THE ASSAY OF ONCODEVELOPMENTAL PROTEINS . THEIR USE IN FOETOPLACENTAL FUNCTION MONITORING AND IN THE DIAGNOSIS AND MONITORING OF MALIGNANT DISEASE .

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AB	Antibody
AB-E	Antibody-Enzyme
AFP	Alpha-foeto-protein
AU/L	Arbitrary units/litre
BSA	Bovine Serum albumin
CEA	Carcino embryonic antigen
CPAP	Carcino-placental alkaline phosphatase
ELISA	Enzyme linked immunosorbent assay
EMIT	Enzyme multiplied immunoassay technique
FSH	Follicle stimulating hormone
HCG	Human chorionic gonadotrophin
HPL	Human placental lactogen
HR P	Horse-radish peroxidase
HS A	Human Serum Albumin
IRP	International Reference Preparation
IUGR	Intra uterine growth retardation
LFD	Light for dates
LH	Luteinizing hormone
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NIBSC	National Institute for Biological Standards and Control
NSB	Non Specific Binding
OPD	Ortho-phenylene diamine
PAPP-A	Pregnancy Associated Plasma Protein A
PBS	Phosphate Buffered Saline
PEG	Poly-Ethylene Glycol
PIH	Pregnancy Induced Hypertension
RIA	Radioimmunoassay
RID	Radial immunodiffusion
TBS	Tris-buffered saline
TSH	Thyroid Stimulating Hormone

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CONTENTS

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SUMMARY

Historical aspects and general concepts regarding the onco-developmental proteins are reviewed together with a review of the specific proteins studied.

Radioimmunoassays for hPL, hCG and AFP are described together with studies of the assay parameters. The methods were shown to be suitable for use in this thesis.

General concepts of enzyme-immunoassays are described. A previously described ELISA for SP_1 was investigated and further sensitized for use in monitoring patients with tumours producing SP_1.

Enzyme immunoassays for PAPP-A and CPAP were developed and validated for use in monitoring pregnant patients and patients with tumours producing PAPP-A. Problems specific to the assay of PAPP-A and SP_1 are described.

Certain factors influencing ELISA are considered - the choice of a suitable solid phase, the leakage of antibody from the solid phase and the reduction of NSB by use of protein or detergent.

Plastic microtitre plates were shown to be suitable as a solid phase although antibody leakage was confirmed. NSB was reduced to acceptable levels.

An antigen excess effect was noted during time course experiments on various antigens. A possible explanation is provided and the importance of time course studies in assay devlopment is emphasized.

Studies into the effects of molecular heterogeneity of SP_1 on ELISA were performed.

Methods of automated data reduction were developed after investigating the kinetics of various stages of the assays. The time courses were shown to obey previously described mathematical relationships and computer programs were developed to provide curve fitting for automating assays.

Having established assay conditions, the application of the measurement of these proteins to pregnancy and oncology were studied.

Reference ranges of hPL, SP_1 and PAPP-A were established for the third trimester of pregnancy. Investigations into the concentrations of these proteins in patients producing a light for dates baby, patients with a poor weight profile during pregnancy, patients with pregnancy induced hypertension and pregnant diabetic patients were performed.

HPL was shown to be a useful predictor of intra uterine growth retardation and conversely could be used for reassurance in patients with poor weight profiles who produce normal weight babies.

Previous reports of elevated PAPP-A concentrations in patients with hypertension were not confirmed. A possible relationship between SP_1 and carbohydrate metabolism in pregnancy was shown.

In the field of oncology, other than established markers such as AFP and HCG, only CPAP was shown to be of possible use in patients with seminoma or ovarian carcinoma. The percentage of patients with positive results was low however and any use would be in a monitoring situation only.

The importance of test assessment is discussed and possible development of markers in the field of oncology are alluded to.

CHAPTER 1

THE ONCODEVELOPMENTAL PROTEINS

1.1 Historical Aspects

The human foeto-placental unit produces a wide variety of proteins. In addition, many of these have now been found to have analogues produced by neoplastic cells. Thus the term "onco-developmental" (encompassing placental, foetal and oncological) proteins was coined to describe this group.

The exclusive foetal, placental or neoplastic production is now open to question however (Searle et al 1978, Duberg et al 1982, Rosen 1982).

Although many of these proteins have only been recognized in the last 25 years, one pregnancy protein (human chorionic gonadotrophin) has been known for many years (Zondek and Ascheim 1927).

Several years later, in 1934 an increase in serum alkaline phosphatase in normal pregnancy was recognized (Coryn 1934) which was later shown to be of placental origin (Messer 1967).

Some 30 years after Ascheim's original work, the first recorded mention of a foetal protein - α foeto protein is found (Bergstrand and Czar 1957).

This was followed a year later by Thornes who demonstrated, using a gel diffusion technique, that pregnancy serum contained a group of proteins which were not present in serum from a non pregnant population (Thornes 1958). Using starch gel electrophoresis, Smithies (1959) was able to show that in the α_2 region there was a distinct band from about 10% of sera from pregnant women. He referred to this band as the Pregnancy Zone. Subsequently, Afonzo and Farnham (1962) and Cooper (1963) were able to demonstrate that this "Pregnancy Zone" protein identified by Smithies was present in over 80% of pregnant subjects.

Table 1 I Classification of the Oncodevelopmental Proteins

Trophoblastic specific	Pregnancy Associated	Foetal
Chorionic gonadotrophin (hCG)	Pregnancy associated α_2 glycoprotein (α_2 PAG)	Carcinoembryonic antigen (CEA)
Human placental lactogen (hPL)	Sex hormone binding globulin (SHBG)	Alpha foetoprotein (AFP)
Pregnancy specific β_1 glycoprotein (SP_1)	Placental proteins PP_1 PP_2 PP_3 PP_4 PP_6 PP_7	
Placental Protein 5 (PP_5)		
Pregnancy Associated Plasma Protein A (PAPP-A)*		
PAPP-B*		

* ? Pregnancy Associated

The isolation in 1962 of human placental lactogen (Josimovich and Maclaren 1962) opened the way to the application of circulating concentrations of placental proteins to the monitoring of placental function.

Similarly the discovery of the Regan isoenzyme of alkaline phosphatase (Fishman et al 1968) opened the way to the biochemical monitoring of neoplastic processes, widening the search for other markers.

In 1970, Tatarinov and Masyukevich (1970), and independently in 1971, Bohn described the first of the "new placental proteins", Pregnancy Specific β_1 Glycoprotein (Schwangerschafts - protein 1, SP_1).

Since this time considerable interest has been generated in the pregnancy proteins. Thus in 1980 Bohn reported that 10 proteins were known (1980 a). Two years later it was twice that number (Klopper 1982).

With the increasing number of onco developmental proteins, some form of classification became necessary such as the one shown in Table 1I (after Horne and Nisbet 1979). However this type of classification is not entirely satisfactory. Thus SP_1 has been shown to be produced by fibroblasts in vitro (Rosen 1982) and epithelial cells cultured from human amniotic fluid (Heikinheimo et al 1980). Similarly production of pregnancy associated plasma protein A (PAPP-A) by non trophoblastic cells in vivo has been shown (Duberg et al 1982).

Such classifications as exist must not be regarded as rigid but require updating as knowledge increases.

1.2 The Placental Proteins

1.2.1 General Concepts

It is known that placental cells contain a wide range of proteins and small peptides. Some are produced by this organ, whilst others appear simply as a consequence of the placenta containing a large volume of maternal blood and thus reflect the general elevation of these proteins in pregnancy.

As stated before, many placental proteins are not truly specific to the placenta. Nonetheless measurement of these may be relevant to studies on this organ in that:-

i) They reflect aspects of the function of an organ which is the only lifeline to the foetus.

ii) Their measurement is widely used as a clinical index of the adequacy of this function.

iii) The placenta has many features in common with a wide range of tumours of non-placental origin.

1.2.2 The Site of Production of Placental Proteins

Until recently, identification of the production site of placental proteins was by immunohistochemical localization. However, demonstration of the presence of a protein in a tissue does not distinguish synthesis from storage. Also, immunohistochemistry is fraught with problems of non-specificity - a problem even more obvious when applied to the placenta (Gau and Chard 1976).

Nevertheless, the syncytiotrophoblast appears to be the principal site of production in late pregnancy. It is possible that in early pregnancy the cytotrophoblast may

