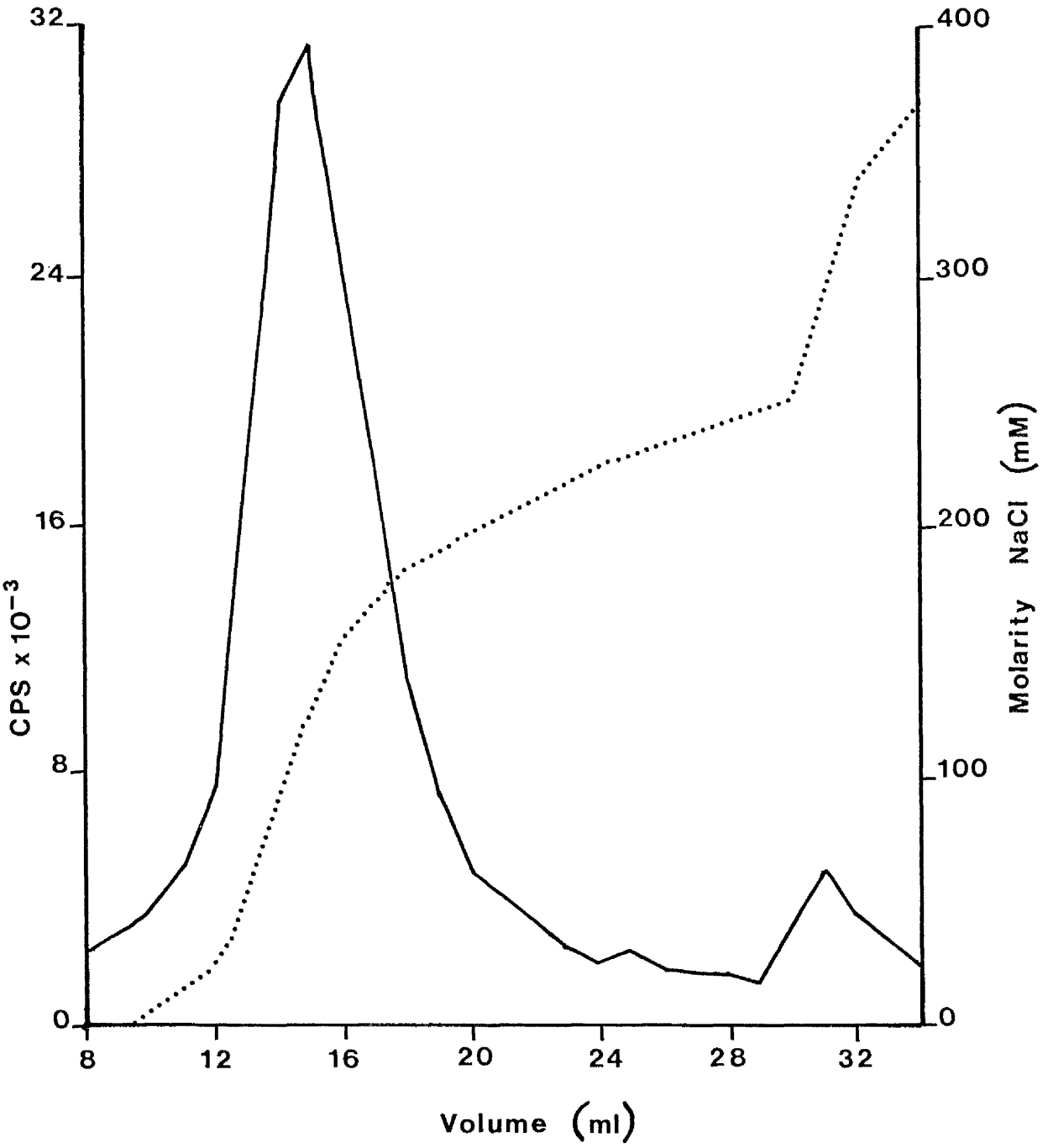

a
1 2 3 4 5 6 7
187k
181k
50k
40k
0 1 3 5 15 30
min
b
1 2 3 4 5 6 7 8
187k
181k
50k
40k
13 14 15 16 30 31 32
ml
c
1 2 3 4 5 6 7 8
420k
400k
13 14 15 16 30 31 32
ml

content. Aliquots of the incubation mix were withdrawn after 0, 1, 3, 5, 15 and 30 min, and the degree of degradation analysed by PAGE, autoradiography and densitometry (Figure 4:9a; Table 4:2). At zero incubation time (i.e. before addition of trypsin), PAPP-A and α_2M bands were clearly visible on the autoradiograph at 187,000 and 181,000 respectively (Figure 4:9a; track 2), as were other lower molecular weight contaminants. As the incubation progressed a reduction in the intensity of the PAPP-A polypeptide at 187,000 was observed, until after 30 min only a very faint band remained. A reduction in the α_2M polypeptide at 181,000 was also apparent from 0-1 min. After 1 min the intensity of the α_2M band remained approximately constant (Table 4:2). Two bands at 50,000 and 40,000 (approx), increased in intensity as the incubation progressed (Figure 4:9a; tracks 3-7). These bands were not present in the PEP starting material (0 min, track 2).

SDS polyacrylamide gel electrophoresis of the fractions of maximum counts after ion exchange chromatography (Figure 4:10; 13-16 ml and 30-32 ml eluted), was carried out under reducing and non-reducing conditions (Figure 4:9b and c). The first peak of radioactivity (Figure 4:10 ; 12-18 ml) contained the ^{125}I-α_2M (Tracks 2-5; Figure 4:9b and c), and other lower molecular weight contaminants. Faint bands were also visible in the fraction of maximum radioactivity from the second peak (Figure 4:10, 29-34 ml eluted; Figure 4:9 b and c, tracks 6-8). These corresponded to reduced and non-reduced PAPP-A respectively. A faint α_2M band was also visible in the peak PAPP-A fractions under non-reducing conditions (Figure 4:9c; track 7), but not in the equivalent fraction under reducing conditions.

Figure 4:10 Ion exchange chromatography of iodinated PEP. The proteins were eluted in an NaCl gradient (- - -) and the whole fraction counts recorded (———), after 30 mins incubation with trypsin.

Figure 4:10


32
24
16
8
0
CPS x 10⁻³
400
300
200
100
0
Molarity NaCl (mM)
8
12
16
20
24
28
32
Volume (ml)

3:4 DISCUSSION

Approach experiments

i) To determine the trypsin binding activity of a partially purified preparation of PAPP-A

In these experiments ^{125}I-labelled trypsin was added to preparations of α_2M and partially purified PAPP-A. Gel filtration was carried out on these mixtures to separate the high molecular weight proteins and ^{125}I-trypsin:protein complexes from albumin and other low molecular weight contaminants, such as unbound ^{125}I-trypsin and free ^{125}I. The PAPP-A fraction used in this experiment was contaminated with albumin (Figure 4:1c), therefore equivalent concentrations were added to the gel filtered solutions of α_2M/trypsin and trypsin to monitor the effect of its presence on the assay.

Two peaks of radioactivity were eluted when PAPP-A/trypsin and trypsin alone were fractionated (Figure 4:2). These peaks coincided (approx) with 2 of the peaks observed after gel filtration of α_2M/trypsin. A peak of radioactivity at 13-19 ml eluted (Figure 4:2) was observed after the α_2M/trypsin fractionation only, and was coincident with the maximum α_2M concentration (17 ml eluted). On fractionation of α_2M/trypsin, 75.5% and 59% of the anti-α_2M and TCA precipitable counts occurred in the α_2M/PAPP-A range, compared with 32% and 16% of the anti-PAPP-A and PTA precipitable counts after gel filtration of PAPP-A/trypsin. As PAPP-A and α_2M eluted in the same range, and from the precipitation results (Figure 4:3a and b), it would appear that PAPP-A does not bind ^{125}I-trypsin in this assay, whereas α_2M does.

The PTA/TCA precipitation results suggest that the albumin present in the gel filtration experiments was associating with the ^{125}I-trypsin. This observation is not unexpected as a characteristic feature of serum albumin is its ability to bind a large number of different biological substances such as long chain fatty acids and steroids, as well as synthetic substances such as dyes (e.g. Cibacron blue) and drugs (Andersson, 1979).

From the results of this experiment the following improvements were suggested. The immune precipitation results (Figure 4:3b) after gel filtration of PAPP-A/trypsin and trypsin showed that the immobilised anti-PAPP-A was precipitating albumin/trypsin complexes and free ^{125}I-trypsin. The specificity of these associations was not determined. However, to eliminate any possible contaminating non-pregnancy specific antibodies (see Sutcliffe et al, 1980a) the PAPP-A antiserum was reabsorbed with adult male serum and a fresh batch of immobilised anti-PAPP-A prepared. Also, as an added precaution, 100 µl of adult male serum were mixed with each ml of immobilised anti-PAPP-A prior to further immune adsorption analyses, to ensure complete blocking of any remaining non-pregnancy specific antibodies. Subsequent immuno-adsorption experiments confirmed the success of these measures (see, for example, Figure 4:8).

As only a single PAPP-A/trypsin binding assay had been carried out and because of the problems encountered with the anti-PAPP-A immunoadsorptions, it was decided to repeat the experiment using a more pure preparation of PAPP-A. Consequently the purification protocol reported in Chapter 3 was developed. However, as the resultant PAPP-A enriched preparation was contaminated with a significant amount of α_2M, the protocol of the trypsin binding assay

had to be changed accordingly, and an ion exchange chromatography step incorporated instead of the gel filtration in order to separate these two proteins.

ii) Validation of the trypsin binding assay incorporating ion exchange chromatography

On ion exchange chromatography (Figure 4:4), α_2M eluted maximally at 120 mM NaCl and PAPP-A at 270 mM, though some α_2M was also detected here. These results are in good agreement with those already published despite the difference in the methods of purification (Bischof, 1979; Sutcliffe et al, 1980b).

The results of the initial experiment using α_2M and ^{125}I-trypsin showed that this was indeed a valid assay for assessing trypsin binding (Figure 4:5). The immobilised anti-α_2M used in this particular experiment was rather 'old' and hence its capacity for precipitation was not very high compared with subsequent immunoadsorption experiments using 'fresh' conjugates (see, for example, Figure 4:7). However, it was apparent that α_2M:trypsin complexes could be precipitated in the expected region, as predicted by AACE, TCA precipitations and previous ion exchange experiments. A few α_2M:trypsin complexes (too few to be detected by AACE), were also eluted in the small peak of radioactivity at 27.5 ml eluted (295 mM NaCl; Figure 4:5), as indicated by immunoadsorption (9.2% c.f. a ^{125}I-trypsin control which gave 6.5% precipitation with anti-α_2M). The reason for the elution of the small amount of α_2M at this molarity is not known, but it was not considered to be a problem, since the immune adsorption values would enable α_2M:trypsin complexes to be distinguished from any PAPP-A:trypsin complexes which would also elute in this range.

Trypsin binding properties of PEP

When mixed with iodinated trypsin, α_2M and PAPP-A eluted from the ion exchange column at substantially different points, which enabled the analyses of their respective trypsin binding capacities. However, as previously observed some α_2M was eluted in the fractions containing PAPP-A (see section (ii) in Approach experiments). In Figure 4:7, there is a close parallelism between the concentration of α_2M and the number of ^{125}I-trypsin counts in the fractions. Further, the majority of these counts were precipitated with anti-α_2M (Figure 4:7). This indicated that these fractions contained α_2M:trypsin complexes. In the fractions containing PAPP-A less than 10% of the counts were precipitable with anti-PAPP-A, a value comparable with the precipitation control (Figure 4:8). Thus there was no evidence for trypsin being bound to PAPP-A.

The effect of trypsin on the PAPP-A enriched product

PAPP-A enriched product (PEP) was incubated with a 3:1 molar ratio of trypsin to α_2M content, to ensure that more than enough trypsin was available to saturate all the α_2M binding sites and to interact with PAPP-A (α_2M binds 1-2 molecules of trypsin; Starkey & Barrett, 1977). The results from autoradiography and densitometry of the aliquots withdrawn during the incubation period, suggest that PAPP-A was being degraded by the trypsin (Figure 4:9a and Table 4:2). As the incubation progressed the band at 187,000 decreased in intensity (Table 4:2), while bands at 40,000 and 50,000 (thought to be tryptic fragments; Figure 4:9a), increased. The band at 181,000, corresponding to α_2M, showed an initial decrease in intensity between 0-1 min

(Table 4:2). On reduction α_2M:trypsin complexes are broken down into fragments of 85,000 mol wt (see Hall and Roberts, 1978), due to the tryptic cleavage of the 'bait' region in α_2M (see Chapter 1, section 4:1). During the remainder of the incubation period the intensity of this band remained approximately constant (Table 4:2). Starkey and Barrett (1977) noted that irrespective of the ratio of trypsin to α_2M, the binding reaction was complete in one minute and no further change occurred over a subsequent 30 min incubation. The results obtained here are also in keeping with these observations. The trypsin not bound by α_2M continued to degrade the PAPP-A throughout the incubation period. The results from the ion exchange chromatography showed that α_2M (α_2M:trypsin complexes and free α_2M), was present in the incubation mix, together with some native PAPP-A (Figure 4:9b and c). Both proteins eluted at the expected molarities.

It is apparent from the autoradiography and densitometric analyses (Figure 4:9a and Table 4:2), that the α_2M and PAPP-A in PEP show disproportionate radiolabelling, with the amount of ^{125}I-labelled α_2M being 3.4 times greater than ^{125}I-labelled PAPP-A (Table 4:2). The reason for this difference is not clear. On ion exchange of ^{125}I-PEP, after the 30 min trypsin incubation (Figure 4:10), the number of counts in the fraction of maximum radioactivity in the 'α_2M' peak (15 ml eluted; 31.4×10^3 cps) was 6.4 times greater than the number of counts in the fraction of maximum radioactivity in the PAPP-A peak (31 ml eluted; 4.9×10^3cps). The disproportionate radiolabelling of these 2 proteins in PEP cannot account for the total difference observed in the peak heights. Faint bands at approximately 40,000 and 50,000 (thought to be tryptic fragments of PAPP-A), are apparent in

this region (tracks 2-5; Figure 4:9b), but not in the corresponding PAPP-A region (tracks 6-8). This would seem to suggest that radiolabelled, tryptic fragments of PAPP-A are eluting in the 'α_2M' range. Together with the disproportionate radiolabelling of PEP, this observation would seem to account for the difference in the amount of radioactivity in the α_2M and PAPP-A peaks after ion exchange chromatography.

Previously, in the trypsin binding experiments, the molar concentrations of α_2M in the PEP sample were in excess of the trypsin molar concentrations. Therefore, it could be argued that the α_2M was binding to the majority of the trypsin, leaving only a small amount unbound to interact with PAPP-A and hence no PAPP-A:trypsin complexes were detected. However, in the incubation studies described above, conditions were such that the molar concentrations of trypsin exceeded those of α_2M and PAPP-A (1.5:1). Hence sufficient trypsin was present to saturate the α_2M binding sites and interact with PAPP-A too. Even under these conditions, PAPP-A:trypsin binding was not detected, and PAPP-A remained susceptible to tryptic digestion throughout the incubation period. These observations, together with the ion exchange chromatography and gel filtration results, indicate that PAPP-A, unlike α_2M, does not bind trypsin.

4. PLASMIN BINDING PROPERTIES OF PEP

4:1 INTRODUCTION

α_2M is a major inhibitor of plasmin in blood (Starkey and Barrett, 1977), and the experiments of Bischof (1979) have claimed

that PAPP-A also inhibits plasmin. The plasmin binding properties of the PAPP-A enriched preparation, purified as described in Chapter 3, was therefore investigated. As in the trypsin binding assay, ^{125}I-plasmin was added to PEP. The different protein species were subsequently resolved by gel filtration and ion exchange chromatography.

4:2 MATERIALS AND METHODS

MATERIALS

Plasminogen and fibrinogen (peak 1), were a gift from Dr. Graham Kemp, St. Andrews University. Streptokinase and a further source of fibrinogen (fraction 1), were obtained from Sigma. Gelatin was purchased from BDH and EDTA from Koch Light Laboratories Ltd. Plasminogen (in 50 mM Tris, 12 mM NaCl pH 7.4), was iodinated by the lactoperoxidase method (Bolton, 1977).

METHODS

Approach Experiments

(i) To establish the activity of the ^{125}I-plasmin

Non-iodinated plasminogen (17.5 µg) in 100 µl of 50 mM Tris, 12 mM NaCl pH 7.4 and lactoperoxidase iodinated plasminogen (1.4 µg; 19 µCi/µg) in 200 µl of 50 mM potassium phosphate, 0.2% gelatin pH 7.5, were activated on incubation with 80 units of Streptokinase (2,000 units/ml) for 20 min at 37^{o}C. The fibrinogenolytic activity of the plasmin produced was determined as follows. An aliquot (25 µl) of the activated plasminogen mixtures was incubated for 3 h at 37^{o}C with 0.1 mg of fibrinogen (1 mg/ml in 5 mM EDTA). SDS polyacrylamide gels

(5-15%) and autoradiography were used to detect the presence of iodinated and enzymatically active plasmin, as judged from fibrinogen degradation.

(ii) Separation of bound and unbound components by gel filtration

A 1 ml sample of dialysed PEP (83 μg PAPP-A; 138 μg α_2M) was mixed with 100 μl of iodinated plasminogen (0.5 μg; 19 μCi/μg) and 1 mg of BSA. This solution was applied to a gel filtration column of Sepharose 4B (0.9 x 57 cm), equilibrated with buffer A at 4°C. The sample was eluted for 7 h at 5 ml/hr with the same buffer. The points of elution of PAPP-A and α_2M were determined by AACE. BSA elution was monitored by PAGE and a TCA precipitation was used to locate the ^{125}I-plasminogen.

Plasmin binding properties of PEP

^{125}I-labelled plasminogen (1.4 μg; 19 μCi/μg) were activated with Streptokinase (see section (i) in Approach experiments). Half of the reaction mix (120 μl) was subsequently incubated with 1 ml of dialysed PEP containing 80 μg of PAPP-A and 140 μg of α_2M, for 1 h at 37°C. As an activity control, the fibrinogen degradative properties of the ^{125}I-plasmin were determined by PAGE (see section (i) in Approach experiments).

The mixture of ^{125}I-plasmin and PEP was applied to a Sepharose 4B gel filtration column (see section (ii) in Approach experiments), linked at its lower end to a column of Sepharose DEAE CL6B (0.9 x 0.8 cm). After the protein mixture had been applied, the columns were washed with 27 ml of buffer A at 5 ml/h. The two columns were then disconnected and an 18 ml salt gradient (0-0.4M NaCl in buffer A)

Figure 4:11 Analysis of the fibrinolytic properties of ^{125}I-plasmin. Fibrinogen breakdown was observed on 6-12% SDS-polyacrylamide gels. Track 1, molecular weight standards; track 2, 100 μg of fibrinogen after incubation with 0.2 μg of Streptokinase activated ^{125}I-plasminogen; track 3, 100 μg of fibrinogen after incubation with 3 μg of streptokinase activated plasminogen; track 4, 100 μg of fibrinogen after incubation with 40 μ of streptokinase.