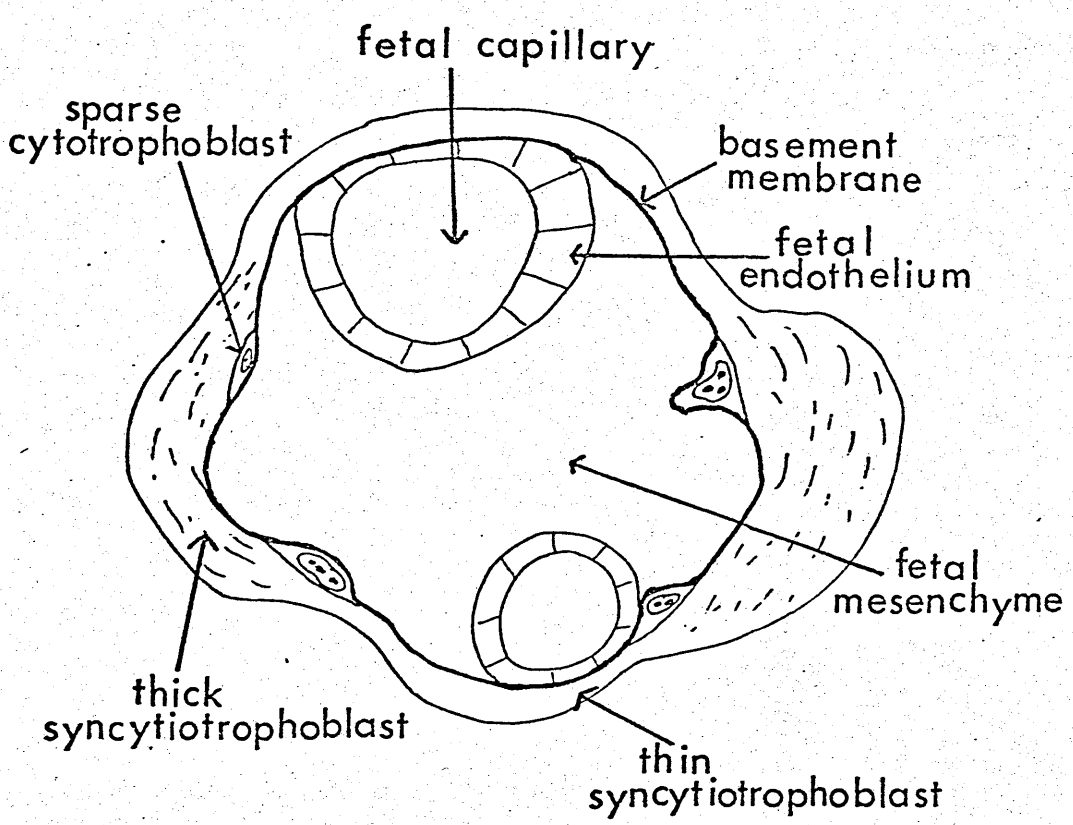

Fig 1i Diagramatic representation of the Syncytiotrophoblast and foetal capillary showing a thick area specialized for protein synthesis and a thin area (Vasculo-syncytial membrane) specialized for transfer.

also contribute - a theory that would be compatible with the fact that the cytotrophoblast is abundant and active at this time and has a somewhat nebulous interface with the syncytium.

McWilliams and Boime (1980) have confirmed the exclusive synthesis of hPL by the syncytiotrophoblast, using an isotopically labelled gene probe to detect specific mRNA. The exact site of protein synthesis has been the subject of a number of studies. Burgos and Rodriguez (1966) observed that there are "thick" areas of syncytiotrophoblast which are rich in endoplasmic reticulum and microvilli; and thin areas without microvilli which appear to be specialized for nutrient and waste material transport (Fig 1 i).

1.3 Production of Oncodevelopmental Proteins by the Normal Adult

It is well recognized that normal adults produce the so called foetal proteins α-foeto protein (AFP) and carcinoembryonic antigen (CEA) in small amounts. A considerable body of evidence now exists to show that the so called "specific" placental proteins are in fact produced by normal non pregnant adults. Allusion to SP_1 production by fibroblasts in vitro (Rosen 1982) has already been made. Similarly, hCG has been isolated from normal testes (Braunstein et al 1975) and more recently these observations have been extended by the demonstration of other "placental" proteins in seminal plasma (Ranta et al 1981, Bischof et al 1983).

The source of these proteins is unknown; they cannot be attributed to the testes or the sperm as levels are identical in vasectomized and non vasectomized subjects. Again, the functional significance is unknown.

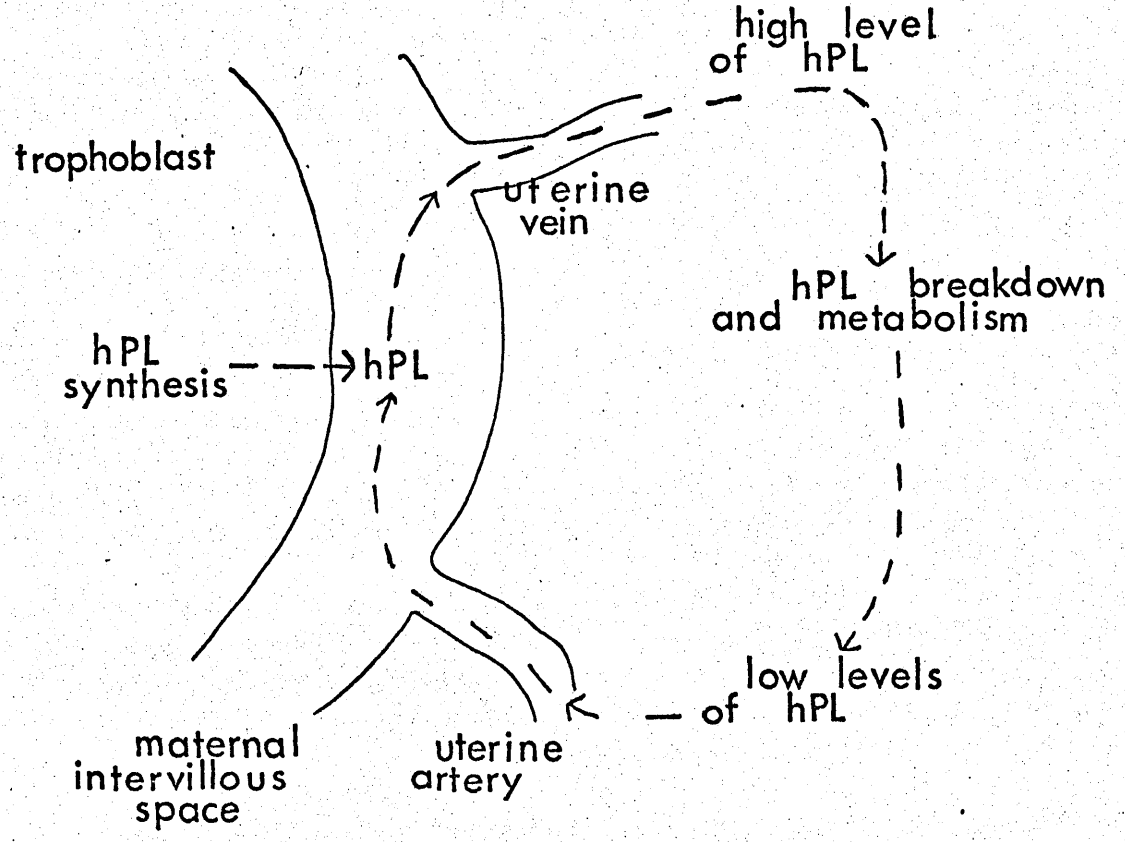

Fig 1 ii Diagram to show how the utero-placental blood flow may control the rate of synthesis of hPL by the trophoblast (After Chard 1982a).

1.4 Control Mechanisms for the production of placental proteins

Few mechanisms have been identified which control the production of placental proteins. Mechanisms have been proposed for the control of hPL release by maternal carbohydrate and lipids, but the evidence is subject to alternative explanations (Pavlou et al 1972).

Chard however has proposed a hypothesis which accounts for all the known facts about placental protein synthesis (Chard 1982a). He suggests that the potential for placental protein production is a direct function of the total mass of the trophoblast, that the rate of release (and secondarily of synthesis) is a function of the concentration in the maternal blood of the intervillous space surrounding the syncytiotrophoblast and that this in turn depends on the rate of blood flow in the intervillous space. (Fig 1 ii). The key implication is that the rate of placental protein production will be closely related to uteroplacental blood flow.

It may also explain why non-placental areas of the trophoblast ie chorion produce only very small quantities of placental proteins as a direct result of their not being in contact with a large fast flowing pool of blood.

1.5 Production of Oncodevelopmental Proteins by Tumours

It is now certain that a small proportion of adult non trophoblastic tumours secrete placental proteins including hCG (Vaitukatis 1977), hPL (Rosen et al 1975) and SP_1 (Grudzinskas et al 1980 a). Since it is clear that "placental" proteins can appear in normal tissues, these findings are

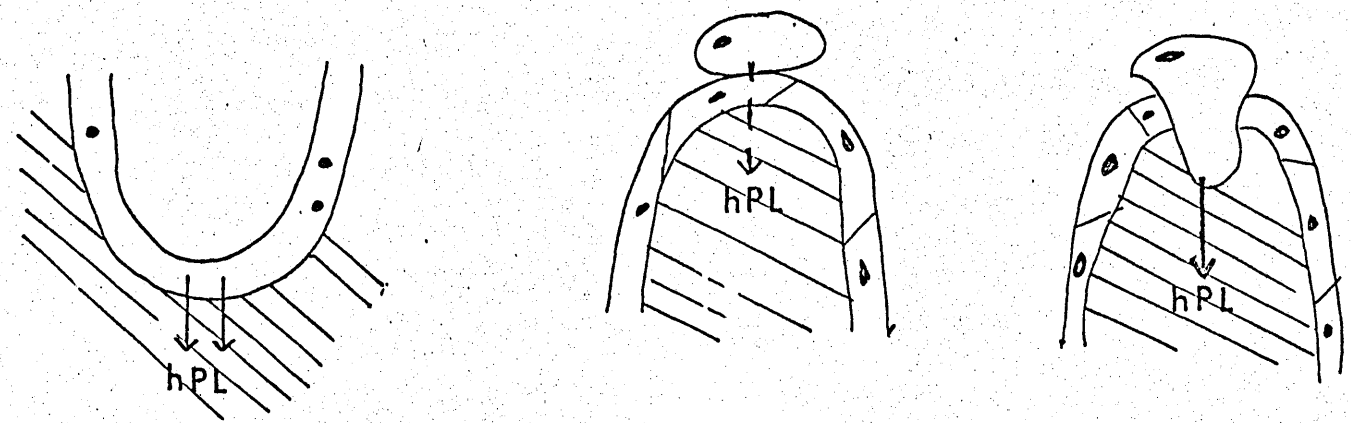

Fig 1 iii A new theory on the Ectopic production of hPL by Tumours (After Chard 1982a).

a) Placenta Trophoblast cells in direct contact with maternal bloodstream - hPL synthesized and released at substantial rate.

b) Normal Tissue Trophoblast type cell separated from blood by basement membrane and endothelium - insignificant release of hPL.

c) Cancer Tissue Trophoblast type cell invading bloodstream in a cancer-hPL synthesized and released at substantial rate.

rather less surprising - but do not of themselves explain the relatively high levels found in the circulation of some patients with tumours.

The discovery of products such as AFP, CEA and ectopically produced hormonal products in patients with various malignancies led to the development of the repression - derepression theory In essence the theory states that the abberent gene expression of tumour cells is not random, but represents a reversion of the cell progeny to a more primitive, less differentiated state, ie that genes "switched off" in the differentiated cell remain "switched on" in the more primitive form. With increased assay sensitivity, it appears that developmental genes may be "turned on" slightly in the normal adult.

Chard however proposes an extension of this hypothesis concerning synthesis in the placenta to explain the observations. There may be numerous cells in adult tissue capable of synthesising "placental specific" proteins but these are either not secreted or are only secreted in very small quantities because they are not in contact with the bloodstream. In the case of a tumour with invasion of blood vessels, cells of this type may establish direct contact with blood and thus form a "mini placenta" (Fig 1 iii). This contrasts markedly with the older repression - depression theories of ectopic protein production, and does not entirely explain the observed facts eg in trophoblastic tumours such as hydatidiform mole there is a striking dissociation between the production of different placental proteins, this being related to the degree of malignancy (Lee et al 1981).

At present, no one theory fits all the known observations, but they provide useful models for further investigation.

1.6 Review of the Proteins studied

1.6.1 Human Chorionic Gonadotrophin (hCG)

The placenta is a source of various peptides which play an active role in the maintenance of pregnancy.

This concept followed the demonstration in blood and urine of pregnant women of a gonadotrophin (Ascheim and Zondek 1927), now known to be human chorionic gonadotrophin (hCG).

HCG is a glycoprotein with a molecular weight of about 39000 (Morgan et al 1975) and a carbohydrate content of about 30%. The molecule consists of two dissimilar non covalently linked subunits designated α and β. The α subunit is common to human luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) and consists of 89 to 92 amino acid residues in identical sequences, although the carbohydrate moieties may be somewhat different (Morgan et al 1975).

The β subunit confers immunological identity upon the molecule and in contrast to the α subunit there are distinctive yet similar amino acid sequences. Whilst there are similarities between the β subunit of hCG and those of FSH and TSH, extensive similarity exists between β hCG and β LH. Of the first 115 amino acid residues, 80% are identical, but hCG has an additional distinctive COOH terminal 30 amino acid residues (Birken and Canfield 1977).

The difference in β subunits has proved useful in raising an antiserum that does not recognize LH. Either purified

β subunit (Vaitukaitis et al 1971) or a conjugated peptide (synthetic or natural) from the unique COOH terminal section (Louvet et al 1974) can be used as an immunogen.

HCG has a strong luteotropic function in the human female and plays an important role in maintaining the function of the corpus luteum during the early stage of pregnancy via stimulation of adenyl cyclase activity.

A variety of uses for hCG in laboratory investigation of disease have been documented. The detection of hCG appears to be a specific indicator for the presence of viable trophoblast. hCG can be detected in the maternal serum as early as 6 days after fertilization ie before implantation; and production of hCG by the blastocyst has been suggested (Saxena et al 1974). Maximal serum hCG concentrations are found between 8 and 10 weeks after the last menstrual period, after which concentrations decrease by 90% and remain so throughout the remainder of pregnancy. Thus hCG estimation in maternal urine and serum has been widely used as a pregnancy test and in the prognosis of pregnancies with first trimester bleeding (Van Leusden 1976). HCG has proved of little use in monitoring foeto-placental function however.

HCG estimation is also a sensitive marker in gestational trophoblastic disease and is used both in diagnosis and monitoring of patients with the disease (Lee et al 1982).

Measurement of hCG is also used in diagnosis and prognosis of disease in patients with a variety of gonadal and non gonadal tumours such as tumours of the stomach, liver, lung, kidney, pancreas, ovary and testes (Vaitukaitis et al 1976, Lange et al 1976).

Human Placental Lactogen (hPL) was independently identified by Ito & Higashi (1961) and Josimovich & Maclaren (1962). The latter group alluded to an immunological relationship between hPL and growth hormone and later, sequencing studies showed a considerable degree of homology (85%) between the amino acid sequences of human growth hormone and hPL (Sherwood et al 1971).

HPL has a molecular weight of about 22,000 but unlike many other trophoblast protein products contains no carbohydrate (Li et al 1973).

Using a fluorescent antibody technique, hPL has been localized in the cytoplasm of the syncytiotrophoblast (Sciarra et al 1963). HPL is detectable in maternal serum as early as 5 weeks after conception; the concentration rising steadily until about 36 weeks of gestation after which a plateau or slight decrease occurs.

A number of biological activities have been proposed for hPL including lactogenesis, growth promotion, effects on carbohydrate and lipid metabolism, stimulation of the corpus luteum, erythropoiesis, inhibition of fibrinolysis and immunosuppresion (Chard 1982 b). Gaede et al (1978) however argue against a vital function in pregnancy as clinically normal pregnancies with no detectable hPL have been recorded. It has thus been suggested that although various activities can be shown in experimental systems, the placental proteins are merely waste products of the general activities of the placenta as as independent organism (Gordon and Chard 1979).

The problem of defining a biological role for hPL is reflected by equal difficulty in defining the physiological control mechanisms. There is little evidence of control mechanisms of the type which apply to endocrine products of the normal adult eg no releasing or inhibiting factors have been identified. The hypothesis given previously for placental protein production (1.4) suggests that the potential for hPL synthesis is a function of the total mass of trophoblast.

The half life of hPL in the maternal circulation as estimated by its rate of disappearance after delivery of the placenta is 10 - 20 min (Pavlou et al 1972), and it appears that there is no circadian rhythm in maternal serum concentrations. However, there are random fluctuations over a 24 hour period which are greater than those attributable to assay imprecision. Thus the diagnostic significance of hPL levels is enhanced if serial results are available.

HPL concentrations in maternal serum have been used in the investigation of a wide variety of pathological conditions.

The results are frequently contradictory having been reviewed recently by Chard (1982 b). However it should be appreciated that negative information is also valuable and irrespective of the clinical condition, a value in the upper end of the normal range indicates a relatively small risk to the foetus.

hPL levels are widely used therefore as a measure of placental function and this has posed a number of groups to question whether placental function testing is worthwhile (Gordon et al 1978, Grudzinskas et al 1981). There is still good reason for continuing hPL measurements but further refinement in the application of hPL values is required.

Table 1 II

Properties of Tumour Associated Alkaline Phosphatase

	Non Regan	Kasahara	Regan	Nagao
Developmental Counterpart	early placenta 6-10 weeks	?intestine ?D variant	Term placenta (S,I,F)	Term placenta D variant
Molecular Weight (subunit)			64,000	
pH Optimum	10.1	10.1	10.6	10.6
Heat stability 56°C 30 min	-	+	+++	+++
65°C 5 min	-	-	+++	+++
Amino Acid phenylalanine	+	+++	+++	+++
Sensitivity (5 mM) L homoarginine	+++	±	±	±
L leucine	+	++	+	+++
Electrophoretic Migration	fast	fast	intermediate	intermediate
Reaction with antisera to:-				
Liver ALP	+	-	-	-
Intestinal ALP	-	++	+	+
Term Placental ALP	-	+	+++	+++

1.6.3 Carcino Placental Alkaline Phosphatase (CPAP)

The occurrence of a placental alkaline phosphatase (heat-stable alkaline phosphatase) was recognized relatively early (Coryn 1934). However despite a large volume of literature on the application of placental alkaline phosphatase concentrations in the prediction of foetal problems, controversy still remains and the assay is little used for this purpose (Stigbrand et al 1982).

However the demonstration of the presence of placental alkaline phosphatase as an oncofoetal or carcinoplacental antigen (Regan isoenzyme) by Fishman et al (1968) and the later description of several other tumour related forms (Nakayama 1970, Warnock 1969) have led to the use of this enzyme in the field of tumour markers.

The placenta is known to produce a variety of allotypic alkaline phosphatases and it is now obvious that many tumours produce isoenzymes that share early and late placental alkaline phosphatases (Fishman & Singer 1976). These alkaline phosphatase isoenzymes in tumours are named after the patients in whom they were identified. Table 1 II shows the characteristics of these isoenzymes and their developmental counterparts.

Elevated levels of "placental" alkaline phosphatase have been reported in a variety of malignancies, the highest incidence being for germ cell tumours of the testes, ovary although elevations have also been reported in tumours of the pancreas, lung, breast, colon, kidney, stomach, bladder and lymphatic system (Stigbrand et al 1982). One seemingly hopeful application is as a marker for seminoma where alternative markers such as hCG and AFP are seldom of use.

1.6.4 Pregnancy Specific β_1 Glycoprotein (SP_1)

The identification of PSβ_1G (SP_1) by two groups (Tatarinov 1970 and Bohn 1972) stimulated research into the "new" placental proteins.

SP_1 is a glycoprotein, the carbohydrate content of which is about 28%. Early estimates of the molecular weight suggested values of 90,000-120,000 (Bohn 1972) but it later became apparent that SP_1 was a heterogeneous molecule consisting of a number of molecular forms with varying molecular weights (Teisner et al 1978). The exact situation remains unclear and will be discussed later.

Immunoreactive SP_1 with a molecular weight of 65000 has been detected in pregnancy urine and it is possible that urinary SP_1 may be a fragment of circulating SP_1 (Bohn and Kraus 1977).

No definite physiological role for SP_1 has been determined. Affinity of various steroids to SP_1 has been reported (Bohn 1974). It has also been suggested that SP_1 may be involved in iron metabolism during pregnancy (Lin et al 1974). Other groups have observed an immunosuppressive effect in vitro (Bohn et al 1976) and it has also been suggested that SP_1 may exert an effect on carbohydrate metabolism (Singh et al 1979, Pledger et al 1982). More recently, it has been suggested that the endometrium might be the target organ for any hormonal action this protein possesses (Ahmed & Klopper 1983 a).