Figure 4:12 An autoradiogram of an SDS-PAGE gel under reducing conditions showing; ^{125}I-plasminogen (track 1); 0.2μg streptokinase activated ^{125}I-plasminogen (track 2); and 0.2 μg of streptokinase activated ^{125}I-plasminogen after incubation with 100 μg of fibrinogen (track 3). Exposure 6 days at room temperature.

Figure 4:11

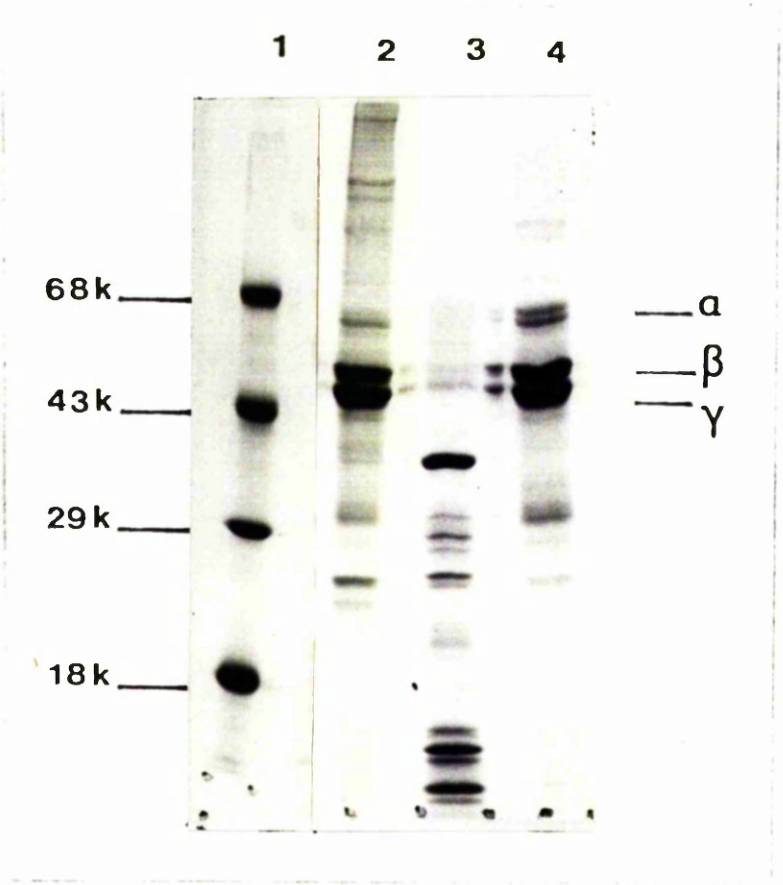
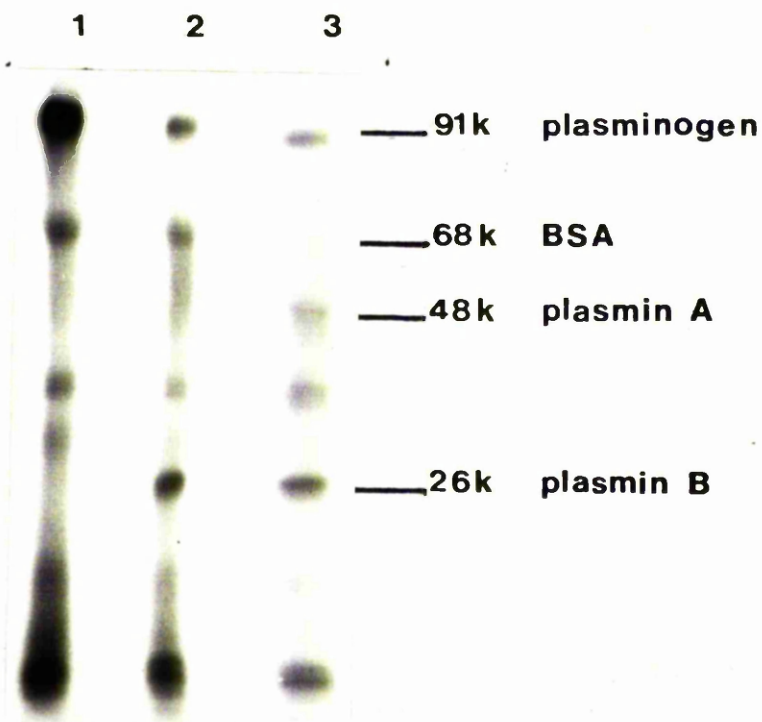


Figure 4:12



applied to the ion exchange column (see General methods section of this chapter). Fractions were analysed by immune adsorption. Their radioactivity and conductivity were also measured, together with the elution points of PAPP-A and α_2M.

4:3 RESULTS

Approach Experiments

(i) To establish the activity of the ^{125}I-plasmin

Before the ^{125}I-labelled plasmin was used in protein binding assays, controls were made to test its enzymatic activity. Fibrinogen breakdown was observed on SDS polyacrylamide gels after incubation with activated ^{125}I-plasminogen (track 2; Figure 4:11). Compared with the control fibrinogen (track 4), there was a reduction in the fibrinogen α doublet and some intensification of low molecular weight bands (track 2). This experiment was carried out with limiting quantities of ^{125}I-plasmin. When unlabelled plasmin was added in excess, the degradation of fibrinogen approached completion (track 3). On autoradiography of ^{125}I-plasminogen, an intense band was observed at 91,000, corresponding to radiolabelled plasminogen (track 1, Figure 4:12). With streptokinase activation the intensity of this band was reduced, and two new bands appeared at 48,000 and 26,000 respectively (tracks 2 and 3).

(ii) Separation of bound and unbound components by gel filtration

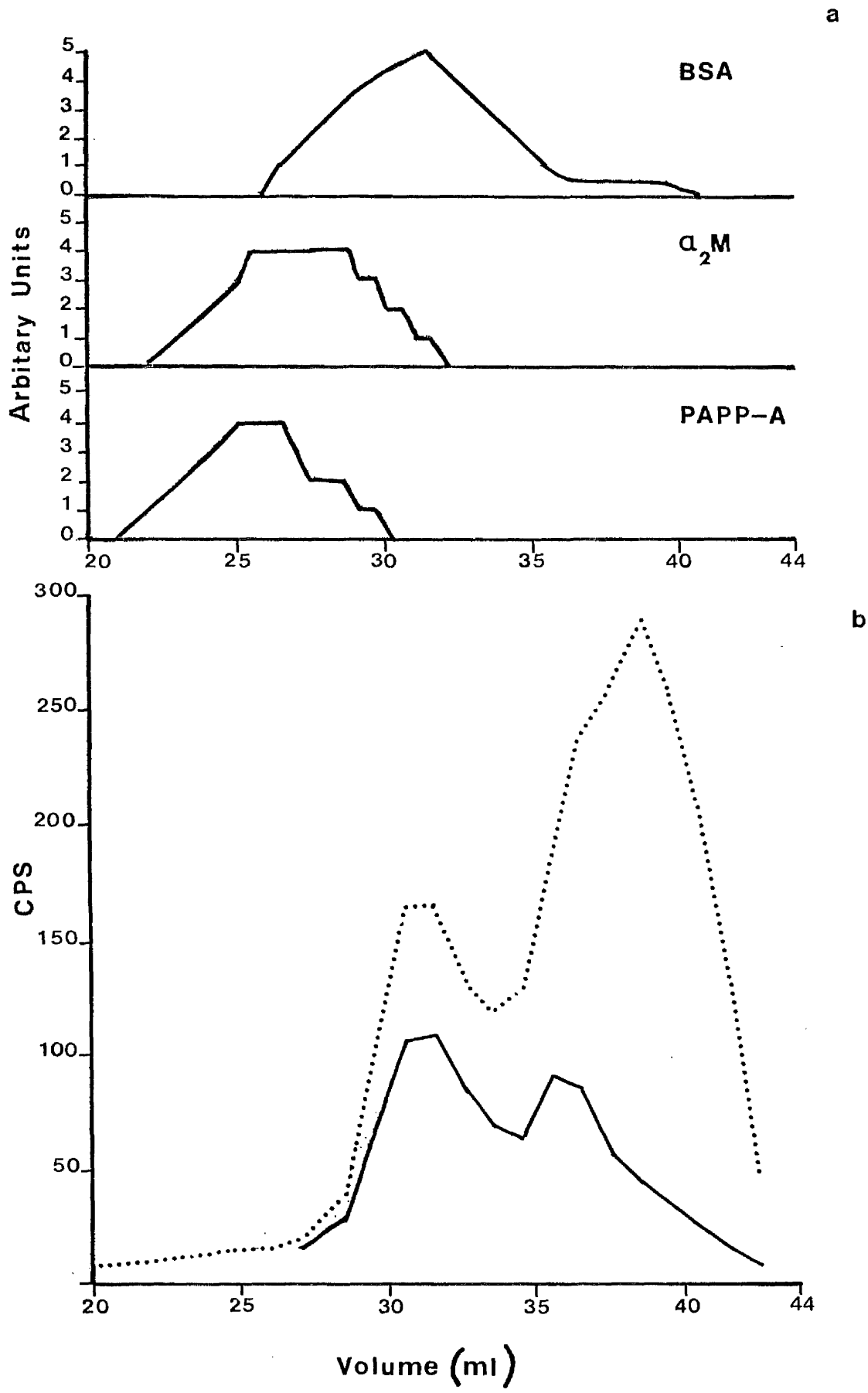
Gel filtration was investigated as a possible method for the separation of plasminogen and unbound plasmin from bound plasmin, α_2M and PAPP-A. A solution of PEP, ^{125}I-plasminogen and BSA was fractionated on a column of Sepharose 4B, and the points of elution of these

Figure 4:13 Gel filtration of a solution of PEP, ^{125}I-plasminogen and BSA on a column of Sepharose 4B.

(a) Elution of BSA (upper panel) was monitored on SDS-PAGE and the intensity of staining of the bands with Coomassie blue plotted. α_2M and PAPP-A elution (middle and lower panels respectively) were monitored on AACE and rocket height plotted.

(b) The elution point of ^{125}I-plasminogen was monitored by determining the elution of the TCA precipitable counts (———). The counts in the unprecipitated fractions were also recorded (- - -).

Figure 4:13


a
BSA
α_2M
PAPP-A
Arbitary Units
0
1
2
3
4
5
20
25
30
35
40
44
b
CPS
50
100
150
200
250
300
Volume (ml)

proteins determined (Figure 4:13). α_2M and PAPP-A were detected from 21-32 ml eluted (maximum PAPP-A at 25-26.5 ml and maximum α_2M at 25.5 - 28.5 ml). BSA began to elute after 26 ml, reaching a maximum at 31.5 ml, and ^{125}I-plasminogen began to elute after 27 ml, reaching its maximum at 31 ml.

Plasmin binding properties of PEP

In this experiment ^{125}I-labelled plasmin was formed by activating ^{125}I-plasminogen with streptokinase and the mixture added to the PEP. This preparation was subsequently fractionated by gel filtration to remove the free plasminogen and plasmin from any bound plasmin, α_2M and PAPP-A. Ion exchange chromatography was used to separate α_2M from the PAPP-A and to resolve any high molecular weight plasmin complexes. Initially, separate ion exchange fractionations were carried out on ^{125}I-plasminogen and streptokinase activated ^{125}I-plasminogen, and their elution maxima were located at 150 mM and 90 mM NaCl respectively.

Radiolabelled plasmin was then incubated with PEP and the mixture fractionated on Sepharoses 4B and DEAE CL6B. Two peaks of radioactivity were observed (Figure 4:14) in the eluate collected from the ion exchange column, after it had been disconnected from the column of Sepharose 4B. The first peak was largest and eluted maximally at 130 mM NaCl. The second peak began to elute at 265 mM NaCl, with the maximum counts at 325 mM NaCl. AACE always showed that most α_2M was eluted between 42-47 ml (Figure 4:15), which corresponded to the fractions making up the largest peak of radioactive plasmin. PAPP-A was eluted between 56-59 ml and some α_2M was also detected there (Figure 4:15).

Figure 4:14 Ion exchange chromatography of a PAPP-A enriched fraction after incubation with ^{125}I-plasmin. The proteins were eluted with an NaCl gradient (- - -) and whole fraction counts recorded (●——●).

Figure 4:15 Immunoadsorption with immobilised anti-α_2M and anti-PAPP-A. Lower panel: ^{125}I-plasmin counts precipitated by immobilised anti-α_2M (△——△) and anti-PAPP-A (□——□). Upper panel: concentrations of α_2M (△——△) and PAPP-A (□——□) determined by AACE.

Figure 4:14

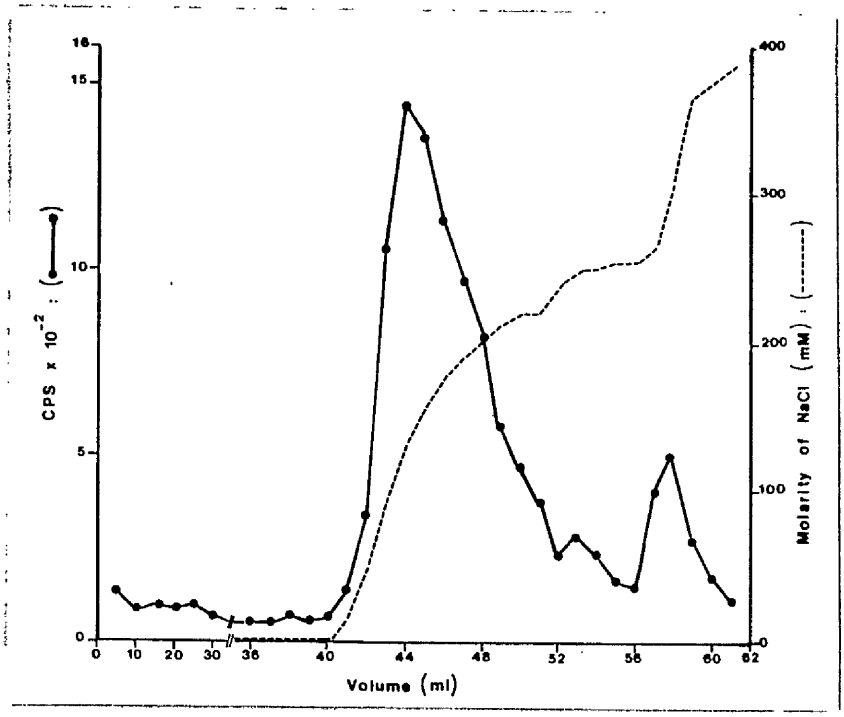


Figure 4:15

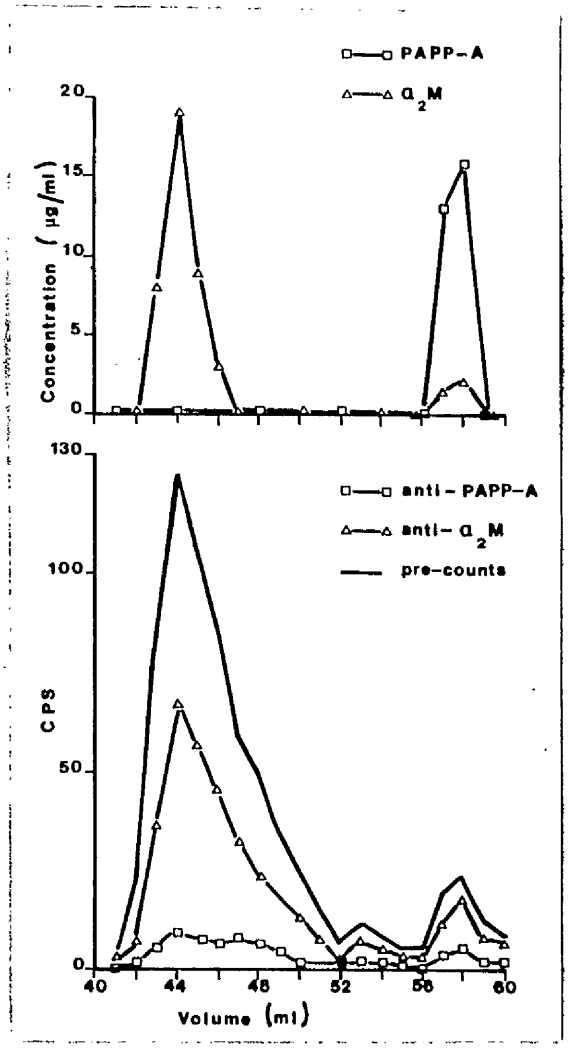
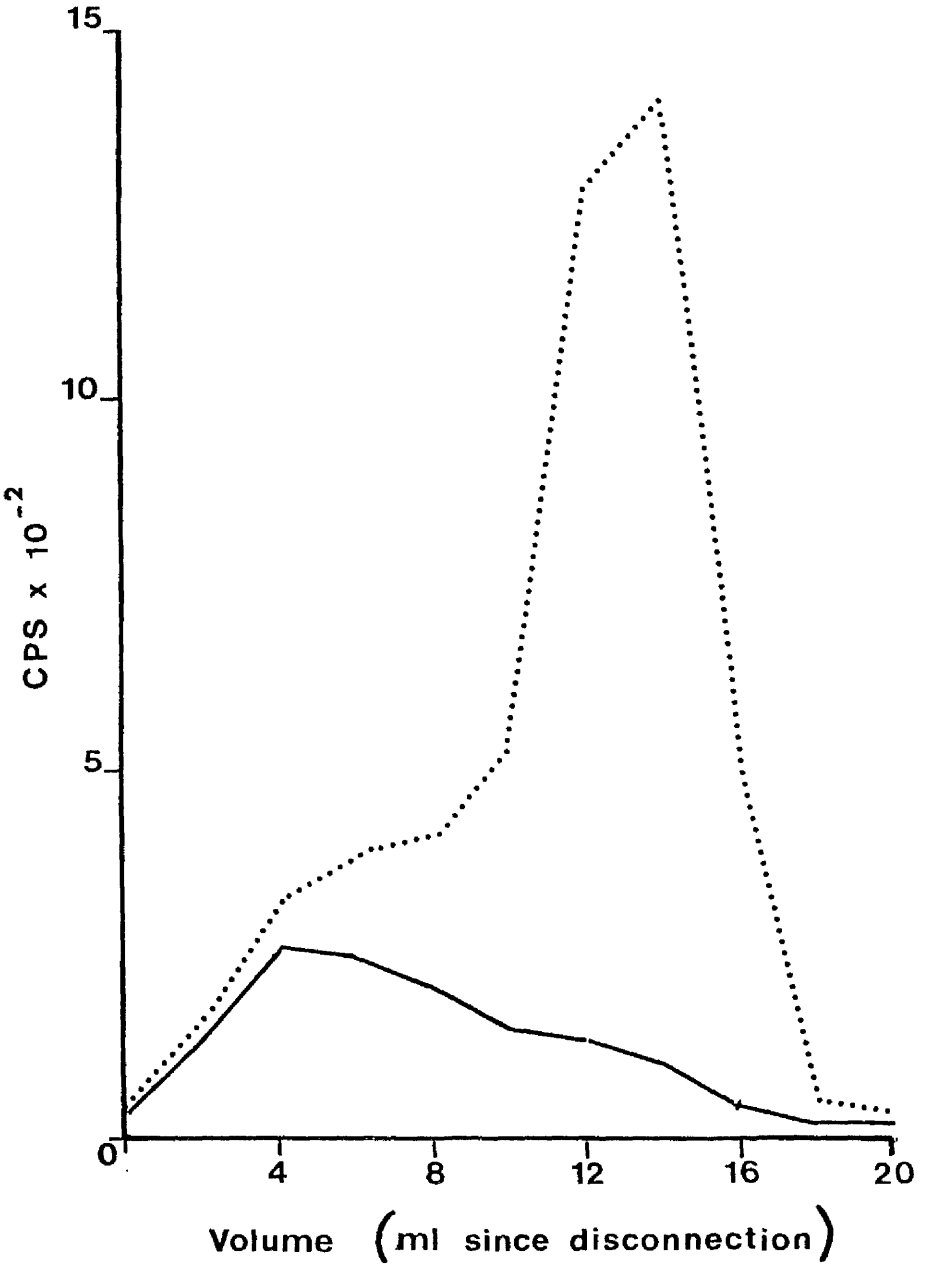


TABLE 4:3 ^{125}I-plasmin counts binding to α_2M in PEP after ion exchange chromatography

Volume eluted (ml)	Total α_2M (µg)	Total PAPP-A (µg)	Total cps	cps per µg α_2M
43-46	39	not detected	3960	101.5
57-58	3.5	29	430	122.9

Figure 4:16 TCA precipitable counts (———) in 100 µl aliquots of the fractions collected from the column of Sepharose 4B after disconnection from the ion-exchange column, during the fractionation of the ^{125}I-plasmin/PEP mix. ^{125}I counts in 100 µl unprecipitated fractions were also recorded (- - -).