Several reports exist suggesting that SP_1 is also present in the circulation of normal males and non pregnant females (Searle et al 1978, Engvall and Yonemoto 1979).

SP_1 has been used as a "pregnancy test" (Ahmed & Klopper 1983 b) and in the prognosis of patients with threatened abortion and ectopic pregnancy (Ho & Jones 1980). In late pregnancy the data is extensive although specific groups of clinical abnormalities are sparse. Associations between SP_1 and IUGR (Gordon et al 1977a), SP_1 and fetal distress, SP_1 and maternal hypertension or diabetes mellitus (Grudzinskas et al 1979) have been investigated. As no common baseline was taken in various studies, it is difficult to form an overall impression of the usefulness of this test in the assessment of foetal wellbeing however.

Several attempts to utilize SP_1 as a tumour marker have also been made, in tumours with trophoblastic elements (Horne et al 1977) and in non trophoblastic tumours (Grudzinskas et al 1980 a). The presence of SP_1 in normal sera detracts from the use of this protein in any diagnostic mode but there is still the possibility of use in a monitoring situation, where other markers are of little use. With the discovery of the molecular heterogeneity of SP_1 and the recognition of the components in differing assay systems, doubt has been expressed over previous work in a clinical context. The possibility of assaying $SP_1 \alpha$ separately from $SP_1 \beta$ may offer wider clinical application for this protein.

1.6.5 Pregnancy Associated Plasma Protein A (PAPP-A)

PAPP-A is a glycoprotein with a molecular weight of about 750,000 (Lin et al 1974) but is distinct from other high molecular weight α_2 glycoproteins. The origin of PAPP-A remains in doubt - it is detectable in non pregnant subjects, in endometrium and seminal plasma (Duberg et al 1982,

Bischof et al 1982 a, Bischof et al 1983). The sudden increase in PAPP-A levels in pregnancy suggests that the production and/or metabolism of this protein is hormone dependent and that early in pregnancy some event triggers production. In contrast to many pregnancy proteins, PAPP-A concentrations continue to rise to term, suggesting that PAPP-A production is not related to placental mass, or that a source other than the placenta is responsible.

As with many other pregnancy proteins, no definitive actions are known for PAPP-A although a variety of interesting hypotheses have been made. In vitro, pure PAPP-A inhibits the activity of the complement system although this may be linked to the fact that PAPP-A binds heparin (Toop & Klopper 1983).

If the observed in-vitro inhibition of complement activity has any physiological importance, it implies that PAPP-A acts as an immunosuppressor. Taking all the known facts into account, Bischof proposes that the protein is carried by the maternal blood stream until it reaches the intervillous space, where it binds to the surface of the syncytiotrophoblast. Tightly bound to the microvilli, PAPP-A would exert its immunosuppressive effects by inhibiting complement activity and by reducing the transformation rate of lymphocytes into lymphoblasts. These effects would lead to an increased tolerance of the foetal allograft by the mother through the local impairment of the maternal immune defences such as cell lysis, immunoadherence, macrophage recruitment and phagocytosis.

The applications of PAPP-A measurement in pregnancy apart from one aspect seem at present unpromising. PAPP-A seems of little

use in detecting retarded foetal growth, although this may be due to the failure to remove hypertensive patients from the IUGR population (Bischof et al 1980). Similarly it seems of little use in monitoring diabetic pregnancies (Lin et al 1977).

One area where considerable interest has been generated is in pre-eclampsia and antepartum haemorrhage. Indeed it has been suggested that PAPP-A levels are elevated in advance of premature labour, antepartum haemorrhage and hypertension (Klopper et al 1980, Hughes et al 1980). If these findings are correct, then measurement of PAPP-A will play a central role in monitoring "at risk" pregnancies.

1.6.6 Alpha Foeto Protein (AFP)

Whilst the proteins discussed so far are products of the maternal component, AFP in contrast is produced by the foetal component and can be detected as early as 29 days after conception, being produced by both the yolk sac and the foetal liver.

AFP is a glycoprotein composed of a single polypeptide chain with a molecular weight of 69,000 but may exhibit heterogeneity (Alpert and Perencevich 1975). It resembles albumin in its amino acid composition but differs in being a glycoprotein containing about 4% carbohydrate. It is likely that there was a common ancestor for AFP and albumin.

The major use of AFP in pregnancy has been in screening programmes to detect foetal neural tube defects (UK collaborative study on AFP in relation to NTD (1979)). More recently interest has been generated in applying AFP measurements in the prediction of foetal growth retardation (Wald et al 1980, Brock et al 1982).

In the field of tumour monitoring, much use has been made of AFP in following patients with hepatoma. Equally in conjunction

of ovary and testes (Pedersen et al 1978). However, not all patients with teratoma are AFP or hCG positive, and patients with seminoma are seldom marker positive. Therefore, there is a need for other markers in these conditions.

CHAPTER 2

DESCRIPTION OF THE RADIOIMMUNOASSAYS EMPLOYED

2.1 Introduction

The assay of proteins in body fluids has been a subject of investigation for many years. Whilst chemical methods allowed quantitation of the major protein fractions and enzymes, specific protein assay was largely dependent upon the evolution of immunological techniques.

Whilst "in vivo" assays (bio-assays) were available for some proteins with hormonal activity, the majority of proteins could not be assayed until suitable in vitro techniques became available.

The antigen-antibody reaction common to all immunoassays was first described in 1897 (Kraus) and formed the basis of a variety of immunoassays (Chow 1947, Ouchterlony 1948, Oudin 1949, Feinberg 1957, Mancini 1965). Extension of immunoassay with the application of electrophoretic techniques (Graber 1953, Laurell 1966) led to a wider usage. None of these techniques however were well suited to rapid, precise and sensitive assays of large numbers of patient specimens.

2.2 Radio-labelled Assays

The introduction of radioimmunoassay (RIA) (Yalow & Berson 1960) therefore had an explosive impact upon endocrinology and specific protein assay. These methods now represent a common analytical approach to the measurement of a vast range of biological substances often present in minute amounts.

The distinguishing concept of conventional RIA (Saturation Assay) is that by allowing the substance to be measured (P) to react with a specific receptor (Q) of limited capacity, substance P

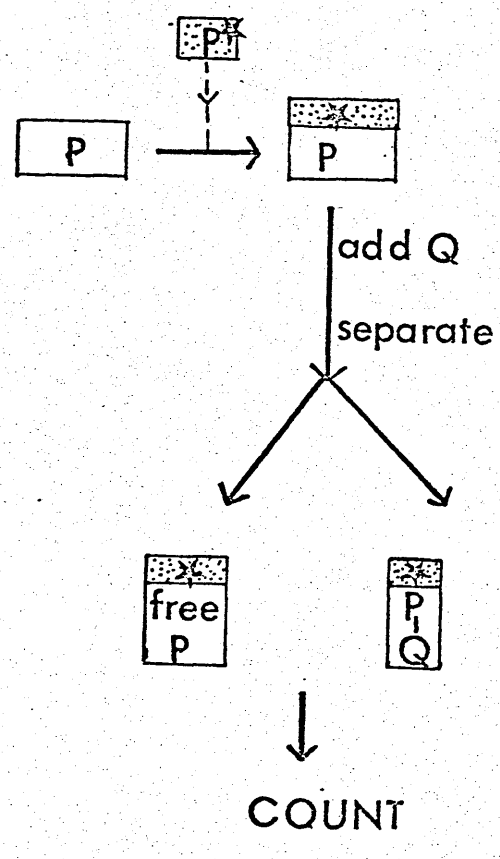

Fig 2 i The Saturation (Radioimmuno) Assay

P is the substance to be measured, and P* its radioactively labelled analogue. Q is the binding material.

by saturating Q is partitioned into two moieties, such that the ratio of the two varies as a function of the total amount of P present. An unknown amount of P may thus be quantitated by comparing its distribution with distributions yielded by standard amounts of P. Identification of the two components relies on addition of radioactive material (usually) identical to P. Following physical separation of the bound (PQ) and residual free P moieties, the partition of radioactivity between them is observed (Fig 2i). Suitable receptors are often antibodies and this type of RIA obeys the general antigen-antibody concept. Because of the limited amount of Q an alternative name for this assay type is the Competitive Assay.

However radioimmunoassays have problems associated with them. Disadvantages such as the use of radioactive tracers with short shelf life, the legal implications of the use of radioactivity and the expensive counting equipment often required led to the development of alternative labels for these assay systems, such as enzymes.

2.3 The Choice of Analytical Methods

The objectives of the work described in this thesis were the investigation of foeto-placental function and the diagnosis/monitoring of patients with cancer. A number of assays connected with this work were already available. Where existing radioimmunoassays were available, their reaction conditions were re-established before use for this thesis.

2.4 Radioimmunoassay of human Placental Lactogen (hPL)

2.4.1 Introduction and Principle

The radioimmunoassay of hPL used in this study was that routinely in use in the department. The method based on that of Letchworth

(1971 a), had undergone extensive investigation prior to the commencement of this study in an attempt to ensure a precise and robust assay and also to standardize the assay in terms of the International Reference Preparation (IRP 73/545) that was now available (Cotes and Das Gaines 1978), this trend being encouraged by National Quality Assurance Schemes.

Certain aspects of the routine assay were confirmed personally before use as detailed below.

hPL labelled with I^{125} and an antibody raised against hPL are incubated with standards, quality control material or patient's sera. After equilibrium is reached, free and bound antibody are separated by selective precipitation using propan-2-ol. The precipitate is centrifuged, the supernatant discarded and the radioactivity associated with the pellet counted on a γ counter.

2.4.2 Method of Iodination

A solid phase lactoperoxidase method was used (Karonen et al 1975).

7.33 µg hPL (iodination grade), 10 µl pH 7.4, 0.5 mol/l phosphate buffer, 10 µl working solution solid phase lactoperoxidase and 10 µl (1 mCi) I^{125} were mixed gently in a small test tube and 5 µl of hydrogen peroxide (10 µl of 30 vol H_2O_2 diluted in 100 ml water). After vortexing and incubating for 15 minutes at room temperature, a further 5 µl of hydrogen peroxide was added, vortexed and incubated for a further 15 minutes. 200 µl of 0.05 Mol/l phosphate buffer containing 2.5 g/l BSA were then added.

Table 2 I

Incorporation of I^{125} assessed by paper electrophoresis of hPL iodination mixture

Strip Section (cm)	Counts	
1	28545	
2	444216	
3	849483	
4	125497	hPL
5	117252	Total counts = 1927763
6	166694	
7	110783	
8	61705	
9	23679	
10	11530	
11	19275	
12	321439	
13	478531	
14	16294	Free Iodine
15	6474	Total Counts = 868184
16	3570	
17	3369	
18	1609	
19	787	
20	973	
21	1026	

$\therefore$ Total counts = 1927763 + 868184 = 2795947

$$\% \text{ incorporation} = \frac{\text{Counts in hPL}}{\text{Total counts}} \times 100$$

$$= \frac{1927763}{2795947} \times 100$$

$$= 69\%$$

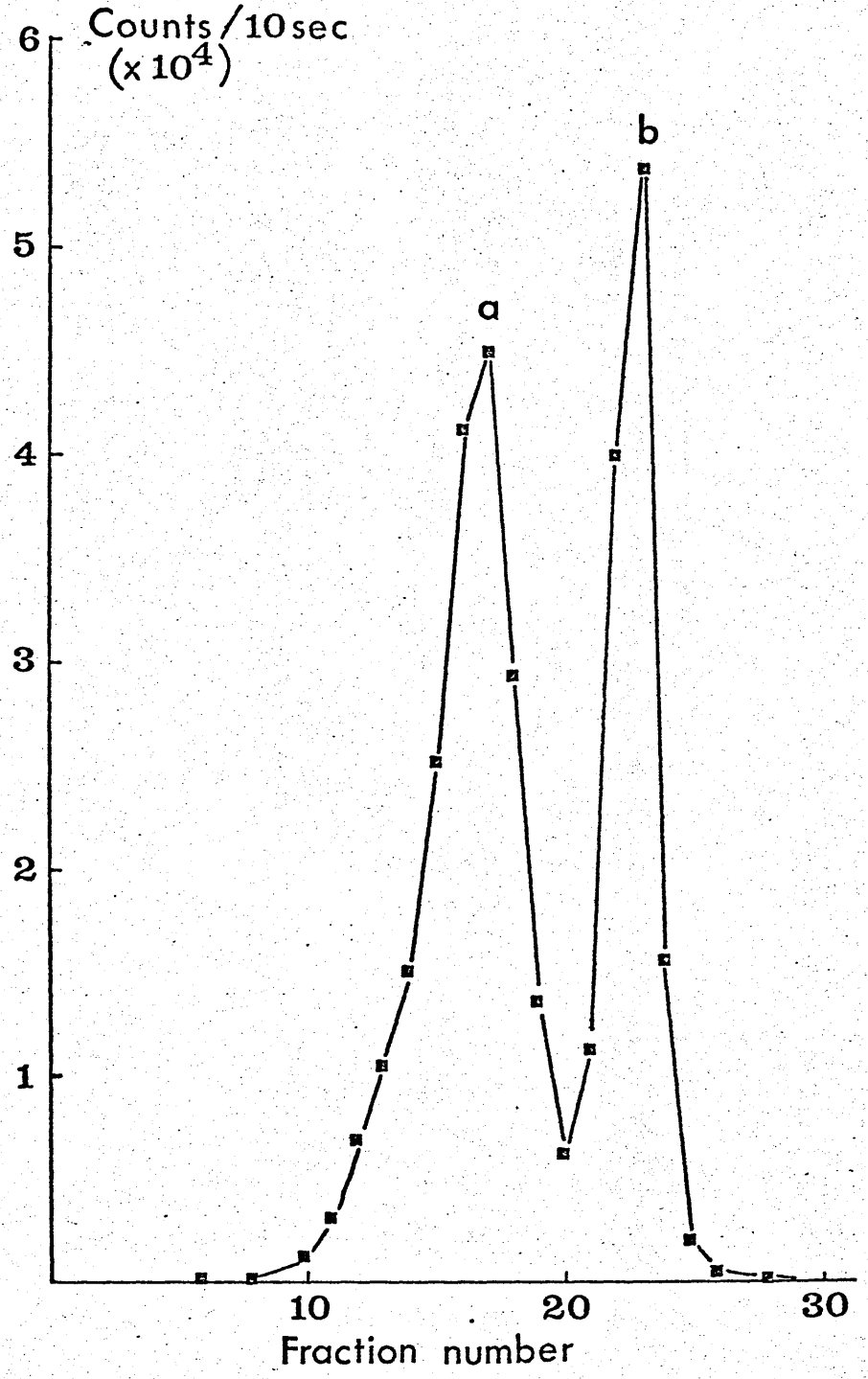

Fig 2 ii A Typical hPL Iodination Profile

Peak (a) represents labelled hPL and peak (b) is unreacted iodine.

2 x 10 µl aliquots of the iodination mixture were applied to a filter paper strip (Whatman 3 MM) marked in 1 cm sections for electrophoresis to assess incorporation, the remaining iodination mixture being transferred to a 60 cm x 1 cm column of Sephadex G150, previously equilibrated with 0.05 Mol/l phosphate buffer containing 2.5 g/l BSA. Fractions of approximately 1 ml volume were collected.

Two or three fractions of I^{125} hPL containing the highest counts were pooled. For use, a 50 µl aliquot of this pool was diluted in a suitable volume of assay diluent to give a count of about 12 000 counts/20 secs/50 µl. The volume of assay diluent required was usually about 3 ml. Bo and maximal binding were assessed for this material.

2.4.3 Assessment of a Typical hPL Iodination

(i) Electrophoresis of iodination mixture

Paper electrophoresis of the mixture was performed on a 10 µl aliquot of the iodination mixture. After drying the paper strip, it was cut into 1 cm sections and the radioactivity present in each section counted. Table 2 I shows the results obtained.

(ii) Gel filtration of iodination mixture and assessment of label dilution

Figure 2 ii shows the profile of counts obtained from the fractions collected from the Sephadex G150 column.

Fractions 16 and 17 were pooled, diluted to 3 ml in assay diluent and a 50 µl aliquot of the diluted label counted. 12,300 counts were accumulated in 20 seconds. This dilution of label was then used for assay.

Table 2 II

Assessment of hPL Label Characteristics

Tube No.	Description	Counts	Mean	% of Total
1	Total	12257	12229	——
2	Counts	12200		
3	Bo	9792	9761	80%
4		9730		
5	Bmax	11397	11299	92%
6		11200		
7	NSB	720	677	5.5%
8		633		

Duplicate tubes were prepared for the following:-

Total counts

Bo

Bmax (antibody concentration 10 x that routinely used)

NSB (no antibody)

Following the normal assay protocol, the bound radioactivity in each tube was counted and percentage Bo, Bmax and NSB were calculated (Table 2II).

2.4.4 Method of Assay

50 µl of standard, quality control sera or test sera were mixed with 300 µl assay diluent (barbitone buffer pH 8.6) containing 250 mg human albumin and 50 µl of diluted I^{125} hPL. 100 µl of anti hPL (diluted 1/250 in assay diluent) was then added, the tube contents mixed on a vortex mixer and incubated at room temperature for 30 minutes. Total count tubes (50 µl I^{125} hPL only) and NSB tubes (no antibody) were also prepared.

After incubation,1 ml of propan-2-ol was added to all tubes except "total counts", mixed and stood for 5 minutes. The tubes were then centrifuged for 10 minutes at 1100 g, the supernatant aspirated and the bound fraction counted on a γ counter. Calculation of test results was performed by comparison of counts in the test samples with counts in the standards using a suitable curve fitting procedure (Chapter 5).

2.4.5 Investigation of Factors Affecting the Assay of hPL

i) Optimal Antiserum Dilution

Various dilutions of antiserum were prepared in assay diluent. Standard materials were processed according

antiserum dilutions. A plot of % B/Bo against concentration (logarithmic scale) was made.

ii) Optimal Precipitant Volume

Replicate tubes were prepared containing 50 µl of either 0 standard or 6.8 mU/l standard. 300 µl of assay diluent and 50 ul of I^{125} hPL were added to each tube, followed by either 100 µl of diluted antiserum or 100 µl of diluent (for NSB estimation). Following incubation for 30 minutes at room temperature, precipitation was carried out using varying volumes of propan-2-ol. Duplicate binding and NSB tubes were used for each volume of precipitant considered. After centrifuging and aspirating the supernatant, the bound radioactivity was counted. A plot of % (counts/total counts) after NSB correction against precipitant volume was made.

iii) Comparison of hPL standards in horse serum and male serum

Standard materials were prepared in Wellcome Horse Serum No. 3 to cover the range 0-11.3 miu/l. Equivalent concentrations were also prepared in a pool of human (male) serum. Both sets of standards were assayed according to the protocol given.

2.4.6 Characteristics of hPL Radioimmunoassay

i) Recovery was calculated by adding known amounts of standard material to horse serum. After assay, the hPL concentration found was expressed as a percentage of the theoretical concentration.

ii) Within batch precision

Two pools of pregnancy serum were assayed 10 times in duplicate in a single assay.