Figure 4:16



In the fraction of maximum radioactivity (44 ml eluted), 55% of the ^{125}I-plasmin counts were precipitable by the immobilised anti-α_2M compared with 7% by the anti-PAPP-A. Of the radioactivity in the smaller peak (at 58 ml), 75% of the counts were precipitable by the anti-α_2M and 21% by the anti-PAPP-A (Figure 4:15). A further 2 peaks of radioactivity were observed in the fractions collected from the column Sepharose 4B, after disconnection from the ion exchange column (Figure 4:16). The first peak which eluted between 0-4 ml after disconnection, was smallest, and 84% of the counts in this peak were TCA precipitable. In the second peak (4-18 ml), 8% of the counts were TCA precipitable. The 2 peaks were therefore judged to be radiolabelled plasminogen plus plasmin, and free iodine respectively.

4:4 DISCUSSION

Approach Experiments

Streptokinase activation of iodinated plasminogen produced iodinated and enzymatically active plasmin. On autoradiography of an electrophoresed (SDS PAGE) sample of labelled plasminogen (Chloramine T iodinated), the major band of radioactivity was detected at 91,000 mol wt (track 1, Figure 4:12), which corresponded to the expected molecular weight for plasminogen (83,800-93,000; Robbins, 1978). On streptokinase activation of ^{125}I-plasminogen the intensity of the plasminogen band was reduced and two new bands were observed at 48,000 and 26,000 mol wt respectively (track 3; Figure 4:12). When plasmin is reduced (see Robbins, 1978), 2 chains are formed, a heavy or A chain (48,800-69,000 mol wt), and a light or B chain (24,000-26,000 mol wt). The 2 additional bands observed on activation of ^{125}I-plasminogen were

therefore judged to be the A and B chain derivatives of iodinated plasmin. The reduction in the α doublet of fibrinogen on SDS-PAGE (track 2; Figure 4:11) after incubation with this ^{125}I-plasmin demonstrated that it was also enzymatically active.

The plasminogen used in these experiments was iodinated by the lactoperoxidase method (Bolton, 1977). Chloramine T iodinated plasminogen was initially used, but the BSA present in the iodination column buffer was found to have a molecular weight similar to the α doublet of fibrinogen. This made it difficult to detect any reduction in these bands due to plasmin degradation.

The results of the gel filtration experiment (Figure 4:13), showed that it was possible to separate plasminogen and any unbound plasmin from the high molecular weight proteins. BSA (68,000 mol wt) was used as an approximate molecular weight marker for plasmin (75,000-81,000 mol wt; Robbins, 1978). As the elution ranges for these proteins were not discrete, a 'cut-off' point after the elution of 27 ml was arbitrarily decided upon. Most of the PAPP-A, and at least half of the α_2M were eluted before 27 ml (Figure 4:13), whereas the majority of the BSA and plasminogen were eluted after this point. Originally, a column of lysine Sepharose (which binds plasminogen) had been used to remove the plasminogen from the PEP/plasmin preparations (instead of gel filtration). However, it appeared that α_2M and possibly PAPP-A were also binding to this column (data not shown). For this reason it was decided not to include the lysine Sepharose step. Even though the activated plasminogen mixture could have been treated this way prior to incubation with PEP, unbound plasmin and possibly some plasminogen would still have been present in the ion

exchanged preparation. The subsequently included gel filtration step was able to separate both of these proteins from the α_2M, PAPP-A and plasmin complexes.

Plasmin binding properties of PEP

In the presence of iodinated plasmin, α_2M and PAPP-A eluted from the ion exchange column at substantially different points, which enabled the analyses of their respectively plasmin binding properties (Figure 4:15). The results with immobilised antisera are comparable to those in the trypsin binding assays (Figures 4:7 and 8). The elution profile of α_2M closely followed that of the radioactivity and on immunoadsorption with anti-α_2M, the fraction containing the greatest amount of α_2M, precipitated the most ^{125}I-plasmin. From the observed number of counts and the concentration of α_2M between 43-46 ml eluted (Figure 4:15), it is possible to calculate the number of ^{125}I-plasmin counts per µg of α_2M (101.5 cps/µg Table 4:3). If PAPP-A were to bind ^{125}I-plasmin in a manner similar to α_2M, this figure can then be used to calculate the expected number of counts in the PAPP-A range (Figure 4:15). Allowing for the differences in their molecular weights, the expected number of ^{125}I-plasmin counts per µg of PAPP-A is calculated to be 101.5 x 181,000/187,000 = 98.2 cps/µg. Since 29 µg of PAPP-A were eluted between 57-58 ml (Figure 4:15, Table 4:3), the number of ^{125}I-plasmin counts expected to bind to PAPP-A is 29 x 98.2 = 2848 cps. However, the observed number of counts between 57-58 ml is 430 cps of which a substantial fraction (67%) are immune precipitable with anti-α_2M. Indeed, the plasmin binding activity of α_2M in the fractions between 43-46 ml (Table 4:3), suggests that the 3.5 µg of

α_2M in the fractions at 57-58 ml would be expected to bind 101.5 x 3.5 = 355.25 cps, or 82.6% of the total plasmin counts in these fractions. On these calculations the remaining 75 cps are not bound to α_2M and they could, therefore, be available to bind PAPP-A. However, these counts are only 2.6% of the 2848 cps expected if PAPP-A were to bind plasmin in a manner similar to that of α_2M. It therefore seems improbable that PAPP-A binds ^{125}I-plasmin to any significant degree.

The present results are not consistent with the view that PAPP-A inhibits plasmin in an homologous way to α_2M (Bischof, 1979, 1980). The reason for this inconsistency is not clear. Bischof's results (1979, 1980) are based on a caseinolytic activity assay, but it is improbable that PAPP-A could inhibit the enzyme without binding strongly to it. The disadvantage with the activity assay is that it does not preclude the possibility that the inhibitory activity is due to a component other than PAPP-A. Both Bischof (1979) and I have found it difficult to remove contaminating α_2M from preparations of PAPP-A. This stresses the importance of binding assays and of using sensitive methods to assess the purity of preparations. Tube gel electrophoresis and immunodiffusion (Bischof, 1979, 1981), may not be suitably sensitive. It is not possible to be certain that the present purification scheme has not denatured or inactivated the PAPP-A in the PEP. However, the α_2M in PEP was active (as it bound both trypsin and plasmin), and the more severe conditions used in previous methods were avoided. This would seem to suggest that the PAPP-A was also in a potentially active state.

5. COMPLEMENT INHIBITORY PROPERTIES OF PEP

5:1 INTRODUCTION

An immunosuppressive role has been put forward for PAPP-A as it is reported to be an inhibitor of complement mediated red cell lysis and lymphocyte transformation (Bischof, 1981; Bischof et al, 1982b). A complement fixation assay was carried out using PEP as a source of PAPP-A. As α_2M has no effect on complement mediated red cell lysis (James, 1980), it was judged that the presence of this protein in PEP was unlikely to interfere with the complement fixation assay.

This data was obtained by Jack Hunter, an assistant to my supervisor, using samples of PEP I purified using the method developed in Chapter 3. His contribution is gratefully acknowledge, as is some technical help from Isabel Edwards during the large scale purification of PEP.

5:2 MATERIALS AND METHODS

MATERIALS

Sheep red cells were obtained from 50% whole blood, 50% Alsevers solution (Gibco, Europe). Guinea pig complement (CT01/02) and rabbit haemolytic serum for sheep cells were obtained from Wellcome.

METHODS

Complement fixation assays were carried out using the haemolytic assay of Mayer (1961). Typically, 10^8 sheep erythrocytes were used in a final volume of 1.5 ml of complement fixation diluent containing 15 µl of 1/2000 dilution of rabbit anti-sheep haemolysin. After incubation at 37°C for 15 min, 200 µl of complement was added, diluted 1/200 (guinea pig complement) or 1/50 (adult human male complement). Both

Figure 4:17 Titration of complement in an haemolysis assay. Various dilutions of complement were added to sensitised sheep red cells and lysis was estimated by absorbance at 415 nm. The values are from two different samples of guinea pig complement.

Figure 4:17

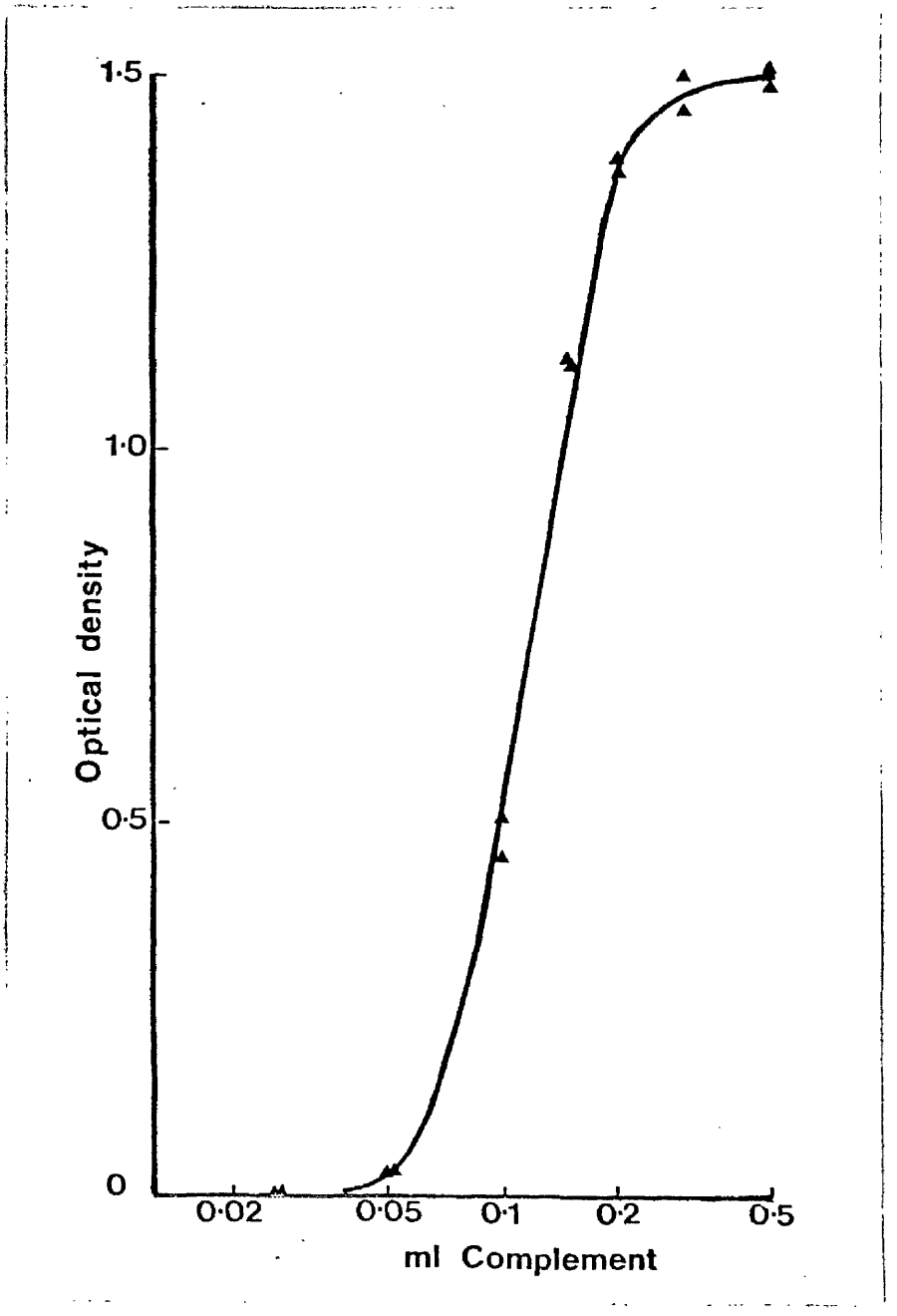


TABLE 4:4 Effect of PAPP-A enriched fractions on complement fixation

Complement source	Protein tested	E^{415}			Mean lysis value
Human serum	None	0.93	0.94	0.96	0.94
	*5 μg PAPP-A + 7 μg α_2M	0.99	1.00		
Guinea Pig serum	None	1.27	1.27	1.28	1.27
	*2 μg PAPP-A + 4 μg α_2M	1.30	1.30	1.30	1.30
	*2 μg PAPP-A + 5 μg α_2M	1.29	1.27	1.28	1.28
	*10 μg PAPP-A + 20 μg α_2M	1.32	1.32	1.34	1.33
	*10 μg PAPP-A + 22 μg α_2M	1.28	1.29	1.29	1.29
	2 μg α_2M	1.27	1.28	1.30	1.28
	10 μg α_2M	1.26	1.28	1.30	1.28
	2 μg αHSA	1.31	1.31	1.32	1.31
	10 μg αHSA	1.31	1.32	1.33	1.32

*PAPP-A enriched fraction.

sources of complement were adsorbed with sheep red blood cells at 20°C before use. The tubes were stood at 37°C for 30 min, centrifuged at 3,000 g for 10 min and the optical density of the supernatant measured at 415 nm. For complement inhibition assays, the quantity of complement was adjusted to limiting concentrations. All test proteins used in inhibition studies had first been dialysed against complement fixation diluent.

5:3 RESULTS

In the experiments to test for possible inhibitory effects of PAPP-A on complement-induced red cell lysis, it was necessary to determine that a limiting concentration of complement was present in the assay. Figure 4:17 shows a typical complement titration curve for this purpose. Table 4:4 shows the level of haemolysis with limiting quantities of guinea pig or human complement in the presence or absence of various preparations of α_2M and PAPP-A. The PAPP-A used in these assays was from a sample of PEP. No evidence of inhibition of complement-mediated red cell lysis was detected for any preparation containing either of these two proteins.

5:4 DISCUSSION

In repeated assays no evidence could be found that PAPP-A is able to inhibit the lysis of sheep red blood cells by guinea pig or human complement. The finding that α_2M is not inhibitory is consistent with the previous knowledge (see James, 1980), and suggests that were PAPP-A homologous to α_2M, it would not necessarily be expected to have anti-complementary properties. In these experiments the PAPP-A concentrations varied from 1.3 μg/ml to 6.7 μg/ml in the fixation assay,

that is some 15-60 fold less than the mean concentration of PAPP-A in maternal serum at term (Sutcliffe et al, 1980a; 1982b). However, as the source of complement itself was diluted by 1/1500 to 1/375 and was present in limiting concentrations in the assay, this was not considered to be an objection to the experiment.

These findings on PAPP-A cannot be reconciled with those of Bischof (1981) who reported anti-complementary activity for PAPP-A. Indeed no such activity was found in the PAPP-A made by Sutcliffe et al (1980b), though Bischof (1981) detected complement-inhibitory activity in this material. This contradiction suggested that this preparation of PAPP-A (Sutcliffe et al, 1980b) may have been in some way unstable or subject to progressive inactivation, possibly due to the chaotropic conditions used for its purification. These considerations prompted the development of the method of purification described in Chapter 3.

6. FIBRONECTIN AND GELATIN BINDING PROPERTIES OF PEP

6:1 INTRODUCTION

Fibronectin, also known by its earlier name of cold insoluble globulin, is a high molecular weight glycoprotein of approximately 450,000 (depending on the source), found in a soluble form in blood and other body fluids (e.g. amniotic fluid). For recent reviews see Mosher (1980) and Ruoslahti et al (1982a and b) and references therein. Insoluble forms of this protein also exist, which are located in connective tissue, at the cell surface and in basement membranes. The differences observed among fibronectins from different sources are mainly due to variations in glycosylation of the molecule, with the

polypeptide portions being approximately the same (Ruoslahti et al, 1982a). Fibronectins appear to exist as disulphide bonded multimeric aggregates at the cell surface (which contributes to their insolubility), whereas soluble fibronectin mainly consists of dimers. These different forms of fibronectin are involved in a variety of seemingly unrelated events ranging from cell surface phenomena to blood coagulation. A summary of the major fibronectin interactions are presented in Table 4:5.

The distribution of PAPP-A in placental tissue has recently been studied using immunoperoxidase staining (Sutcliffe, Khalef and Horne, in preparation). PAPP-A was identified in Nitabuch's layer (at the utero-placental interface), chorionic plate, placental septae, syncytiotrophoblasts and cytotrophoblastic cell islands (see Table 1:3), which are some of the principal zones for the deposition of fibrin and the related fibrinoid material (Boyd and Hamilton, 1970). A similar distribution pattern was also observed by these workers with immunoperoxidase staining for fibronectin, particularly in regions of decidua containing reticular fibres and fibrinoid material.

Nitabuch's layer, also known as Nitabuch's membrane or striae (Boyd and Hamilton, 1970), is a layer of extracellular fibrinoid material containing fibrin/fibrinogen type proteins and a variety of other polypeptides (Sutcliffe et al, 1982a). It forms a boundary very early on in pregnancy, between the fetal and maternal tissues (Boyd and Hamilton, 1970), and is therefore of interest since it might be involved in the maintenance of the fetal allograft. On culturing this layer for one week in the presence of ^{35}S-methionine, radioactive PAPP-A and fibronectin were identified in the culture supernatant (Khalef, Buultjens and Sutcliffe, in preparation).