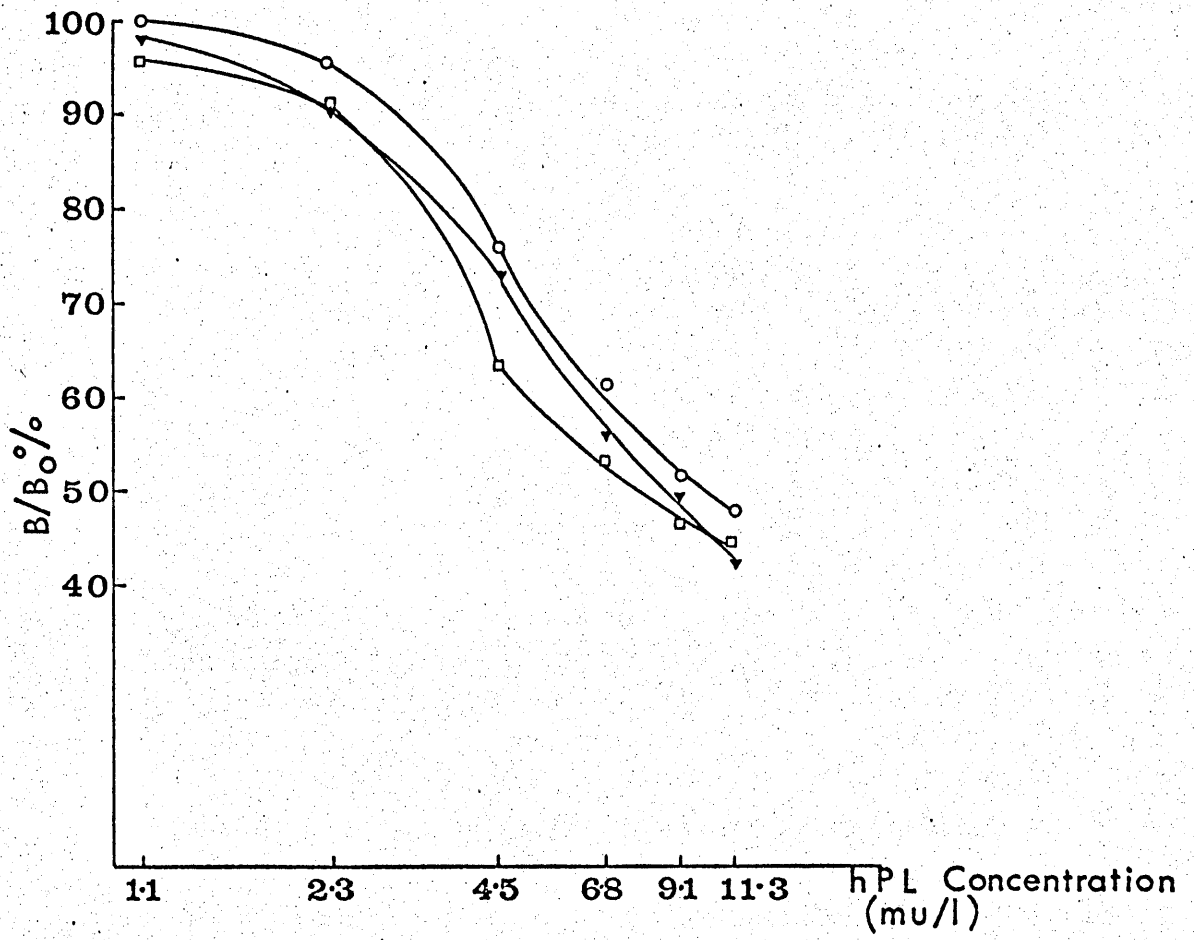

Fig 2 iii The Assessment of Optimal Antiserum Dilution for hPL

(o) antiserum diluted 1/200, (▼) 1/250, and (□) 1/300.

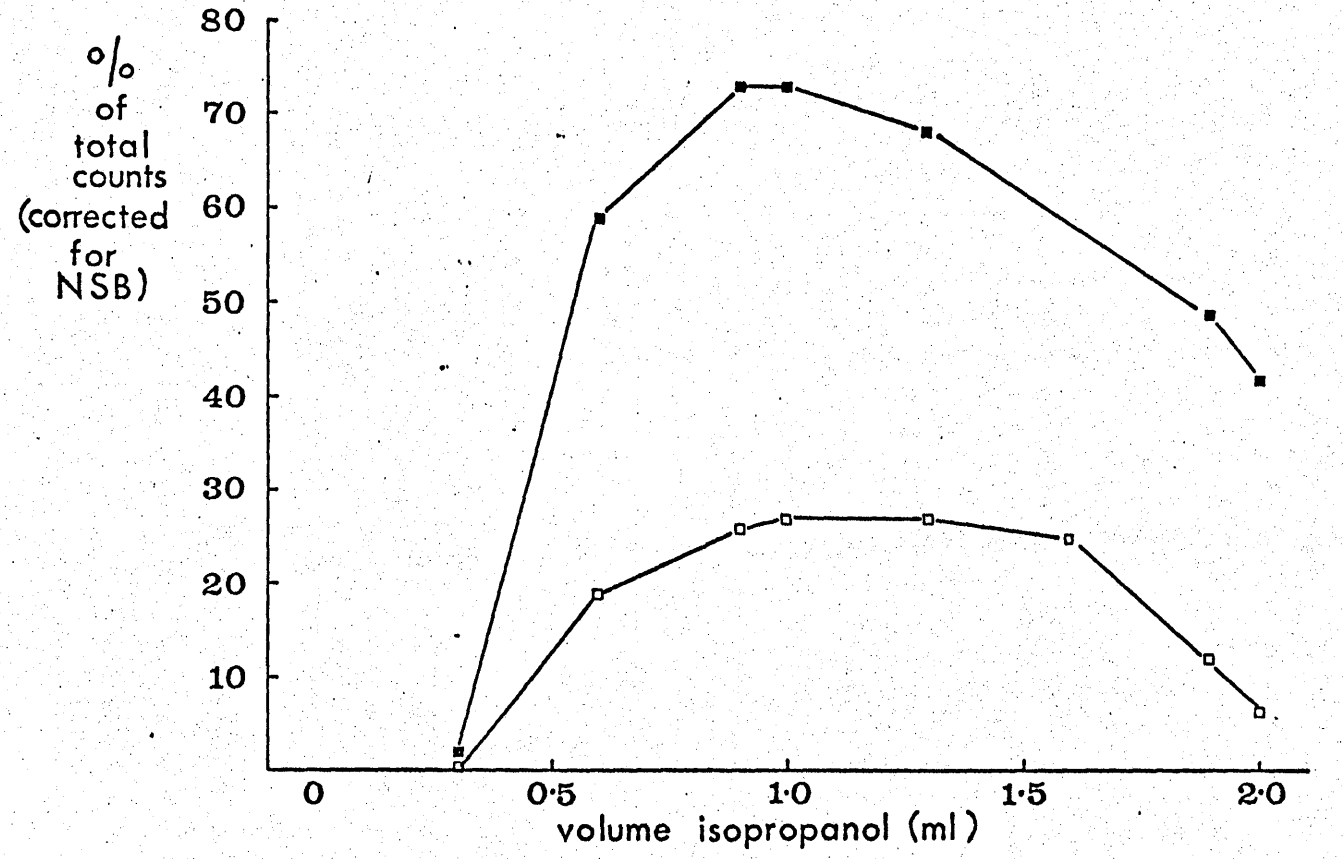

Fig 2 iv The assessment of optimal precipitant volume for Radioimmunoassay of hPL at two antigen concentrations (■ 0 miu/l) and (□ 6.8 miu/l)

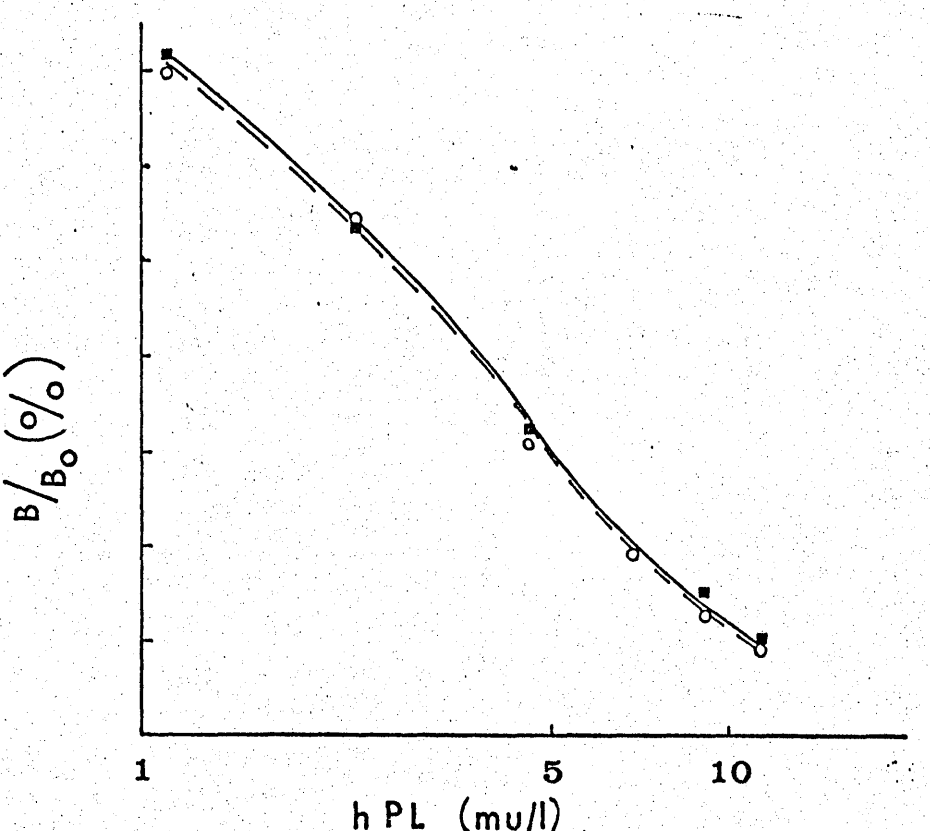

Fig 2 v Comparison of hPL Standards prepared in horse serum (■) and human serum (o).

iii) Overall-precision

Two quality control materials were included in each assay performed (215 assays in total).