TABLE 4:5 Fibronectin interactions[a]

Macromolecule	Interaction with fibronectin
Collagen	Binding: possibly forms extracellular matrices.
Gelatin	Binding: stronger than collagen.
Fibrinogen	Binding: weak.
Fibrin	Binding: weak, but is cross-linked to fibronectin by transglutaminase, therefore both proteins are incorporated into clots. May provide a temporary adhesive matrix for cells to grow into a wound.
Glycosaminoglycans (including heparin)	Binding: thought to be involved in the formation of the extracellular matrix. Heparin binding enhances collagen binding to fibronectin.
Thrombin	Fibronectin acts as a substrate.
Plasmin	Fibronectin acts as a substrate
Transglutaminase (Factor XIII)	Cross-links fibronectin and fibrin.
Urokinase	Fibronectin enhances activation of plasminogen by urokinase.
DNA	Binding
Actin	Binding
Gangliosides	Possible cell surface receptor on eukaryotic cells that fibronectin binds to, mediating cellular adhesion to the extracellular matrix.
Compounds of bacterial cell walls	Fibronectin is involved in bacterial opsonization.
Tissue debris	Fibronectin may serve as a non-specific opsonin promoting removal of insoluble tissue debris by the reticular endothelial system.

(a) Data from Mosher (1982) and Ruoslahti *et al* (1982a & b).

As PAPP-A and fibronectin seem to have a similar distribution in placental tissue and are both associated with reticular fibres around decidual cells, it was considered possible that they might bind to each other or to some common substrate. To investigate this hypothesis, the binding properties of PAPP-A to fibronectin, gelatin and gelatin:fibronectin complexes were investigated.

6:2 MATERIALS AND METHODS

MATERIALS

All solutions, glassware and dissecting instruments were sterilised prior to use and a sterile technique maintained throughout the tissue culturing experiments. Tissue culturing media were made up as follows, using materials obtained from Flow Laboratories or Gibco Bio-Cult Ltd. Complete medium: to 150 ml of sterile H_2O the following were added; 18 ml, 10 x Eagle's medium (Glasgow modification); 6.7 ml, 7.5% sodium bicarbonate; 2 ml, 200 mM glutamine; 20 ml, Fetal Bovine serum (FBS; mycoplasma and virus screened); 2 ml, 100 mM sodium pyruvate; 2 ml, 100 x non-essential amino acids; 2 ml 100 x Penicillin/streptomycin. Methionine minus medium: as for complete medium, except that 10 x Eagle's medium (Glasgow modification) minus methionine was used and the FBS was omitted. Culture medium: (2% FBS); was made by diluting complete medium (10% FBS) 1 in 5 with methionine minus medium (0% FBS). ^{35}S-methionine (Amersham) was diluted with culture medium to give a specific activity of 10 μCi/μl.

Triton-X 100 and gelatin were obtained from BDH, and fibrin clot was prepared as described in Sutcliffe *et al* (1982a).

Scintillation counts were measured in a SL30 Liquid Scintillation

Spectrometer (Intertechnique). All samples were prepared in a 10 ml volume of high efficiency emulsifier cocktail (Biofluor, New England Nuclear).

METHODS

Culturing Nitabuch's layer

The uteroplacental surface of a freshly delivered placenta (less than 1 h old), was washed free of contaminating blood with PBS. Samples of Nitabuch's layer, which was observed as a colourless, translucent material on the surface, were removed by blunt dissection, and washed with methionine minus medium. Similarly, a piece of tissue from the villous placenta was also removed and this was used as a control in the culturing experiments. The samples of Nitabuch's layer and villous placenta were cut into pieces about 2-3 mm square by 1 mm thick and incubated (individually) in 0.5 ml of culture medium, to which 50 μCi of ^{35}S-methionine were added. After 24 h at 37^{o}C, the supernatants were harvested. Aliquots were analysed on SDS polyacrylamide gels (5-15%) and by autoradiography. A TCA precipitation was also carried out on these supernatants.

Further samples of Nitabuch's layer were removed from the fresh placenta by scraping the utero-placental surface with a scalpel blade, and washed by centrifugation in PBS (5,000 g for 5 min). The resulting pellets were washed by vigorous agitation in a solution of 0.5% (v/v) Triton-X 100 in PBS (for 3 min using a Fison's Whirlimixer), and by centrifugation (5,000 g for 5 min). This procedure was repeated until all traces of blood were removed from the pellets and the discarded supernatants. A 0.5 mm square piece of pellet was then homogenised in

500 µl of 1% (w/v) SDS. Aliquots of the homogenates (5, 25 and 50 µl) were reduced with 1% (v/v) β-mercaptoethanol and analysed on SDS polyacrylamide gels (5½-12%).

Fibronectin binding assay

A sample of the supernatant from cultured Nitabuch's layer (300 µl) containing ^{35}S-fibronectin, was incubated for 1 h at 37°C with 1 ml of dialysed PEP (89 µg PAPP-A; 130 µg α_2M), plus 100 µl of lactoperoxidase iodinated PEP (0.9 µg PAPP-A, 2.7 µg α_2M; 11 µCi/µg). This was then applied, at room temperature, to a gel filtration column of Sepharose 4B (0.45 x 57 cm). The column was equilibrated with PBS and the sample eluted with the same buffer at a flow rate of 5 ml/h. The radioactivity (^{125}I and ^{35}S) in the fractions was measured, together with the elution points of PAPP-A and α_2M. Aliquots of the fractions were analysed by SDS-PAGE (5-15%). TCA precipitations were carried out and the protein bound ^{125}I and ^{35}S located.

Interactions of PEP with gelatin and gelatin:fibronectin complexes

PEP-gelatin interactions

A 750 µl sample of dialysed PEP, containing 170.5 µg of PAPP-A and 173.3 µg of α_2M, was incubated at room temperature for 1 h on a column of Sepharose 4B (0.5 x 4 cm), to which 15 mg of gelatin had been coupled. The column was equilibrated with PBS and the unbound protein eluted with 4 ml of the same buffer. This was followed by 4 ml of buffer B. The fractions (1 ml) were analysed for their PAPP-A and α_2M content.

TABLE 4:6 Incorporation of ^{35}S-methionine into the proteins in the supernatant, after culturing Nitabuch's layer

Culture supernatant	Pre-counts (cpm)	Precipitated[(a)] (counts (cpm)	Incorporation %
Nitabuch's layer (4 separate cultures, from one placenta)	337458	26453	7.8
	388115	23679	6.1
	382515	17335	4.5
	272073	21962	8.1
Villous placenta (2 separate cultures, from one placenta)	479859	8233	1.7
	522778	4054	0.8

(a) TCA precipitations on 10 µl of supernatant.
Averages of duplicate precipitations.

PEP-gelatin:fibronectin interactions

^{35}S-fibronectin in 500 µl of supernatant from cultured Nitabuch's layer was incubated for ½ h at room temperature on a column (0.5 x 4 cm) of gelatin (15 mg) coupled to Sepharose 4B. The column was equilibrated with PBS. After the incubation 5 ml of PBS were applied to the column to elute any free ^{35}S and unbound protein. A 750 µl sample of dialysed PEP (130.5 µg PAPP-A; 124.5 µg α_2M) was subsequently applied to the gelatin Sepharose/fibronectin column, where it was incubated at room temperature for 1 h. Unbound protein was eluted with a further 5 ml of PBS and the concentration of PAPP-A and α_2M in these fractions (1 ml) determined.

TCA precipitation of ^{35}S-labelled proteins

Duplicate aliquots (10-50 µl) of labelled protein solutions were spotted onto filter paper discs (Whatman no. 1), and left to dry. Proteins were precipitated by gently stirring the discs in ice cold 5% (w/v) TCA for 30 min. Unprecipitated counts and TCA were removed by washing with 4-5 volumes of methanol. The washed and dried discs were placed in scintillation vials with 10 ml of Biofluor and the precipitated counts measured.

6:3 RESULTS

Culturing Nitabuch's layer

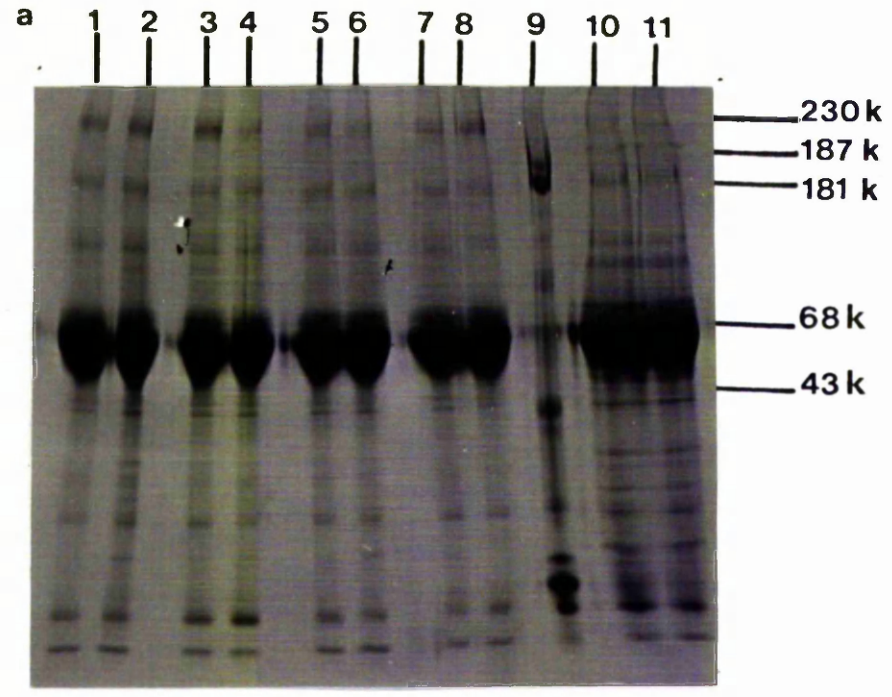
Of the ^{35}S-methionine counts in the supernatant from cultured Nitabuch's layer, 4.5-8.1% were precipitable with TCA, compared with 0.8-1.7% of the counts in the control supernatants (Table 4:6). Analysis on SDS polyacrylamide gels (Figure 4:18a) revealed many bands,

Figure 4:18 Analysis of the supernatant after culturing Nitabuch's layer for 24 h with ^{35}S-methionine.

(a) SDS-PAGE under reducing conditions, stained with Coomassie blue. Tracks 1-8, aliquots of 8 different culture supernatants from Nitabuch's layer (odd no. tracks are radiolabelled, even no. tracks are not). Track 9, PAPP-A (187 k) and α_2M (181 k) standards; tracks 10 and 11, control supernatants from villous placenta (track 10, ^{35}S-labelled; track 11, unlabelled).

(b) Autoradiogram of gel shown in figure 4:18a. Tracks 2-5, Nitabuch's supernatant, showing a radiolabelled polypeptide at 230 k. Track 6, villous placenta control supernatant. Track 1, an aliquot of Nitabuch's supernatant (see Figure 4.18a) stained with Coomassie blue. Exposure; 4 days at room temperature.

Figure 4:18



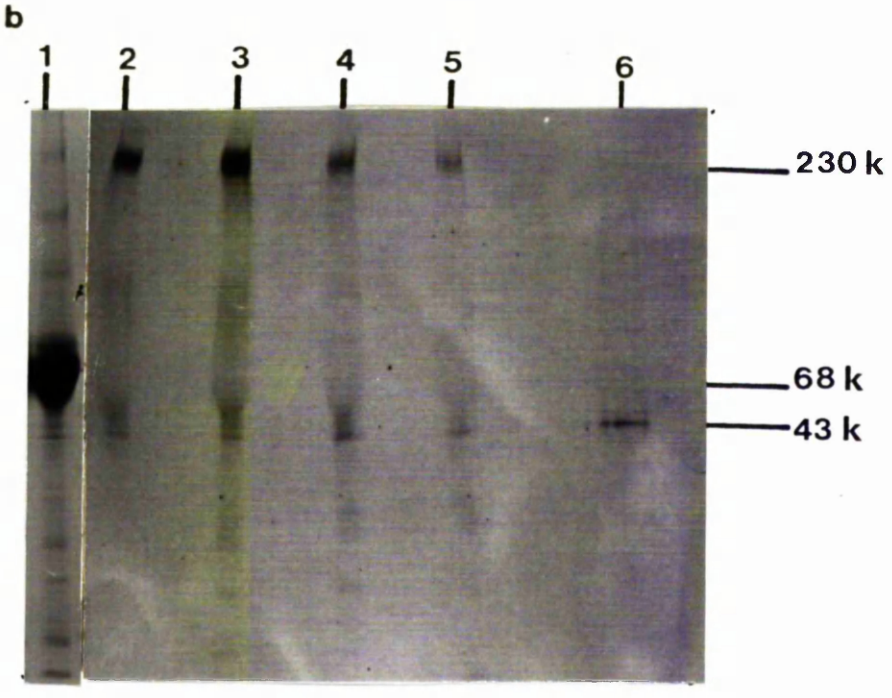
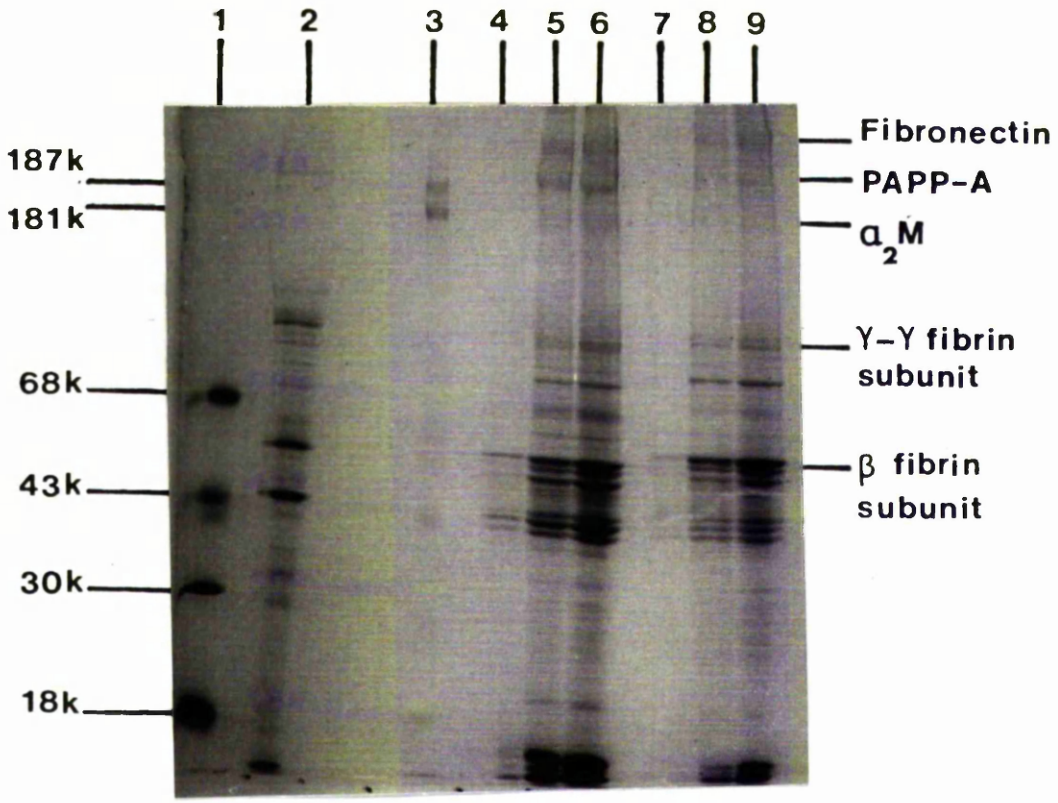


Figure 4:19 Analysis of the scrapings taken from Nitabuch's layer on SDS-PAGE under reducing conditions. Track 1, molecular weight standards; track 2, fibrin clot standard; track 3, PAPP-A (187 k) and α_2M (181 k) standards. Tracks 4-6 and 7-9, samples (5, 25 and 50 µl) of homogenised Nitabuch's scrapings from 2 different placentae.

Figure 4:19


1
2
3
4
5
6
7
8
9
187k
181k
68k
43k
30k
18k
Fibronectin
PAPP-A
α_2M
γ-γ fibrin subunit
β fibrin subunit

some of which were common to both types of supernatant. For example the heavily stained band at 68,000 (mol wt) and the lighter stained bands at 181,000, 43,000 and 30,000. A high molecular weight polypeptide (230,000 approx) was observed in the supernatant from Nitabuch's layer (tracks 1-8; Figure 4:18a). This band was also present in the supernatant from the villous placenta control, but to a lesser extent (tracks 9 and 10). Radiolabelling of this polypeptide was observed on autoradiographs for the Nitabuch's supernatant only (tracks 1-4: Figure 4:18b). Other lower molecular weight polypeptides (less than 68,000) observed on the autoradiographs of the Nitabuch's supernatant were more weakly labelled. A polypeptide with molecular weight of about 43,000 showed some ^{35}S incorporation in the control supernatant (Figure 4:18b, track 5) and a similar band in the Nitabuch's supernatant showed comparable labelling (tracks 1-4).

Polyacrylamide gels of Nitabuch's scrapings showed a different banding pattern on staining with Coomassie blue (Figure 4:19). However, the high molecular weight bands at 230,000 and 181,000 (mol wt) were again apparent, though the latter was only faint (tracks 4-7; Figure 4:19). A third high molecular weight band, not present in the supernatant from cultured Nitabuch's layer, was observed at 187,000 (tracks 4-7). The intensity of the Coomassie staining of this band was comparable to that seen for the 230,000 mol wt polypeptide.

Fibronectin binding assay

As this experiment involved radioisotopes emitting different types of radiation (^{35}S, β-radiation; ^{125}I, γ-radiation), it was

Figure 4:20 Correction curve for the fibronectin binding assay: The gamma counts in solutions of ^{125}I-PEP were recorded and plotted against the number of counts per second recorded for the same solutions in the liquid scintillation counter.