2.4.7 Results and Discussion

Figure 2 iii shows the curves with varying antiserum dilutions. A dilution of 1/250 was chosen for the assay.

From the precipitation studies (Fig 2 iv) it can be seen that at volumes of isopropanol of less than 1 ml incomplete precipitation of the bound fraction was achieved, shown by the increasing percentage counts precipitated. At isopropanol volumes greater than 1.3 ml however, the corrected counts precipitated decrease due to an increase in the NSB component, ie free label is being precipitated in addition to bound label. The optimum isopropanol volume was thus defined as 1 ml.

No difference was observed between the standard curves prepared in male serum and horse serum (Fig 2 v). Thus measurement of hPL in human specimens by comparison with standards in horse serum is acceptable.

Recovery over the range of hPL concentrations commonly encountered in the 3rd trimester of pregnancy varied between 89% and 108%.

Within batch precision at mean hPL concentrations of 3.9 mU/l and 6.0 mU/l was shown to be 6.9% and 6.0% CV respectively whilst the CV for overall precision varied between 12% at a concentration of 2.7 mU/l and 8.9% at a concentration of 6.4 mU/l.

The assay therefore provides a suitable means of measuring hPL concentrations in the range commonly encountered in the third trimester of pregnancy.

2.5 Radioimmunoassay of human Chorionic Gonadotrophin (hCG)

2.5.1 Introduction and Principle

The radioimmunoassay of hCG was carried out using a kit manufactured by NMS Pharmaceuticals Ltd, Newport Beach, California (Kit Code NMS 1023) and supplied through RIA (UK) Ltd, Washington, Tyne and Wear.

A fixed amount of labelled antigen (I^{125} hCG) competes with the sample, standard or control for a fixed number of binding sites in a β specific antiserum (rabbit anti β hCG). After this incubation, the bound antigen is precipitated using a second antibody (goat anti-rabbit gamma globulin). The I^{125} bound to anti β hCG is precipitated and counted. The kit is standardized against the 2nd International Reference Preparation (WHO), Holly Hill, England.

Before use in this study, certain characteristics of the kit were verified.

2.5.2 Method of Assay

Duplicate tubes were prepared containing 100 µl of standard or test serum. 100 µl of anti hCG was then added and the tubes incubated at room temperature for 30 min. 100 µl of I^{125} hCG was then added to each tube. All tubes were then incubated overnight at room temperature, after which 200 µl of second antibody were added and the tubes incubated for 30 mins at room temperature. Immediately prior to centrifugation 2.0 ml of cold phosphate buffered saline was added. The tubes were then centrifuged at $4^{\circ}C$ (3000 rpm) for 15 minutes. The supernatant was aspirated and the bound fraction counted.

2.5.3 Investigation of Method Characteristics

i) Assessment of Cross Reaction with other Peptide Hormones

Standards for follicle stimulating hormone (FSH 81/L), luteinizing hormone (LH 81/L) and thyroid stimulating hormone (TSH 68/38) were obtained from the National Institute for Biological Standards and Control, Holly Hill, London.

Each standard was reconstituted according to the protocols supplied and further dilutions of this solution were made in PBS to give standard ranges:-

FSH	0 - 400 mU/L
LH	0 - 500 mU/L
TSH	0 - 75 mU/L

The diluted standards were then assayed following the routine assay protocol supplied by the manufacturers of the kit. A plot of percentage B/Bo was made.

ii) Limit of Detection

This parameter was assessed by assaying 10 duplicate Bo's in 1 assay as described previously (Pledger et al 1981).

iii) Precision

Within batch and overall precision were calculated using quality control materials supplied in the kit, and a pool of pregnancy serum for overall precision.

iv) Normal Range

The normal range for hCG in a non pregnant population was assessed by assaying 27 male sera and 26 female sera drawn from laboratory staff.

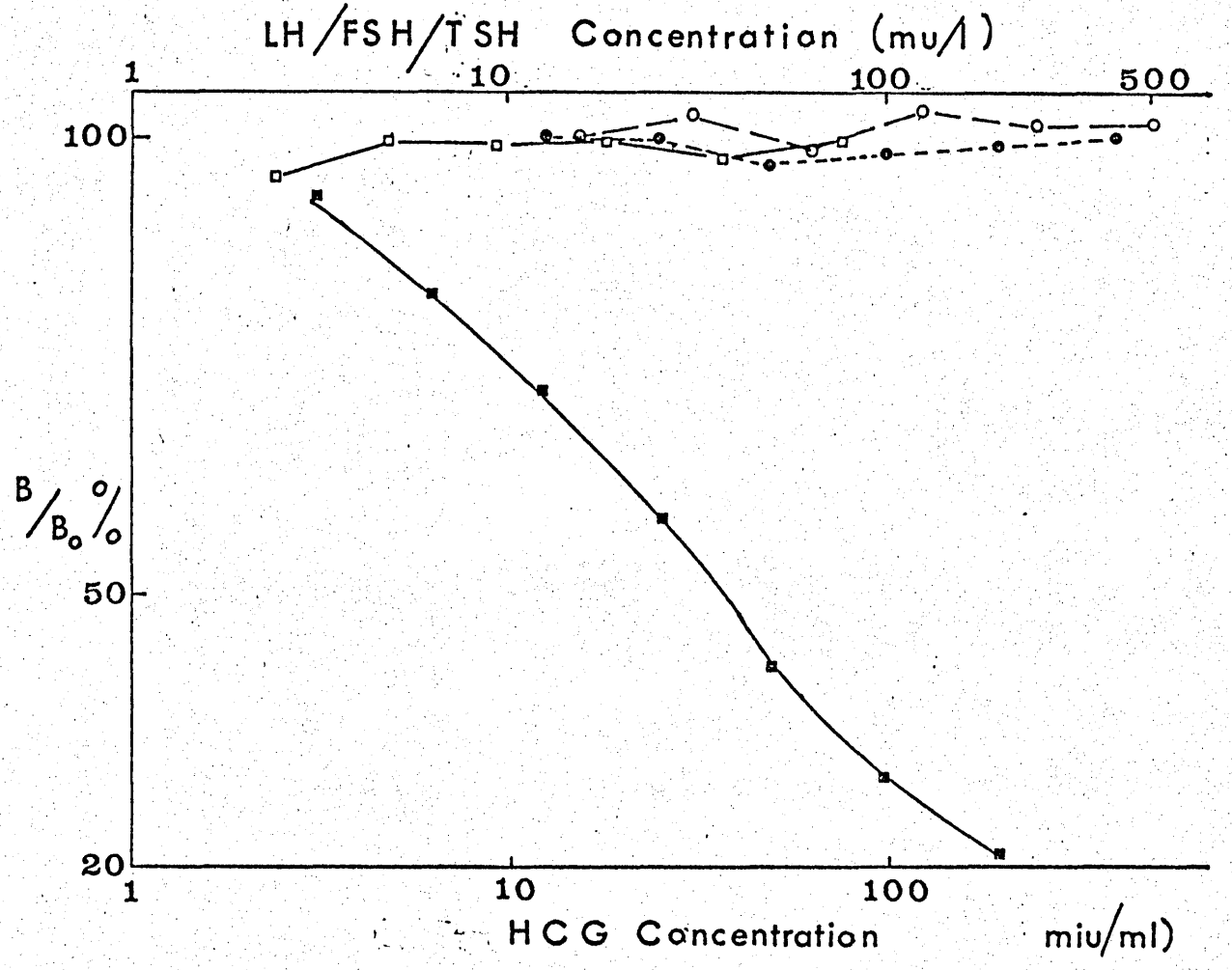

Fig 2 vi The Cross Reaction of LH, FSH and TSH in the Radioimmunoassay of human chorionic gonadotrophin.

■ hCG □ TSH ○ LH ● FSH

2.5.4 Results and Discussion

Fig 2 vi shows the results of the cross reaction studies. There was no significant cross reaction with any of the peptide hormones considered over the working range of the assay.

The limit of detection was assessed as 2.06 miu/ml which is in agreement with the manufacturer's quoted value of 2.0 miu/ml.

Most normal sera had hCG concentrations less than the limit of detection of the assay, and no sera had hCG concentrations greater than 5 miu/ml. The normal range was therefore defined as 0-5 miu/ml which is in accordance with the kit manufacturer's estimate.

The limit of detection is acceptable within this context and the assay is also precise (CV varying between 9.4% and 7.0% at mean concentrations of 7.6 miu/ml and 60.5 miu/ml).

2.6 Radioimmunoassay of Alpha Foeto Protein

2.6.1 Introduction and Principle

The assay used was that routinely in use in this department.

AFP labelled with I^{125} and unlabelled AFP (standards, tests) together with an antibody raised against AFP are incubated in barbitone buffer containing 11.2% polyethylene glycol, overnight at room temperature.

The following day, the tubes are centrifuged, the supernatant discarded and the bound radioactivity counted.

The assay was standardized against IRP 72/227 obtained from NIBSC.

2.6.2 Method of Assay

Duplicate tubes were prepared containing 100 µl antibody, 100 µl labelled AFP, 50 µl standard, serum or quality control, 600 µl PEG. The tubes were incubated at room temperature overnight and the following day were centrifuged at 2000 g for 20 minutes, the supernatant aspirated and the bound radioactivity counted.

2.6.3 Assay Characteristics and Discussion

Within batch and overall precision were estimated using horse serum spiked with foetal cord serum. Within batch precision was 7.9% at a concentration of 45.3 u/ml and overall precision was 9% at a concentration of 50.0 u/ml.