Figure 4:20

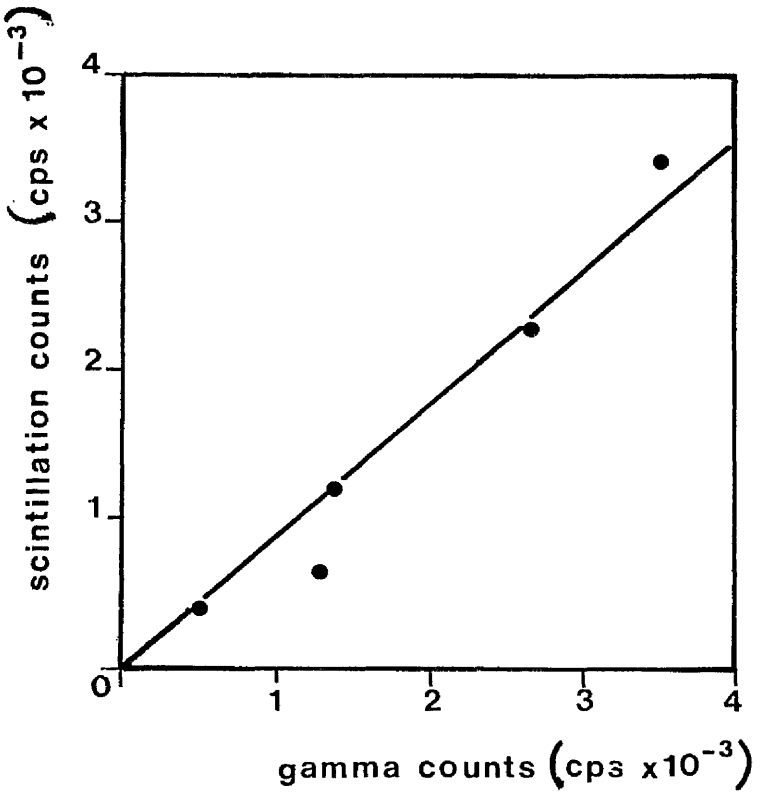
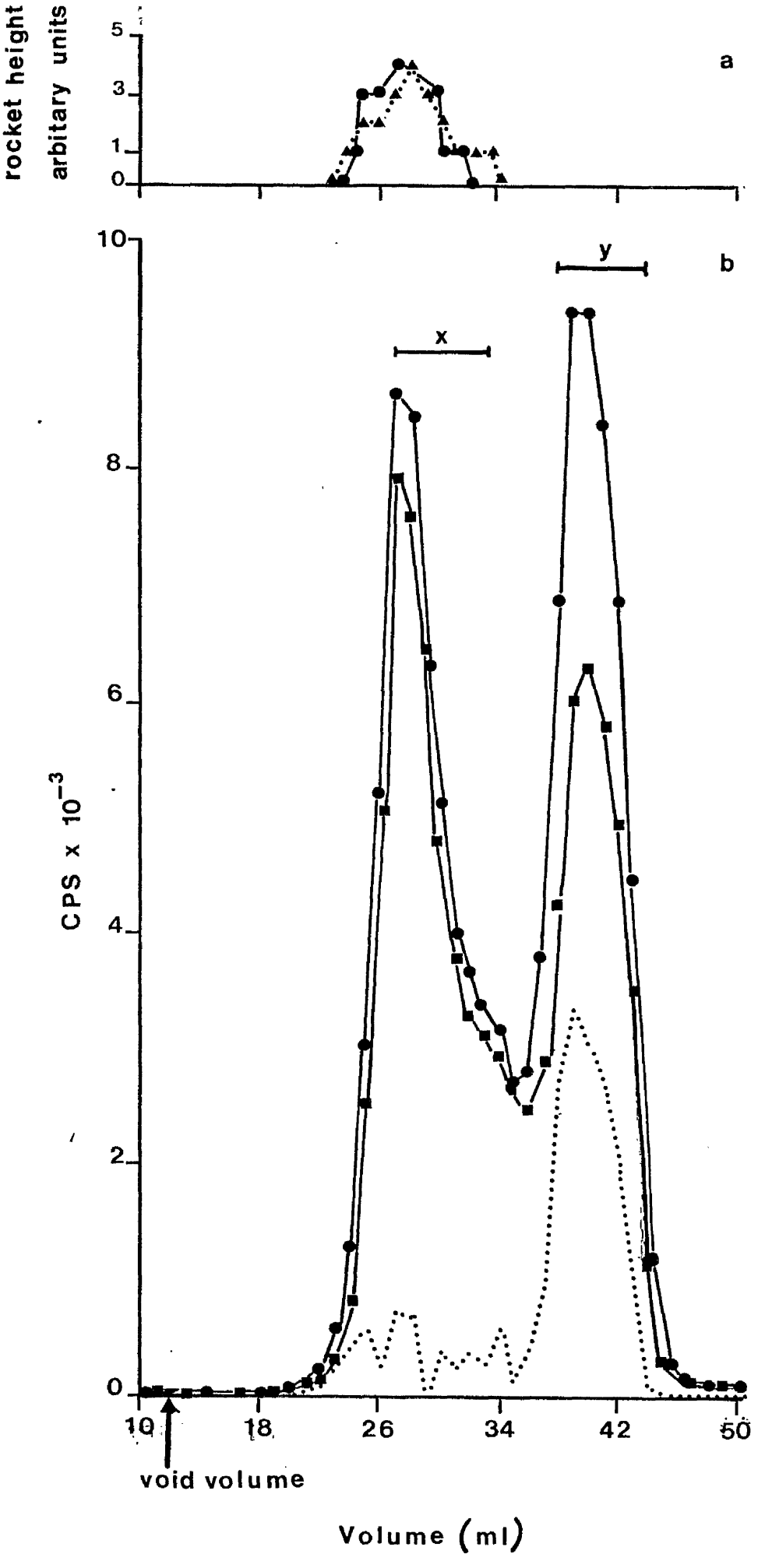


Figure 4:21 Fibronectin binding assay. Gel filtration of ^{125}I-PEP and ^{35}S-fibronectin after incubation for 1 h. Sept 4B

(a) Concentration of α_2M (▲- - -▲) and PAPP-A (●——●) as determined by AACE.

(b) The elution profiles of the ^{35}S-counts (●——●) and ^{125}I-counts (■——■) were recorded. The correction curve in Figure 4:20 was used to determine the "corrected" ^{35}S-counts (- - -). The fibronectin elution range (x) was determined from SDS-PAGE (see Figure 4.22). The elution range of free ^{125}I and ^{35}S (y) was determined by a TCA precipitation.


rocket height
arbitary units
5
3
1
0
a
10
8
6
4
2
0
b
y
x
CPS x 10^{-3}
10
18
26
34
42
50
void volume
Volume (ml)

necessary to determine how the presence of one isotope affected the detection of the other, in a mixture of the two. This was ascertained by recording both the gamma and scintillation counts measured in various dilutions of the individual isotopes. No solution containing ^{35}S-fibronectin gave a reading above background when measured in the gamma counter. However, a substantial number of counts were recorded when ^{125}I-PEP solutions were measured in the liquid scintillation counter, and these were plotted against the number of gamma counts recorded for the same solutions (Figure 4:20). An approximate 1:1 relationship was observed, in that one cps recorded by the gamma counter was recorded as one cps by the liquid scintillation counter.

The supernatant from cultured Nitabuch's layer containing ^{35}S-fibronectin (plus other proteins such as BSA from the culture medium), was incubated with ^{125}I-PEP and unlabelled PEP. The proteins were then separated by gel filtration. Two peaks of radioactivity were observed in the eluate for the ^{35}S (corrected and uncorrected) and ^{125}I counts (Figure 4:21b). The counts in the first peak (21-35 ml) reached their maximum at 27 ml eluted for both isotopes. The second peak (35-45 ml) eluted maximally at 39-40 ml and 40 ml for the ^{35}S and ^{125}I counts respectively. These were judged to be free ^{125}I and ^{35}S counts as very few of them were TCA precipitable (6% or less). AACE assays showed that α_2M and PAPP-A eluted in the first peak of radioactivity (23-34 ml; Figure 4:21a). SDS-PAGE located the fibronectin elution range to approximately 27-33 ml eluted (Figure 4:22).

Interactions of PEP with gelatin and gelatin:fibronectin complexes

PAPP-A and α_2M were initially incubated on a column of gelatin Sepharose to determine their gelatin binding capacity. The results of

Figure 4:22 SDS-PAGE analysis of the column eluate after gel filtration of ^{125}I-PEP/^{35}S-fibronectin. Tracks 1-3, aliquots of fractions at 26, 28 and 33 ml eluted, respectively. Track 4, PAPP-A, α_2M and other molecular weight standards (see methods section).

Figure 4:22

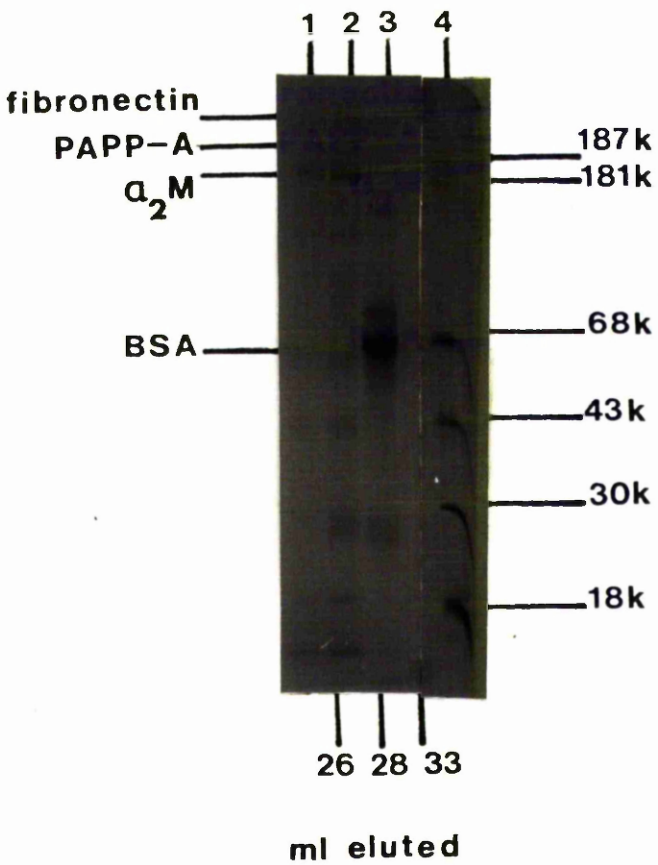

1 2 3 4
fibronectin
PAPP-A
α_2M
BSA
187k
181k
68k
43k
30k
18k
26 28 33
ml eluted

TABLE 4:7 PEP/Gelatin interactions

Fraction collected	Column buffer	Concentration of α_2M (µg/ml)	Concentration of PAPP-A (µg/ml)
1	PBS	38.0	51.3
2	"	60.3	56.7
3	"	34.3	41.3
4	"	11.0	4.0
Total not binding		143.6 (82.9%)	153.3 (89.9%)
5	Buffer B	9.3	4.0
6	"	7.3	2.7
7	"	5.7	2.7
8	"	3.7	2.7
Total binding		26.0 (15.0%)	12.1 (7.1%)
Total protein recovered		169.6 µg (97.9%)	165.4 µg (97.0%)

Figures in parentheses = % recovery

TABLE 4:8 PEP/Fibronectin:Gelatin interactions

Fraction collected	Cps (in 100 µl)	Concentration of α_2M (µg/ml)	Concentration of PAPP-A (µg/ml)
1	21630	-	-
2	128825	-	-
3	19218	-	-
4	2850	-	-
5	476	-	-
Total cps eluted	172999 (61.22%)		
6	170	16.0	16.3
7	95	34.3	39.3
8	68	28.3	29.3
9	33	14.7	17.7
10	19	13.0	13.0
Total eluted	385 (0.14%)	106.3 µg (85.4%)	115.6 µg (88.6%)
Total remaining on column.	109212 (38.64%)	18.2 µg (14.6%)	14.9 µg (11.4%)

Figures in parentheses = % recovery.

this experiment are summarised in Table 4:7. Of the protein loaded 153.3 μg (89.9%) of the PAPP-A and 143.6 μg (82.9%) of the α_2M were recovered from the column during the PBS elution (fractions 1-4). On washing the gelatin Sepharose with 0.3 M NaCl in PBS, a further 12.1 μg (7.1%) of the PAPP-A and 26 μg (15%) of the α_2M were recovered (fractions 5-8).

Radiolabelled fibronectin was then incubated on a similar column. Unbound counts were washed off the column with PBS (Table 4:8, fractions 1-5). These amounted to 61.2% of the total counts loaded (Table 4:8). A further 0.14% of the counts were eluted after the PEP incubation (fractions 6-10), along with 115.6 μg (88.6%) of the PAPP-A and 106.3 μg (85.4%) of the α_2M originally applied.

6:4 DISCUSSION

The incorporation of ^{35}S-methionine into the supernatant proteins of cultured Nitabuch's layer was observed on polyacrylamide gels and autoradiography (Figure 4:18a and b). A polypeptide with molecular weight of 230,000 (approx) incorporated the most ^{35}S (Figure 4:18b). This was judged to be fibronectin, as the protein in this band had previously been shown to bind to gelatin and anti-fibronectin immobilised on Sepharose (Khalef, Buultjens and Sutcliffe, in preparation). The identity of the other more weakly labelled proteins present in the supernatant was not determined. No PAPP-A or α_2M synthesis was observed after culturing for 24 h.

The polyacrylamide gel analysis of Nitabuch's scrapings from two different placentae, after staining with Coomassie blue, is shown

in Figure 4:19 (tracks 4-7). Fibronectin (230,000) was again apparent, as was a faint band at 181,000 thought to be α_2M. However, a high molecular weight band, not present in the superantant of cultured Nitabuch's layer, was also observed. The molecular weight of this band was estimated to correspond to that of PAPP-A (i.e. 187,000). This band showed a similar intensity of staining to the fibronectin band, which suggests that they were present in approximately the same concentration. Some of the smaller polypeptides had molecular weights corresponding to those observed in the fibrin standard, such as the β and γ-γ dimer subunits (track 2, Figure 4:17). Further analyses of these and the other bands was not carried out.

As both PAPP-A and fibronectin were present in analysed samples of Nitabuch's scrapings, since they show a similar distribution in placental and decidual tissue and since they are both synthesised by Nitabuch's layer (Khalef, Buultjens and Sutcliffe, in preparation), it was considered possible that these two proteins might bind to each other or to some common substrate such as the collagen in the reticular fibres (type III). This type of association is observed in basement membranes, where collagen is found in conjunction with two non-collagenous glycoproteins (Kefalides, 1975). These proteins are secreted by the cell and interact extracellularly to form the desired structural arrangement required for each tissue (see Chapter 5, Figure 5:1). If PAPP-A and fibronectin were to bind to each other a complex with a molecular weight of well over one million (450,000 + 750,000) would be formed. Such a high molecular weight complex could be resolved from free PAPP-A and fibronectin by gel filtration. Differential radiolabelling of these 2 proteins would allow the identification of any

such high molecular weight complexes (even at low concentrations), present in the column void volume, and distinguish them from the unbound components. Such was the rationale behind the gel filtration experiment carried out on the ^{35}S-fibronectin after incubation with ^{125}I-PEP.

The elution profile for the ^{125}I and ^{35}S counts (corrected and uncorrected) were made up of 2 peaks of radioactivity (Figure 4:21), the first peak (21-35 ml eluted) being composed of the protein bound counts and the second (35-45 ml eluted), the free ^{125}I and ^{35}S (TCA non-precipitable counts). PAPP-A, α_2M and fibronectin were detected in the first peak only. The order of their elution confirmed that gel filtration had indeed occurred, since the fibronectin (450,000 mol wt) began to elute after the α_2M and PAPP-A (620,000-820,000 mol wt), and before the BSA (68,000 mol wt; tracks 2 and 3, Figure 4:22). Neither PAPP-A nor fibronectin were detected by AACE or SDS-PAGE and autoradiography in the earlier fractions (10-23 ml eluted). It was concluded therefore, that under these conditions PAPP-A and fibronectin do not associate to form a high molecular weight complex.

Since fibronectin has a high affinity for gelatin (see Mosher, 1980), the binding properties of PAPP-A, and α_2M, to gelatin and fibronectin:gelatin complex were investigated. Gelatin is produced when collagen is denatured by boiling, thus fibronectin bound to gelatin = Sepharose was used as a model for the binding of insoluble fibronectin to collagen. Denatured collagens are more efficient binders of fibronectin than their native forms, but of the native forms, collagen type III, as found in reticular fibres, is the most active (Ruoslahti, 1982a). Initially, the capacity of the gelatin Sepharose

to bind PAPP-A and α_2M was determined (Table 4:7)。 The majority of the PAPP-A and α_2M loaded onto the column was eluted during the PBS wash (89.9% and 82.9% respectively)。 Some, however, did appear to bind to the gelatin (7.1%, PAPP-A; 15%, α_2M), but most of this was eluted in the subsequent salt wash (Table 4:7)。 The binding was thus thought to be of an ionic nature, rather than a covalent nature。 The total recovery of PAPP-A and α_2M was 97% and 97.9% respectively, therefore it was concluded that the gelatin Sepharose showed no capacity to bind PAPP-A or α_2M。 (Fibronectin, which does bind to gelatin Sepharose, requires 4M urea in 50 mM Tris-HCl pH 7.5; 2M urea in 1M NaCl; glycine HCl NaI buffer pH 2.6 or ethylene glycol to dissociate it from gelatin ; Ruoslahti, 1982a)。

After incubation of Nitabuch's supernatant on a similar column, 38.6% of the counts loaded remained on the gelatin Sepharose after washing with 5 ml of PBS (Table 4:8)。 These were taken to be bound ^{35}S-fibronectin counts。 The recoveries of PAPP-A and α_2M (88.6% and 85.4% respectively; Table 4:8), after subsequent incubation of PEP on the fibronectin:gelatin Sepharose, were very similar to those observed in the absence of fibronectin (Table 4:7)。 From these results itwould appear that the similarities in the distribution of PAPP-A and fibronectin throughout placental tissue cannot be attributed to these proteins forming an association with each other or with collagen, nor by PAPP-A binding to a fibronectin : collagen complex。 Fibronectin is known to bind to many proteins (see Table 4:5), such as heparin, fibrinogen, fibrin and other components of the fibrinolytic andhaemostatic systems (Mosher, 1980), and any of these molecules could act as a common binding substrate for fibronectin and PAPP-A。 It has been reported that PAPP-A

has a high specific affinity for heparin (Sinosich et al, 1981). Fibronectin binding to collagen is enhanced in the presence of heparin (Ruoslahti, 1982b), and the binding of heparin (heparin sulphate or hyaluronic acid) to fibronectin:gelatin complexes conveys upon them greater stability than in the absence of heparin (Ruoslahti, 1982a). PAPP-A binding of collagen may therefore be dependent on the presence of other molecules such as heparin. Further studies of this type are required therefore, before any definite statements on the interactions of PAPP-A and fibronectin can be made.

CHAPTER 5 : DISCUSSION AND CONCLUSIONS

DISCUSSION AND CONCLUSIONS

"In Science the primary duty of ideas is to be useful and interesting even more than to be true"

W. Trotter (1941)

In this last chapter, the main conclusions presented in Chapters 2, 3 and 4 will be reviewed and discussed in the light of the experimental objectives (as presented in Chapter 1, section 6). The notion that α_2M and PAPP-A might be related to each other arose because of the similarities observed in their physicochemical properties. Under reducing conditions their isoelectric points were found to be equivalent (Sutcliffe et al, 1980b), and their amino acid, hexosamine and sialic acid compositions similar (see Tables 1:5 and 1:6). However, their total carbohydrate contents differed quite markedly, with a mean value of 19.2% carbohydrate for PAPP-A and 8.6% for α_2M (Sutcliffe et al, 1980b). The neutral carbohydrate content of these two proteins was determined to see if this could account for the observed difference in their total carbohydrate content. If so, this would be consistent with the view that the molecular charges on these proteins are similar, as implied by the equivalence in their isoelectric points. The neutral carbohydrate content of PAPP-A (using a galactose standard) was found to have a mean value of 12.9%, compared with a value of 3.9% for α_2M (Table 2:2). The observed difference in the neutral carbohydrate (9.0%) accounts therefore, for a high proportion (70-96%, see Chapter 2:4), of the difference in total carbohydrate. It could be argued that this result supports the hypothesis that PAPP-A represents a modified form of α_2M (at either the level of the gene or post-transcriptionally), being equivalent to a molecule of α_2M plus additional neutral carbohydrate. However, electro-

phoresis results (Sutcliffe et al, 1980b), indicate that the molecular weight of the PAPP-A polypeptide exceeds that of α_2M by about 3.3%. Since their total carbohydrate contents differ by 10.6%, this would suggest that the PAPP-A polypeptide is smaller than that of α_2M. It would appear therefore, that the notion that PAPP-A is equivalent to a molecule of α_2M plus additional neutral carbohydrate is too simplistic. If indeed, these proteins are related, then it seems more likely that PAPP-A represents a modified form of α_2M which has had a portion of its polypeptide chain removed and carbohydrate added. It could be argued that polyacrylamide gel electrophoresis is not a sufficiently accurate method for assessing molecular weight, and determination of the sedimentation equilibrium point of PAPP-A, for example, might give a molecular weight value for PAPP-A that is more in keeping with this hypothesis. Gordon and Chard (1979), have suggested that all placental proteins are analogues of proteins present in the normal adult. Human pituitary growth hormone (prolactin), for example, is the non-pregnant analogue of human placental lactogen (hPL) (Josimovich, 1977) and the pregnancy-associated alpha fetoprotein (AFP) is thought to be the fetal counterpart of adult serum albumin (see Law and Dugaiczyk, 1981; and references therein). However, it is important to note that the theories of homology for these proteins are based on detailed analysis not just of their physicochemical properties, but also an analysis of their primary structure at the amino acid and/or DNA levels. Amino acid analysis of hPL and prolactin has shown that these two hormones have 163 out of 190 amino acid residues in common (Josimovich, 1977). Of the remaining 27 residues, 24 are "highly acceptable" alternatives in terms of the genetic code. Homology between the primary structure of AFP and serum

albumin was deduced from a complete c-DNA sequence and from the fact that AFP can be organized into a 3-domain structure almost identical to that of serum albumin (Law and Dugaiczyk, 1981). It is interesting to note that attempts to peptide map α_2M and PAPP-A with V8 protease and cyanogen bromide (Sutcliffe et al, 1980a; Gore and Sutcliffe, in press), have failed to produce evidence of homology. This cannot be taken to be indicative of a total lack of homology, but a lack of homology in those sequences flanked by methionine, aspartic acid and glutamic acid residues. However, it does not lend support to a theory of homology. Previous attempts to find antigenic cross-reactivities between these two molecules have also failed to yield evidence of homologies (Sutcliffe et al, 1980a & b). The observed similarities in their physicochemical properties do not constitute strong evidence for homology. Both proteins are serum macroglobulins whose tetrameric structures are to be expected from their size, and whose very similar isoelectric points are typical of many other proteins.

PAPP-A was obtained in an enriched state under mild conditions of purification involving Cibacron blue dye-ligand chromatography, negative antibody affinity chromatography and gel filtration (see Chapter 3). The product was a mixture of PAPP-A and α_2M (the PAPP-A enriched preparation or PEP). The PAPP-A produced by this method was not as pure as that obtained after positive affinity chromatography (Sutcliffe et al, 1980b; Folkersen et al, 1981a). However, this was largely an operational factor, since the purity of the PAPP-A could be markedly increased by reducing the volumes loaded onto the columns and restricting the pool size to those fractions containing the maximum concentration of PAPP-A. The problem of the contaminating α_2M was greatly reduced

after ion exchange chromatography. This step was not employed however, until after the investigation of the functional properties of PAPP-A, thus avoiding further processing of the PAPP-A and making use of the contaminating α_2M as a positive internal control during the subsequent protease binding experiments. Starkey and Barrett (1977), noted that long, complex purification procedures tended to result in serious inactivation of α_2M, and that it was sometimes necessary to compromise between obtaining α_2M free from all contaminants and retaining the major part of the α_2M in an active form. Such was the rationale behind this method of PAPP-A purification. It was not possible to be certain that the PAPP-A produced by this method was in an essentially native and potentially active state, but the more severe conditions such as high salt concentrations and ammonium sulphate precipitation employed in previous methods were avoided.

The results of the trypsin and plasmin binding assays (Chapter 4, sections 3 and 4), also lend no support to the hypothesis that PAPP-A has a function which is homologous to α_2M. The PAPP-A in PEP bound neither trypsin nor plasmin, whereas the α_2M did. Therefore it is improbable, as binding was not observed, that PAPP-A inhibits either of these enzymes (see Chapter 1:5), though the possibility that it inhibits enzymes other than trypsin or plasmin cannot be excluded. It is usual to carry out a stoichiometric analysis on the inhibition of enzyme activity in conjunction with such binding experiments. It is acknowledged that this type of information would have been useful for accurately assessing the degree of activity of the α_2M in the PEP, thus acting as a point of reference for the quantitative assessment of the activity status of the PAPP-A. Had the PAPP-A shown trypsin or

plasmin binding a stoichiometric analysis would have been performed, as it did not, this type of analysis was not considered necessary.

α_2M does not inhibit complement, if PAPP-A were functionally homologous to α_2M, it would also not be expected to have complementary-inhibitory properties. In repeated assays, no evidence could be found to show that PAPP-A was able to inhibit the lysis of sensitised sheep red blood cells by guinea pig or human complement (Chapter 4; section 5). This negative result however, cannot be interpreted as an indication of functional homology.

The present results are not consistent with the views that PAPP-A inhibits plasmin in a way homologous to α_2M (Bischof, 1979), or that PAPP-A is an inhibitor of complement (Bischof, 1981). How can these conflicting results be reconciled? It could be argued that the PAPP-A in PEP, contrary to expectations, had been sufficiently denatured to prevent its functioning. Indeed, there is no direct proof that the PAPP-A in PEP is physiologically active. However, the presence of native and active α_2M in PEP would seem to suggest that the conditions of purification were sufficiently mild to have maintained the PAPP-A in a similar state. Obviously it is not possible to assess the activity status of the PAPP-A purified by Bischof (1979), but he did use an ammonium sulphate precipitation step (see Table 1:2) which would have had adverse effects on α_2M (see Chapter 1, section 4:1). Bischof's assays (1980, 1981) for the inhibition of plasmin by PAPP-A were based on a caseinolytic activity assay. Such assays are open to misinterpretation as any observed inhibition may be due to a contaminant and not to the protein being tested as an inhibitor. Both Bischof (1979), and I have found it difficult to remove contaminating α_2M from preparations

of PAPP-A. This stresses the importance of binding assays and of using sensitive methods to assess the purity of preparations. Tube gel electrophoresis and immunodiffusion (Bischof, 1979, 1981), may not be suitably sensitive. Sutcliffe et al (1980a), detected some anti-α_2M specificities in their sheep anti-PAPP-A serum, which had been raised against purified preparations of PAPP-A (Sutcliffe et al, 1978). This would seem to suggest that there was some α_2M in these preparations.

The problem of contamination of PAPP-A by α_2M may underly some of the disagreements in the literature concerning the radioimmunoassays of PAPP-A. Sinosich et al (1982), for example, were unable to detect PAPP-A in the blood of the 100 non-pregnant individuals they tested, whereas Bischof et al (1981), using a comparable radioimmunoassay, detected PAPP-A in non-pregnant subjects at a concentration of 0.1 μg/ml. This discrepancy could be explained if the radiolabelled PAPP-A used by Bischof et al was contaminated by α_2M and if the antiserum contained anti-α_2M specificities. α_2M in non-pregnant sera would then compete with the small amount of ^{125}I-α_2M in the tracer in the assay, which would give the impression that there was PAPP-A in the non-pregnancy sera competing with the ^{125}I-PAPP-A for binding sites. Similarly, Sinosich et al (1982) did not detect PAPP-A in the umbilical circulation, whereas Bischof's colleagues did (0.1 μg/ml; Duberg et al, 1982). It is interesting to note that the concentration of "PAPP-A" detected in both non-pregnancy and umbilical chord sera was 0.1 μg/ml, it is possible that this reflects the concentration of the contaminating α_2M.

If the PAPP-A that Bischof (1979) purified is contaminated with α_2M, this would account for the plasmin inhibitory properties assigned to PAPP-A by Bischof (1979), and possibly even the urokinase

inhibition too (though there is some doubt as to whether α_2M inhibits urokinase; Starkey and Barrett, 1977). However, the complement inhibitory properties of PAPP-A observed by Bischof (1981), cannot be explained in these terms. It is curious that Bischof (1981) detected the inhibition of complement using the PAPP-A purified by Sutcliffe et al (1980b), as they themselves were unable to detect such activity (Dr. R.G. Sutcliffe, personal communication). The reason for the observed inhibitory capacity of both preparations of PAPP-A is not understood.

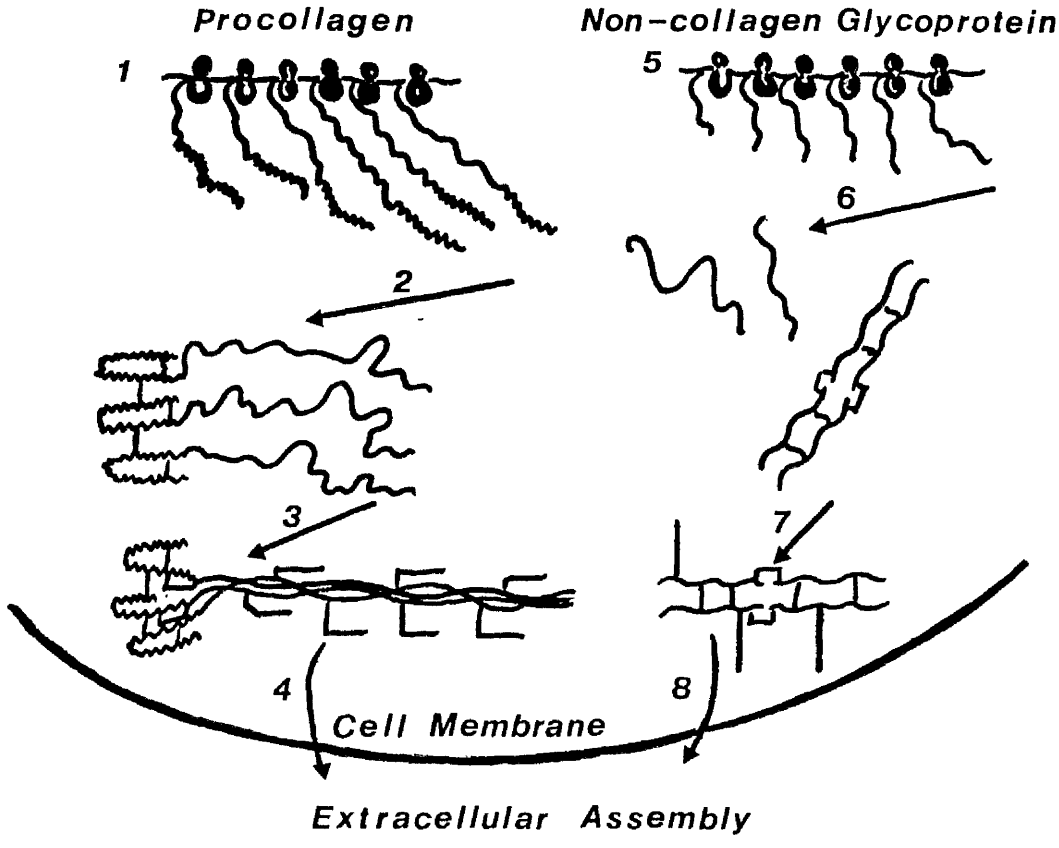
The immunoperoxidase localization studies on PAPP-A and fibronectin carried out by Sutcliffe, Khalef and Horne (in preparation), shows that these two proteins have a very similar distribution pattern in placental tissues, particularly in Nitabuch's (fibrinoid) layer at the utero-placental interface and in association with the reticular fibres observed around decidual cells (see Chapter 1, section 3:3). SDS polyacrylamide gel analysis of scrapings from Nitabuch's layer (Chapter 4:6), showed that PAPP-A, fibronectin and other lower molecular weight polypeptides were present in this layer. On culturing Nitabuch's layer for 24 h with ^{35}S-methionine, radiolabelled fibronectin was located in the culture supernatant (Chapter 4:6). Recent results have shown that if this layer and chorionic plate tissue are cultured for 1 week under the same conditions, radiolabelled PAPP-A is also synthesised (Khalef, Buultjens and Sutcliffe, in preparation). Experiments were carried out to see if PAPP-A and fibronectin bound to each other or to a common substrate of collagen, (in the form of gelatin; see Chapter 4:6). PAPP-A did not appear to bind to fibronectin, gelatin or to a fibronectin: gelatin complex. However, it is currently believed (Khalef, Buultjens

and Sutcliffe, in preparation), that PAPP-A can primarily be regarded as a protein contributing to the structure of the extracellular matrix in the utero-placental and chorionic plates, and in certain fibrotic areas of placental villous trunks. The observation that there is a specific interaction between PAPP-A and heparin (Sinosich et al, 1981), would seem to give further weight to this view of PAPP-A. McKay and Laurell (1980) have pointed out that proteins which bind to immobilised heparin can be divided into 4 functionally related groups, namely; coagulation proteins, protease inhibitors, complement system proteins and cell surface proteins. As PAPP-A does not appear to inhibit plasmin, trypsin or complement mediated haemolysis it seems unlikely that PAPP-A fits into any of the first 3 categories, thus leaving the cell surface protein group. This group includes fibronectin, and could therefore, be possibly extended to contain proteins of the extracellular matrix as fibronectin is also located there (and since it is difficult to define the precise demarcation barrier between these two areas). In the presence of heparin, more stable fibronectin:gelatin complexes are formed (Ruoslahti, 1982a), and fibronectin forms co-polymers with collagen of types I and III (as found in reticular fibres), when incubated with heparin (Mosher, 1980). It would be very interesting as a future line of investigation, to see if PAPP-A and heparin form associations with fibronectin and/or collagen mixes.

Figure 5:1 shows a schematic diagram of the intracellular events in the synthesis of extracellular matrices. It is possible that this represents the events taking place in the cytotrophoblastic cells of the chorionic plate and Nitabuch's layer, leading to the incorporation of PAPP-A and fibronectin into the reticular fibres,

Figure 5:1 A schematic diagram of the intracellular events in the synthesis of basement membrane. A possible model for the synthesis of PAPP-A and fibronectin leading to their incorporation in the extracellular matrix. (Adapted from Kefalides, 1975).

Figure 5:1



1. Translation & Hydroxylation

2. Release of Chains & Assembly

3. Glycosylation & Helix Formation

4. Secretion

5. Translation

6. Release of Chains & Assembly

7. Glycosylation

8. Secretion

extracellular matrices and fibrotic areas in the villous trunks. What role might these areas play in gestation? Sutcliffe et al (1982a), suggested that the layer of fibrin/fibrinoid material at the utero-placental interface (i.e. Nitabuch's layer, which stains for PAPP-A) since it is so extensively distributed over the delivered placenta, may have a role in the separation of the placenta from the uterus during parturition. In contrast the reticular fibres observed around the uterine decidual cells, which stain for PAPP-A, may possibly be involved in maintaining a tight junction between the placenta and uterus (Khalef, Buultjens and Sutcliffe, in preparation). It may be that the characteristic increase in PAPP-A concentration in maternal serum right up to term, reflects the involvement of PAPP-A in the maintenance of the placental-uterine unit. The long half-life of PAPP-A might be attributable to the presence of PAPP-A around the uterine decidual cells persisting into the post partum period, or to some Nitabuch's layer remaining on the wall of the uterus, continuing to synthesize PAPP-A after parturition.

The experimental objectives of this thesis were to purify PAPP-A under mild conditions in an attempt to obtain it in a physiologically active form; to further compare α_2M and PAPP-A physicochemically and physiologically; and to compare the activity of the PAPP-A (purified in this manner), to the activity of that purified by other workers. On the basis of the results in this thesis there seems little reason to consider that PAPP-A is homologous or analogous to the serum protein α_2M, in function or finer structure. The gross similarities in their tetromeric structure are to be expected from their size, and their very similar isoelectric points are typical of many other proteins.

The evidence indicates that PAPP-A should no longer be considered as a protein involved in immunosuppression or fibrinolysis, but primarily as a protein contributing to the structure of the extracellular matrix in the utero-placental and chorionic plates, and in certain fibrotic areas of placental villous trunks.

Should the observed similarities between α_2M and PAPP-A be dismissed as mere anomalies? Van Leuven (1982), has noted that fibrin-monomers, fibronectin and α_2M are the major (if not the only) substrates in plasma for factor XIII (transglutaminase, a cross linking enzyme), and that both fibronectin and α_2M interact with cellular receptors on macrophages and fibroblasts. He concludes that, "this is a set of high-molecular weight, glycoproteins, having major biochemical features in common and interacting, very specifically, with cells". It would be of great interest to see if PAPP-A too is a substrate for factor XIII, for if it is, then the relationship between PAPP-A and α_2M may well be clarified, and PAPP-A added to this group of proteins.

BIBLIOGRAPHY

AITKEN, R.J., BEACONSFIELD, R., GINSBURG, J. (1979)
"Origin and formation of the placenta" In "Placenta - A neglected experimental animal" Ed. by Beaconsfield, P. & Villee, C.
Pergamon Press - Oxford pp.152-163.

ALEXANDER, B., MEYERS, L., KENNY, J., GOCOSTEIN, R., GUREWICH, V., GRINSPOON, L. (1956)
"Blood coagulation in pregnancy"
New England Journal of Medicine 254: 358-362.

ALEXANDER, P. (1972)
"Fetal 'Antigens' in Cancer"
Nature 235: 137-140.

ANDERSSON, L-O (1979)
"Serum Albumin" (A.) Biochemistry In Plasma Proteins
Ed. Blomback, B. & Hanson, L.A.
John Wiley & Sons Ltd., pp43-54.

ASTEDT, B. (1972)
"Fibrinolysis. A, in pregnancy labour, puerperium and during treatment with sex hormones. B, in human ontogenesis and in human organ culture"
Acta Obstetric Science (supp. 1) 18: 1-45.

ASTRUP, T. MULLERTZ, S. (1952)
"The fibrin plate method for estimating fibrinolytic activity"
Archives of Biochemistry and Biophysics 40: 346-351.

ATTWOOD, H.D. & PARK, W.W. (1961)
"Embolism to the lungs by trophoblast"
Journal of Obstetrics & Gynecology of the British Commonwealth 68: 611-617.

BAGSHAWE, K.D. (1974)

"Tumour associated antigens"

British Medical Bulletin 30: 68-73.

BARRETT, A.J. & STARKEY, P.M. (1973)

"The interaction of α_2M with proteinases"

Biochemical Journal 133: 709-724.

BARRETT, A.J., BROWN, M.A., SAYERS, C.A. (1979)

"The electrophoretically 'Slow' and 'Fast' forms of the α_2 Macroglobulin molecule"

Biochemical Journal 181: 401-418.

BAUER, R. (1977)

"Morphometry of the placental exchange area"

Advances in Anatomical Embryology & Cell Biology 53: 1-65.

BECKMAN, G., BECKMAN, L., MAGNUSSON, S.S. & VON SCHOULTZ, B. (1974)

"The "pregnancy zone" protein and abortion"

Acta Obstetrica et Gynecologica Scandinavica 53, 177-180.

BEER, A.E. (1975)

"Immunogenetic determinants of the size of the fetoplacental unit and their modus operandi" In "Human Placentation" Ed. Brosens, I.A., Dixon, G. & Robertson, W.B.

Excerpta Medica pp.134-146.

BEER, A.E. & BILLINGHAM, R.E. (1974)

"Host response to intra-uterine tissue of cellular and fetal allografts"

Journal of Reproduction & Fertility (suppl) 21: 59-88.

BEER, A.E. & BILLINGHAM, R.E. (1976)

"The immunobiology of mammalian reproduction"

Prentice-Hall Inc. New Jersey.

BEER, A.E., QUEBBERMAN, J.F., AYERS, J.W.T. & HAINES, R.F. (1981)
"Major histocompatibility complex antigens, maternal and paternal immune responses and chronic habitual abortions in humans"
American Journal of Obstetrics & Gynecology 141: 987-999.

BEISCHER, N.A., BHARGAVA, V.L., BROW, J.B. & SMITH, A.A. (1968)
"The Incidence and significance of low oestriol excretion in an obstetric population".
Journal of Obstetrics & Gynecology of the British Commonwealth 75: 1024-1033.

BELL, S.C. & BILLINGTON, W.D. (1980)
"Major anti-paternal alloantibody induced by murine pregnancy is non-complement fixing"
Nature 288: 387-388.

BELLMAN, N.O., TEBBE, J., LANG, N. & BAUR, M.P. (1980)
"Determination of SP1 and hPL for predicting perinated asphyxia"
In "The human placenta: proteins and hormones".
Proceedings of the Serono Symposium vol 35. Ed. by Klopper, A., Genazzani, A. and Crosignani, P.G.
Academic Press, London. pp.99-108.

BERGQVIST, D. & NILSSON, I.M. (1979)
"Hereditary α_2M deficiency"
Scandanavian Journal of Haematology 23: 433-436.

BILLINGTON, W.D. (1975)
"Immunological aspects of normal and abnormal pregnancy"
In "Human Placentation" Ed. Brosens, I.A., Dixon, G. & Robertson, W.B.
Excerpta Medica pp.147-154.

BILLINGTON, W.D. (1979)

"The placenta and the tumour: variations on an immunological enigma"

In "Placenta - A neglected experimental Animal"

Ed. Beaconsfield, P. & Villee, C. Pergamon Press - Oxford pp.267-283.

BISCHOF, P. (1979)

"Purification and characterization of pregnancy-associated plasma protein A"

Archives Gynecology 227: 315-326.

BISCHOF, P. (1980)

"Observations on the isolation of macromolecules of placental origin"

In"Proceedings of the Serono Symposium Vol 35" Ed. Klopper, A.,

Genazzani, A. and Crosignani, P.G. pp.47-55.

Academic Press, London & New York.

BISCHOF, P. (1981)

"Pregnancy-associated plasma protein-A: an Inhibitor of the complement system"

Placenta 2: 29-34.

BISCHOF, P., BRUCE, D., CUNNINGHAM, P. & KLOPPER, A. (1979)

"Measurement of pregnancy-associated plasma protein A"

Clinica Chimica Acta 95: 243-247.

BISCHOF, P., HUGHES, G., KLOPPER, A. (1980)

"Relationship of obstetric parameters to the concentration of PAPP-A"

American Journal of Obstetrics & Gynecology 138: 494-499.

BISCHOF, P., HAENGGELI, L., SIZONENKO, M.T., HERRMANN, W. & SIZONENKO, P.C. (1981)

"Radio immunoassay for the measurement of pregnancy-associated plasma protein-A"

Biology of Reproduction 24: 1076-1081.

BISCHOF, P., DUBERG, S., HERRMANN, W. & SIZONENKO, P.C. (1982a)

"Amniotic fluid and plasma concentrations of PAPP-A throughout pregnancy: comparison with other fetoplacental products"

British Journal of Obstetrics & Gynecology 89: 358-363.

BISCHOF, P., LAUBER, K., DE WURSTEMBERGER, B. & GIRARD, J.P. (1982b)

"Inhibition of lymphocyte transformation by pregnancy-associated plasma protein A"

Journal Clinical & Laboratory Immunology 7: 61-65.

BISCHOF, P., RAPIN, C.H., WEIL, A. & HERRMANN, W. (1982c)

"Is pregnancy-associated plasma protein A a tumour marker?"

American Journal of Obstetrics & Gynecology 143: 379-381.

BISSONNETTE, J.M. (1982)

"Membrane vesicles from trophoblast cells as models for placental exchange studies"

Placenta 3: 99-106.

BOHN, H. (1980)

"Protein antigens on the human placenta" In "The human placenta: Proteins & hormones". Ed. Klopper, A., Genazzini, A. & Crosignani, P.G. Proceedings of the Serono Symposium Vol 35.

Academic Press - London. pp.23-34.

BOHN, H. & WINCKLER, W. (1977)

"Isolierung und Charakterisierung des Plazenta-Proteins PP5"

Archiv Fur Gynakologie 223: 179-186.

BOLTON, A.E. (1977)

"Radioiodination Techniques" In Reviews of the radio-chemical centre, vol XVII, pp.45-58. Amersham.

BOYD, J.D. & HAMILTON, W.J. (1970)

"The Human Placenta"
Published by Heffer & Sons Ltd. Cambridge, England.

BROCK, D.J.H. & SUTCLIFFE, R.G. (1972)

"Alpha-fetoprotein in the antenatal diagnosis of anencephaly and spina bifida"

Lancet 2(1) 197-199.

CHARD, T. (1976)

"Normality and abnormality" In "Plasma hormone assays in evaluation of fetal wellbeing" Ed. Klopper, A. pp.1-11.

Churchill Livingstone - Edinburgh.

COOPER, D.W. (1980)

"Immunological Relationships between Mother and Conceptus in man"

In "Immunological Aspects of Reproduction and Fertility control"

Ed. by J.P. Hearn. MTP Press Ltd. pp.33-61.

CRAWFORD, G.P.M., OGSTON, D. & DOUGLAS, A.S. (1976)

"The contribution of plasma protease inhibitors to antiplasmin activity in man"

Clinical Science & Molecular Medicine 51: 215-218.

CURRIE, G.A. & BAGSHAWE, K.D. (1967)

"The masking of antigens on trophoblast and cancer cells"

Lancet 1: 708-710.

CURZEN, P. (1968)

"The antigenicity of human placenta"

Journal Obstetrics & Gynecology of the British Commonwealth 75: 1128-1133.

DEWHURST, J. (1981) (ed)

"Integrated obstetrics & Gynecology for Postgraduates" 3rd edition

Blackwell Scientific publications - Oxford. pp.84-92 & 133-145.

DUBERG, S., BISCHOF, P., SCHINDLER, A.M., BEGUIN, F., HERRMANN, W. & SIZONENKO, P.C. (1982)

"Tissue and plasma concentrations of PAPP-A: comparison with other fetoplacental products"

British Journal of Obstetrics & Gynecology 89: 352-357.

DUBOIS, M., GILLIES, K.A., HAMILTON, J.K., REBERS, P.A. & SMITH, F. (1956)
"Colorimetric method for determination of sugars and related substances"
Analytical Chemistry 28: 350-356.

FAULK, W.P. & JOHNSON, P.M. (1977)
"Immunological studies of human placentae: identification and distribution of proteins in mature chorionic villi"
Clinical & Experimental Immunology 27: 365-375.

FAULK, W.P., SANDERSON, A.R. & TEMPLE, A. (1977)
"Distribution of MHC antigens in human placental chorionic villi"
Transplantation proceedings 9: 1379-1384.

FAULK, W.P., YEAGER, C., McINTYRE, J.A. & UEDA, M. (1979)
"Oncofetal antigens of human trophoblast"
Proceedings of the Royal Society (B) 206: 163-182.

FEENEY, R.E. (1971)
"The Non-Bond splitting mechanism of action of inhibitors of proteolytic enzymes - the conservative interpretation"
In"Proceedings of the International Research Conference on Proteinase Inhibitors 1970" Ed. Fritz, H. & Tschesche, H. pp.162-168.

FISHMAN, W.H. (1973)
"Carcinoplacental Isoenzyme Antigens"
Advances in Enzyme Regulation 11: 293-321.

FITZGERALD, M.J.T. (1978)
"Human Embryology"
Harper International Edition : New York. pp.25-32 & 169-175.

FOLKERSEN, J., WESTERGAARD, J.G., HINDERSSON, P. & TEISNER, B. (1979)
"Affinity chromatographic purification of a new high mol wt pregnancy specific protein, SP4" In "Carcino-embryonic Proteins" Vol 2 Ed. Lehmann, F.G. pp.503-506. Elsevier-North Holland, Amsterdam.

FOLKERSEN, J., GRUDZINSKAS, J.G., HINDERSSON, P., TEISNER, B., WESTERGAARD, J.G. (1981a)
"Purification of pregnancy-associated plasma protein-A by a two step affinity chromatographic procedure"
Placenta 2: 11-18.

FOLKERSEN, J., GRUDZINSKAS, J.G., HINDERSSON, P., TEISNER, B. & WESTERGAARD, J.G. (1981b)
"Pregnancy-associated plasma protein A: circulating levels during normal pregnancy"
American Journal of Obstetrics & Gynecology 139: 910-914.

GALBRAITH, G.M.P., GALBRAITH, R.M. & FAULK, W.P. (1980)
"Immunological studies of transferrin & transferrin receptors of human placental trophoblast"
Placenta 1: 33-46.

GALL, S.A. & HALBERT, S.P. (1972)
"Antigenic constituents in pregnancy plasma which are undetectable in normal non-pregnant male or female plasma"
International Archives of Allergy and Applied Immunology 42: 503.

GLEICHER, N., DEPPE, G. & COHEN, C.J. (1979)
"Common aspects of immunologic tolerance in pregnancy and malignancy"
Obstetrics & Gynecology 54(3): 335-342.

GOODFELLOW, P.N. (1982)
"Fetal antigens, cancer and the wooden spoon"
Immunology Today 3: 225-227.

GORDON, Y.B. & CHARD, T. (1979)

"The specific proteins of the human placenta; some new hypotheses"

In "Placental Proteins" pp.1-21. Ed. Klopper, A. & Chard, T.

Springer-Verlag, Berlin.

GORDON, Y.B. & GRUDZINSKAS, J.G. (1978)

"The role of biochemical tests and ultra-sound in antenatal diagnosis"

In "Diagnostic ultrasound applied to obstetrics and gynaecology"

Ed. by Sabbagha, R. Harper and Row, New York.

GORE, C.H. & SUTCLIFFE, R.G. (1983)

"Pregnancy associated plasma protein A: Purification under mild conditions, peptide mapping and tests for possible interactions with trypsin, plasmin and complement"

Placenta - In press.

GRUDZINSKAS, J.G., LENTON, E.A., GORDON, Y.B., KELSO, I.M., JEFFERY, D., SOBOWALE, O. & CHARD, T. (1977)

"Circulating levels of pregnancy specific glycoprotein in early pregnancy"

British Journal of Obstetrics and Gynaecology 84: 740-742.

GRUDZINSKAS, J.G., CHARNOCK, M., OBIEKWE, B.C., GORDON, Y.B. & CHARD, T. (1979)

"Placental protein 5 in fetal and maternal compartments"

British Journal of Obstetrics & Gynecology 86: 642-644.

HALBERT, S.P. & LIN, T-M (1979)

"Pregnancy associated plasma proteins, PAPP-A and PAPP-B"

In "Placental Proteins" Ed. Klopper, A. & Chard, T. pp.89-103.

Springer-Verlag, Berlin.

HALL, P.K. & ROBERTS, R.C. (1978)

"Physical and chemical properties of human plasma α_2 Macroglobulin"

Biochemical Journal 173(1): 27-38.

HAMBERG, U., STELWAGEN, P. & ERVAST, H-S (1973)

"Human α_2M, Characterization and trypsin binding"

European Journal of Biochemistry 40: 439-451.

HARPEL, P.C. (1976)

"Human α_2-Macroglobulin" In "Methods in Enzymology" Vol 45

"Proteolytic Enzymes" Part B. Ed. L. Lorand. pp.639-652.

HARPEL, P.C. (1977)

"The effectiveness of α_2 Plasmin-inhibitor in the presence of α_2M"

Journal Experimental Medicine 146: 1033-1040.

HARPEL, P.C. & HAYES, M.B. (1980)

"Immunoimmobilization of α_2 Macroglobulin-β-Trypsin complexes:

A novel approach for the biochemical characterization of modulator

protease interactions"

Analytical Biochemistry 108: 166-175.

HEARN, J.P. (ed) (1980)

"Immunological aspects of Reproduction and Fertility"

MTP Press Ltd. England.

HEIMBURGER, N. (1975)

"Proteinase Inhibitors of Human Plasma - Their properties and control

functions" In "Proteases & Biological control" Ed. Reich, E.,

Rifkin, D.B., Shaw, E. pp.367-386.

Cold Spring Harbour Conferences on Cell Proliferation, Volume 2.

HEYNER, S. (1973)

"Detection of H-2 antigens on the cells of the early mouse embryo"

Transplantation 16: 675-678.

HORNE, C.H.W., REID, I.N. & MILNE, G.D. (1976)

"Prognostic significance of inappropriate production of pregnancy

proteins by breast cancers"

Lancet VII: 279-282.

HORNE, C.H., TOWLER, C.M., PUGH-HUMPHREYS, R.P., THOMPSON, H.W. & BOHN, H. (1976)

"Pregnancy specific β1 glycoprotein - a product of the syncytiotrophoblast"

Experientia 32: 1197-1199.

HUGHES, G., BISCHOF, P., WILSON, G., SMITH, R. & KLOPPER, A. (1980)

"Tests of fetal well-being in the 3rd trimester of pregnancy"

British Journal of Obstetrics & Gynecology 87: 650-656.

HUNTER, W.H. & GREENWOOD, F.C. (1962)

"Preparation of Iodine-131 labelled human growth hormone of high specific activity"

Nature (Lond) 194: 495-496.

JAMES, K. (1980)

"Alpha-2-macroglobulin and its possible importance in immune systems"

Trends in Biochemical Science 5: 43-47.

JEPPSSON, J-O, LAURELL, C-B (1975)

"Function and Chemical composition of α_1-antitrypsin"

In "Proteases & Biological Control" Ed. by Reich, E., Rifkin, D.B. & Shaw, E. pp.405-414.

Cold Spring Harbour Conferences on Cell Proliferation Volume 2.

JOHNSON, P.M. (1982)

"Challenges to Nature's transplant"

Immunology Today 3: 130-131.

JOHNSON, P.M. & BROWN, P.J. (1981)

"Fcγ receptors in the Human Placenta"

Placenta 2: 355-370.

JOHNSON, P.M., BROWN, P.J. & FAULK, W.P. (1980)

"Immunobiological aspects of the human placenta"

In "Oxford reviews of Reproductive biology" Ed. Finn, C.A.

Vol 2, pp.1-40. Oxford University Press - Oxford.

JONES, W.R. (1976)

In "Immunology of Human Reproduction" Ed. Scott, J.S., Jones, W.R.

pp.375-413. Academic Press - New York.

JOSIMOVICH, J.B. (1977)

"Human Placental Lactogen"

In "Endocrinology of Pregnancy" Ed. Fuchs, F. & Klopper, A.

2nd Ed. Harper & Row, Maryland, pp.191-205.

KEFALIDES, N.A. (1975)

"Basement Membranes: Current concepts of structure and synthesis"

Dermatologica 150: 4-15.

KHALEF, S.A., BUULTJENS, E. & SUTCLIFFE, R.G.

In preparation.

KIRBY, D.R.S., BILLINGTON, W.D., BADBURY, S. & GOLDSTEIN, D.J. (1964)

"Antigen barrier of the mouse placenta"

Nature (Lond) 204: 548-549.

KLOPPER, A. (1969)

"The assessment of placental function in clinical practice"

In "Foetus and Placenta" Ed. by Klopper, A., Diczfalusy, E.

Blackwell Scientific Publications, Oxford. pp.471-555.

KLOPPER, A. (1980)

"The new placental proteins"

Placenta 1: 77-89.

KLOPPER, A., SMITH, R., and DAVIDSON, I. (1979)

"The measurement of trophoblastic proteins as a test of placental function" In "Placental Proteins" Ed. Klopper, A. & Chard, T. Springer Verlag, Berlin. pp.23-41.

KOEHLER, L.H. (1952)

"Differentiation of CHOs by Anthrone Reaction rate and colour intensity" Analytical Chemistry 24: 1576-1579.

KUKULSKA-LANGLANDS,B.M. (1980)

"Studies on human placental proteins"

Ph.D. Thesis University of Glasgow, Scotland.

LAURELL, C.B. (1966)

"Quantitative estimation of proteins by electrophoresis in Agarose Gel containing antibodies"

Analytical Biochemistry 15: 45-52.

LAW, S.W. & DUGAICZYK, A. (1981)

"Homology between the primary structure of α-fetoprotein, deduced from a complete cDNA sequence, and serum albumin"

Nature 291: 201-205.

LETCHWORTH, A.T. (1976)

"Human placental lactogen assay as a guide to fetal wellbeing"

In "Plasma hormone assays in evaluation of fetal wellbeing"

Ed. Klopper, A. Churchill, Livingstone, Edinburgh. pp.147-173.

LETCHWORTH, A.T. and CHARD, T. (1972)

"HPL as a screening test for fetal distress and neonatal asphyxia"

Lancet i: 704-706.

LIN, T.M., HALBERT, S.P. & KIEFER, D. (1973)

"Characterization and purification of human pregnancy associated plasma proteins"

Federation Proceedings 32: 623.

LIN, T.M., HALBERT, S.P. & KIEFER, D. (1974)

"Pregnancy-associated serum antigens in the rat and mouse"

Proceedings of the Society of Experimental Biology & Medicine 145: 62-66.

LIN, T.M., HALBERT, S.P. & SPELLACY, W.N. (1974)

"Measurement of pregnancy-associated plasma proteins during human gestation"

Journal of Clinical Investigation 54: 576-582.

LIN, T.M., HALBERT, S.P., KEIFER, D., SPELLACY, W.N. (1974a)

"Three pregnancy-associated human plasma proteins. Purification, monospecific antiserum and immunological identification"

International Archives of Allergy and Applied Immunology 47: 35-53.

LIN, T.M., HALBERT, S.P., KIEFER, D., SPELLACY, W.N. & GALL, S. (1974b)

"Characterization of four human pregnancy – associated plasma proteins"

American Journal of Obstetrics & Gynecology 118: 223-236.

LIN, T.M. & HALBERT, S.P. (1976)

"Placental localization of human pregnancy-associated plasma proteins"

Science 193: 1249-1252.

LIN, T.M., HALBERT, S.P. & SPELLACY, W.N. (1976)

"Relationship of obstetric parameters to the concentration of four pregnancy-associated plasma proteins at term in normal gestation"

American Journal of Obstetrics & Gynecology 125: 17-24.

LIN, T.M., HALBERT, S.P., SPELLACY, W.N. and GALL, S. (1976)

"Human pregnancy-associated proteins during the post-partum period"

American Journal of Obstetrics and Gynecology 124: 382-387.

LIN, T.M., HALBERT, S.P., SPELLACY, W.N. & BERNE, B.H. (1977)

"Plasma concentrations of four pregnancy-associated plasma proteins in complications of pregnancy"

American Journal of Obstetrics & Gynecology 128: 808-810.

LIN, T.M. & HALBERT, S.P. (1978)

"Immunological relationship of human and subhuman primate pregnancy-associated plasma proteins"

International Archives of Allergy and Applied Immunology 56: 207-223.

LLEWELLYN-JONES, D. (1969)

"Infectious diseases in pregnancy" In "Fundamentals of Obstetrics and Gynaecology" pp.245. Faber and Faber: London.

LOKE, Y.W., JOYSEY, V.C. & BORLAND, R. (1971)

"HLA-antigens on human trophoblast cells"

Nature (London) 232: 403-405.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., RANDALL, R.J. (1951)

"Protein measurement with the folin phenol reagent"

Journal of Biological Chemistry 193: 265-275.

MAYER, M.M. (1961)

"Complement and complement fixation" In "Experimental Immunochemistry Eds. Kabat, E.A. & Mayer, M.M. 2nd ed. p.133.

Springfield, Illinois, Charles C. Thomas.

MENSER, M.A. (1976)

"Immunological aspects of maternal infection"

In "Immunology of Human Reproduction" Ed. Scott, J.S. & Jones, W.R.

Academic Press, London.

MOSHER, D.F. (1980)

"Fibronectin"

Progress Hemostasis & Thrombosis 5: 111-151.

MULLERTZ, S. & CLEMMENSEN, I. (1976)

"The primary inhibitor of plasmin in human plasma"

Biochemical Journal 159: 545-553.

McCORMICK, J.N., FAULK, W.P., FOX, H. & FUDENBERG, H.H. (1981)
"Immunohistological and elution studies of the human placenta"
Journal of Experimental Medicine 133: 1-18.

McINTYRE, J.A. & FAULK, W.P. (1979)
"Role of the major histocompatibility complex in the immunology of trophoblast antigens" In "Placenta - A neglected experimental animal" Ed. Beaconsfield, P. & Villee, C.
Pergamon Press, Oxford. pp.294-305.

McINTYRE, J.A., HSI, B., FAULK, W.P., KLOPPER, A. & THOMSON, R. (1981)
"Immunological studies of the human placenta: function and morphological analysis of PAPP-A"
Immunology 44: no. 3,577-583.

McKAY, E.J. & LAURELL, C-B (1980)
"The interactions of heparin and plasma proteins. Demonstration of different binding site for anti-thrombin III complexes and anti-thrombin III.
Journal of Laboratory & Clinical Medicine 95: 69-80.

OGSTON, D., BENNETT, B., HERBERT, R.J. & DOUGLAS, A.S. (1973)
"The inhibition of urokinase by alpha-2-macroglobulin"
Clinical Science 44: 73-79.

PECK, A.B., MURGITA, R.A., WIGZELL, H. (1978)
"Cellular and Genetic restrictions in the immunoregulatory activity of alpha-fetoprotein. II. AFP-induced suppression of cytotoxic T lymphocytes"
Journal of Experimental Medicine 148: 360-372.

PIJNENBORG, R., ROBERTSON, W.B., BROSENS, I. & DIXON, G. (1981)
"Trophoplast Invasion & the establishment of haemochorial placentation in man and laboratory animals"
Placenta 2: 71-92.

PORATH, J., AXEN, R. & ERNBACK, S. (1967)

"Chemical coupling of proteins to agarose"

Nature (Lond) 215: 1491-1492.

PUTNAM, F.W. (1965)

"Structure and function of the Plasma Proteins"

In "The Proteins: Composition, Structure & Function" Vol III.

Ed. by Neurath, H. pp. 153-267.

Academic Press - New York and London.

RENDINA, G. (1971)

"Experimental methods in Modern Biochemistry" pp.68-74.

W.B. Saunders Company. Philadelphia and London.

ROBBINS, K.C. (1978)

"Plasmin" In "Fibrinolytics & Antifibrinolytics"

Handbook of Experimental Pharmacology Vol 46. Ed. Markwardt, F.

Springer-Verlag, Berlin. pp.317-336.

ROBERTS, R.C., NELLES, L.P., HALL, P.K., SALVESEN, G.S. & MISCHLER, E.

(1982)

"Comparison of the structure and aspects of the proteinase-binding

properties of cystic fibrotic α_2M with normal α_2M"

Pediatric Research 16(6): 416-423.

ROBERTSON, W.B., BROSENS, I.A. & DIXON, G. (1975)

"Uteroplacental vascular pathology" In "Human Placentation"

Ed. Brosens, I.A., Dixon, G., & Robertson, W.B.

Excerpta Medica - Amsterdam, pp.47-65.

ROCKLIN, R.E., KITZMILLER, J., CARPENTER, B., GAROVOY, M. & DAVID, J.R.

(1976)

"Maternal-fetal relation: Absence of an immunologic blocking factor

from the serum of women with chronic abortions"

New England Journal of Medicine 295: 1209-1213.

ROMNEY, S.L., WHARTON, J.T. and FLETCHER, G. (1975)
"Malignant Diseases" In "Gynecology and Obstetrics; the Health Care of Women"
McGraw-Hill Book Company, New York. pp.474-512.

RUOSLAHTI, E., HAYMAN, E.G., PIERSCHBACHER, M., ENGVALL, E. (1982a)
"Fibronectin: Purification, Immunochemical Properties and Biological Activities" In "Methods in Enzymology" 82: 803-831.
Structural and contractile proteins, Part A: Extracellular matrix.
Ed. Cunningham, L.W. & Frederiksen, D.W. Academic Press New York.

RUOSLAHTI, E., PIERSCHBACHER, M., HAYMAN, E.G., ENGVALL, E. (1982b)
"Fibronectin: A molecule with remarkable structural and functional diversity"
Trends in Biochemical Science 77 (vol 7 no. 5): 188-190.

SALEM, H.T., OBIEKWE, B.C., AL-ANI, A.I.M., SEPPALA, M. and CHARD, T. (1980)
"Molecular heterogeneity of placental protein 5 (PP5) in late pregnancy serum and plasma: evidence for a heparin-PP5 polymer"
Clinica Chimica Acta 107: 211-215.

SAUNDERS, R., DYCE, B.J., VANNIER, W.E. & HAVERBACK, B.J. (1971)
"The separation of alpha-2-macroglobulin into 5 components with differing electrophoretic and enzyme-binding properties.
Journal of Clinical Investigation 50: 2376-2383.

SCHULTZ-LARSEN, P., HERTZ, J.B. (1978)
"The predictive value of pregnancy specific β_1 glycoprotein (SP_1) in threatened abortion"
European Journal of Obstetrics, Gynecology and Reproductive Biology 8: 253.

SIMMONS, R.L., LIPSCHULTZ, M.L., RIOS, A. & RAY, P.K. (1971)
"Failure of neuraminidase to unmask histocompatibility antigens on trophoblast"
Nature (New Biology, London) 231: 111-112.

SINOSICH, M.J., TEISNER, B., DAVEY, M. and GRUDZINSKAS, J.G. (1981)
"Pregnancy associated plasma Protein-A: Interaction with heparin in crossed affinity immunoelectrophoresis"
Australian and New Zealand Journal of Medicine 11: 429-433.

SINOSICH, M.J., TEISNER, B., FOLKERSEN, J., SAUNDERS, D.M. & GRUDZINSKAS, J.G. (1982)
"Radioimmunoassay for pregnancy-associated plasma protein A"
Clinical Chemistry 28(1): 50-53.

SMITH, R., BISCHOF, P., HUGHES, G. & KLOPPER, A. (1979)
"Studies on PAPP-A in the 3rd trimester of pregnancy"
British Journal of Obstetrics and Gynecology 86: 882-887.

SMITH, R., COOPER, W. & THOMSON, M.A.R. (1980)
"Pregnancy-associated plasma protein A. A measure of placental ageing"
In "The human Placenta: Proteins and Hormones"
Proceedings of the Serono Symposia 35. Ed. by Klopper, A., Genazzani, A. & Crosignani, P.G. Academic Press, London. pp.109-113.

SMITH, R., THOMSON, M.A.R. & COOPER, W. (1981)
"The relationship between changing values of PAPP-A in late pregnancy and the onset of labour"
Placenta 2(2): 143-148.

SPIRO, R.G. (1960)
"Studies on Fetuin, a Glycoprotein of Fetal serum"
Journal of Biological Chemistry 235: 2860-2869.

SPIRO, R.G. (1970)
"Glycoproteins"
Annual Review of Biochemistry 39: 599-638.

STARKEY, P.M. (1981)

"Evolution of human α_2Macroglobulin"

Acta Biologica et Medica Germanica 40: 1555-1559.

STARKEY, P.M. & BARRETT, A.J. (1977)

"α_2M, a physiological regulator of proteinase activity"

In "Proteinases in mammalian cells and tissues"

Ed. A.J. Barrett. North-Holland Publishing Company, Amsterdam,

New York, Oxford. pp.663-696.

STEINBUCH, M., PEJAUDIER, L., QUENTIN, M. and MARTIN, V. (1968)

"Molecular alteration of α_2 macroglobulin by aliphatic amines"

Biochimica et Biophysica Acta 154: 228-231.

STEVENS, V.C. (1980)

"The current status of anti-pregnancy vaccines based on synthetic fractions of HCG" In "Immunological aspects of reproduction and fertility control" Ed. Hearn, J.P.

MTP Press Ltd., England. pp.203-216.

STIMSON, W.H. (1975)

"Correlation of the blood level of a pregnancy-associated α-macroglobulin with the clinical course of cancer patients"

Lancet 1(2): 777-779.

STITES, D.P., PAVIA, C.S., CLEMENS, L.E., KUHN, R.W. & SIITERI, P.K. (1979)

"Immunologic regulation in pregnancy"

Arthritis & Rheumatism 22: 1300-1307.

SUNDERLAND, C.A., REDMAN, C.W.G. and STIRRAT, G.M. (1981)

"HLA A, B, C antigens are expressed on non-villous trophoblast on the early human placenta"

Journal of Immunology 127: 2614-2615.

SUNDERLAND, C.A., NAIEM, M., MASON, D.Y., REDMAN, C.W.G., & STIRRAT, G.M. (1981)
"The expressions of major histocompatibility antigens on human chorionic villi"
Journal of Reproductive Immunology 3: 323-331.

SUTCLIFFE, R.G., BROCK, D.J.H., NICHOLSON, L.V.B. & DUNN, E. (1978)
"Fetal and uterine-specific antigens in human amniotic fluid"
Journal of Reproduction & Fertility 54: 85-90.

SUTCLIFFE, R.G., KUKULSKA, B.M., NICHOLSON, L.V.B. & PATERSON, W.F. (1979)
"The use of antibody affinity chromatography and other methods in the study of pregnancy associated proteins"
In "Placental Proteins" Ed. Klopper, A. & Chard, T.
Springer-Verlag, Berlin, Heidelberg. pp.55-75.

SUTCLIFFE, R.G., HUNTER, J.B., GIBB, S. and MacLEAN, A.B. (1980a)
"Studies on Human Pregnancy-associated Plasma Protein A"
In "Proceedings of the Serono Symposia" Vol 35.
Ed. Klopper, A., Genazzani, A. and Crosignani, P.G. pp.57-66.
Academic Press, London and New York.

SUTCLIFFE, R.G., KUKULSKA-LANGLANDS, B.M., COGGINS, J.R., HUNTER, J.B. and GORE, C.H. (1980b).
"Studies on human pregnancy-associated plasma protein A"
Biochemical Journal 191: 799-809.

SUTCLIFFE, R.G., DAVIES, M., HUNTER, J.B., WATERS, J.J. & PARRY, J.E. (1982a)
"The protein composition of the fibrinoid material at the human uteroplacental interface"
Placenta 3: 297-308.

SUTCLIFFE, R.G., KUKULSKA-LANGLANDS, B.M., HORNE, C.H.W., MacLEAN, A.B., JANDIAL, V., SUTHERLAND, H.W., GIBB, S., BOWMAN, A.W. (1982b)
"Studies on the concentration of pregnancy-associated Plasma Protein-A during normal and complicated pregnancy"
Placenta 3: 71-80.

SUTCLIFFE, R.G., KHALEF, S.A., HORNE, C.H.W.
In preparation.

SZULMAN, A.E. (1973)
"The A, B and H blood group antigens in human placenta"
New England Journal of Medicine 286: 1028-1031.

TATARINOV, Y.S. & SOKOLOV, A.V. (1977)
"Development of a radioimmunoassay for pregnancy-specific β_1 glycoprotein and its measurement in serum of patients with trophoblastic and nontrophoblastic tumours"
International Journal of Cancer 19: 161-165.

TATARINOV, Y.S., KOZLJAEVA, G.A., PETRUNIN, D.D. & PETRUNINA, Y.A. (1980)
"Two new human placenta-specific α-globulins"
In "The human placenta: Proteins & Hormones" Proceedings of the Serono Symposia, Vol 35, pp.35-46.
Ed. by Klopper A., Genazzani, A. & Crosignani, P.G. Academic Press, London.

TATRA, G. (1979)
"Clinical Aspects of Pregnancy-specific β_1 glycoprotein (PSβG)"
In "Placental Proteins" Ed. by Klopper, A. and Chard, T.
Springer-Verlag, Berlin. pp. 135-142.

TAYLOR, C. and FAULK, W.P. (1981)
"Prevention of recurrent abortion with leukocyte transfusions"
Lancet ii: 68-70.

THAU, R.B. and LANMAN, J.T. (1975)

"Endocrinological Aspects of placental function" In "The placenta" Ed. by Gruenwald, P. MTP, Medical and Technical Publishing Co. Ltd., Lancaster. pp. 123-144.

TOWLER, C.M., HORNE, C.H.W., JANDIAL, V., CAMPBELL, D.M. & MacGILLIVRAY, I. (1976)

"Plasma levels of pregnancy-specific β_1 glycoprotein in normal pregnancy"

British Journal of Obstetrics and Gynecology 83: 775-779.

TOWLER, C.M., HORNE, C.H., JANDIAL, V., CAMPBELL, D.M. & MacGILLIVRAY, I. (1977)

"Plasma levels of pregnancy-specific β_1-glycoprotein in complicated pregnancies"

British Journal of Obstetrics & Gynecology 84: 258-263.

TRAVIS, J., BOWEN, J., TEWKSBURY, D., JOHNSON, D. & PANNELL, R. (1976)

"Isolation of Albumin from Whole Human Plasma and fractionation of Albumin depleted plasma"

Biochemical Journal 157: 301-306.

TROTTER, W. (1941)

"Collected papers of Wilfred Trotter"

Oxford University Press.

VAN LEUSDEN, H.A. (1976)

"Chorionic Gonadotrophin in pathological pregnancy"

In "Plasma Hormone Assays in evaluation of fetal wellbeing" Ed. Klopper, A. Churchill Livingstone, Edinburgh. pp.48-71.

VAN LEUVEN, F. (1982)

Human α_2-Macroglobulin: structure and function"

Trends in Biochemical Science 77 (vol 7 no. 5): 185-187.

VAN LEUVEN, F., CASSIMAN, J.J. and VAN DEN BERGHE, H. (1982)

"Functional Modifications of α_2-Macroglobulin by primary amines"

Biochemical Journal 201: 119-128.

VIRCA, G.D., TRAVIS, J., HALL, P.K. & ROBERTS, R.C. (1978)

"Purification of human α_2M by chromatography on Cibacron blue Sepharose"

Analytical Biochemistry 89: 274-278.

VOISIN, G.A. (1979)

"Immune agents of the facilitation reaction. Their possible role in protection of the placental allograft"

In "Placenta - A neglected experimental animal"

Ed. Beaconsfield, P. & Villee, C. Pergamon Press, Oxford. pp.283-294.

WAHLSTROM, T., TEISNER, B., FOLKERSEN, J. (1981)

"Tissue localization of PAPP-A in normal placenta"

Placenta 2 no. 3: pp.253-258.

WALKER, J. & JAMES, G.B. (1976)

In "Combined Textbook of Obstetrics & Gynecology"

Ed. Walker, J., MacGillivray, I., MacNaughton, M.

Churchill Livingstone, Edinburgh, 9th ed. pp.116-124.

WILD, A.E. (1973)

"Transport of immunoglobulins and other proteins from mother to young"

In "Lysosomes in Biology & Pathology"

Ed. Dingle J.T. Vol. 3 pp.425-464. Amsterdam : North-Holland.

WILD, A.E. (1979)

"Placental antibody transport and immunological protection - their cellular mechanisms" In "Placenta - A neglected experimental Animal" Ed. Beaconsfield, P. & Villee, C.

Pergamon Press - Oxford. pp.306-315.

WILLINGHAM, M.C., MAXFIELD, F.R. & PASTAN, I.H. (1979)

"α_2M binding to the plasma membrane of cultured fibroblasts"

Journal of Cell Biology 82: 614-625.

WINGATE, M.B., IFFY, L., KELLY, J.V., BIRNBAUM, S. (1976)

"Diseases Specific to pregnancy" In "Gynecology & Obstetrics:

The Health care of Women"

McGraw-Hill Book Company, New York. pp.713-776.

WOOD, G.W. (1980)

"Mononuclear phagocytes in the human placenta"

Placenta 1: 113-123.

WYNN, R.M. (1975)

"Development and ultrastructural adaptations of the human placenta"

In "Human Placentation" Ed. Brosens, I.A., Dixon, G. & Robertson,

W.B. Excerpta Medica - Amsterdam. pp. 3-21.