The limit of detection assessed by assaying 10 duplicate Bo's in 1 assay was calculated as 1.8 u/ml.

The assay provides a suitable method for the determination of AFP in patients with neoplastic disease.

The method is acceptably sensitive and precise.

CHAPTER 3

DESCRIPTION OF ENZYME LINKED IMMUNOSORBENT ASSAYS EMPLOYED

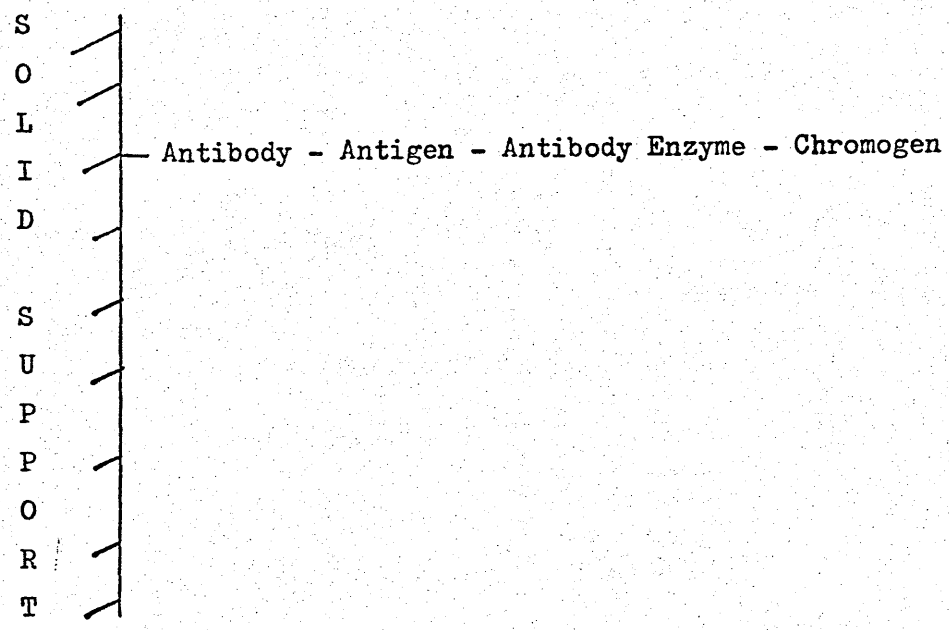

Fig 3 i The Sandwich ELISA

3.1 Introduction

The disadvantages of radioactive labels led chemists and immunologists to investigate alternative non-isotopic labels. These labels included enzymes (Van Weemen and Schurrs 1971), erythrocytes (Adler and Liu 1971), bacteriophages (Haimovich et al 1970), fluorescent groups (Aalberse 1973) and stable free radicals (Leute et al 1972). Of these labels only enzymes gained rapidly in popularity. Whether this was due to an existing knowledge of enzyme kinetics, or the easily remembered acronym (ELISA) Enzyme Linked ImmunoSorbent Assay coined by Engvall and Perlmann is debatable. (1972)

Enzyme immunoassays may be classified into two main types viz homogeneous (exemplified by EMIT assays) and heterogeneous (generally known as ELISA), the distinction being made on the absence or presence of washing stages.

Terminology still poses a problem, various acronyms having been used (Van Weemen and Schurrs 1971, Pinon and Dropsy 1977, Saunders et al 1977, Yorde et al 1976).

The enzyme immunoassays considered in this study will be classified as ELISA, as this acronym encompasses the essential concepts (Fig 3 i).

3.2 Choice of Analytical Technique

Where it was necessary to establish assays for placental proteins which had not previously been assayed on a regular basis in these laboratories, ELISA systems were devised. Because ELISA has not been so widely used as RIA, factors influencing performance have not always been clearly defined. Thus an investigation of some of these factors was also undertaken.

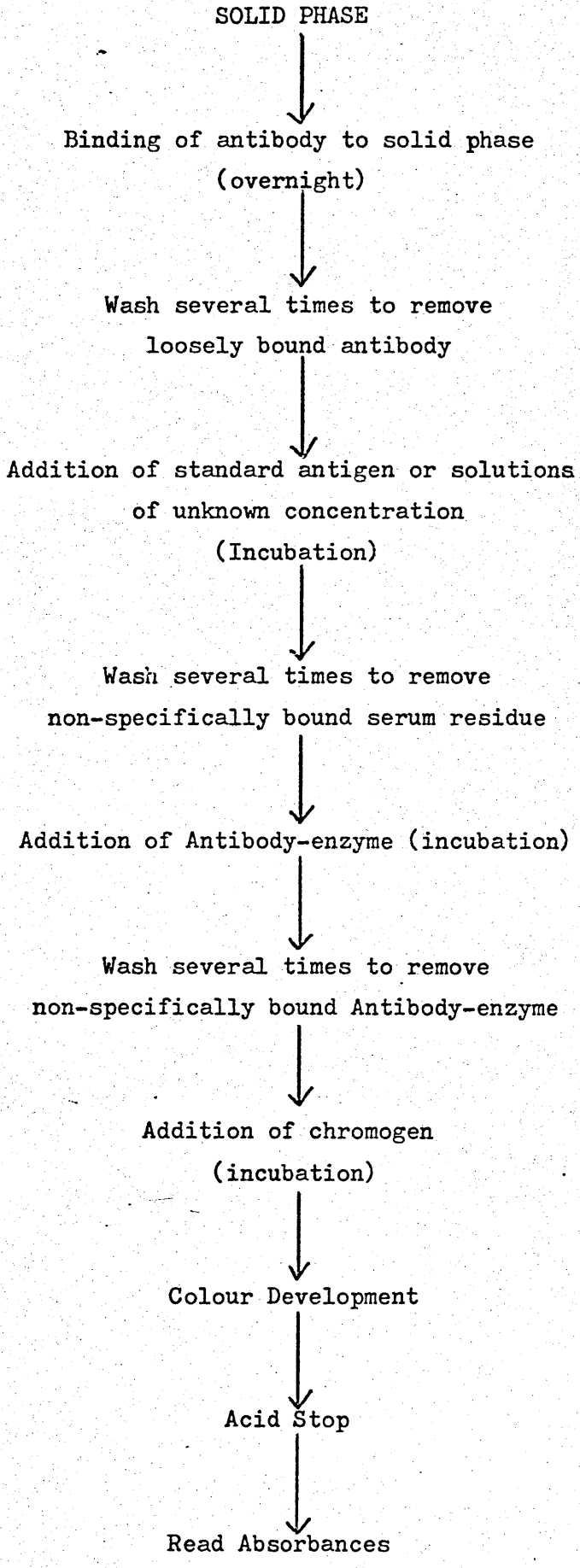

Fig 3 ii Flow Sheet for Sandwich ELISA for antigen assay

3.3.1 The Options Available

A variety of assay types have been described with analogues in conventional radiolabelled assays. These have been well reviewed by a number of authors (Wisdom 1976, O'Sullivan et al 1979) and no further description will be given here.

Sandwich ELISA offers sensitive, specific assays with ease of separation, avoiding problems sometimes seen with interfering factors in competitive ELISA. Only sandwich assays were utilized in this study and this assay type will be considered in more detail.

3.2.2 Sandwich ELISA for antigen

This procedure requires the antigen to have at least 2 binding sites. Antigen is reacted with excess solid phase antibody and after incubation followed by washing, the bound antigen is reacted with excess labelled antibody. After further washing, the bound label is assayed, thus providing a direct measure of the amount of antigen present (Maiolini and Masseyeff 1975). Two assumptions are made in this type of assay:-

a) that an enzyme marker may be attached to an antibody with retention of both immunological and enzymic activity.

b) that an antibody may be linked to a solid phase carrier surface.

In practice, both of these assumptions hold true. The assay concept is shown schematically in Fig 3 ii.

3.3.3 Immobilization of Antibodies

ELISA is distinguished from other types of enzyme immunoassay by the immobilization of antibody, allowing rapid, simple separation of free antigen and antibody enzyme from the immobilized (bound) fraction.

Table 3 I

Criteria for Enzyme Labels

1) Availability of purified low-cost homogeneous enzyme preparations.

2) High specific activity.

3) Presence of residues through which the enzyme can be cross-linked to other molecules with minimal loss of both enzyme and antibody activity.

4) Stable enzyme conjugates.

5) Enzyme absent from biological fluids.

6) Assay method should be cheap, simple, sensitive, precise and not affected by factors present in biological fluids.

7) Enzyme, substrate, co-factors etc should not pose a potential health hazard.

A wide variety of solid phases have been used - PVC (Macdonald et al 1979), polystyrene (Pledger and Belfield 1983), microcrystalline cellulose (Maiolini and Masseyeff 1975), nylon (Hendry and Herrmann 1980) and activated paper discs (Lehtonen and Viljanen 1980).

Most proteins adsorb to plastic surfaces probably as a result of hydrophobic interaction between non-polar protein substructures and the non-polar protein matrix. However, as the antibody is only physically adsorbed, loss of protein during washes and incubations is possible (Lehtonen and Viljanen 1980). Furthermore, adsorbed proteins may undergo denaturation with loss of immunological activity. Further investigation of these factors is warranted.

3.3.4 Choice of Enzyme Label

The enzyme label forms an integral part of ELISA and the choice of enzyme must therefore be made carefully. Ideal enzyme properties are listed in Table 3 I. Few if any enzymes possess all these properties and the choice of label is thus to a large extent dictated by the nature of the assay ie homogeneous or heterogeneous.

Assay sensitivity is also partially dependent on the enzyme label. Thus whilst lysozyme has been used as a label to detect a number of drugs in urine at the mg/l range (Rubenstein et al 1972) it would not be a suitable label for assays requiring high sensitivity, due to its low specific activity.

The most widely used enzymes have been horse-radish peroxidase (HRP), β galactosidase and alkaline phosphatase.

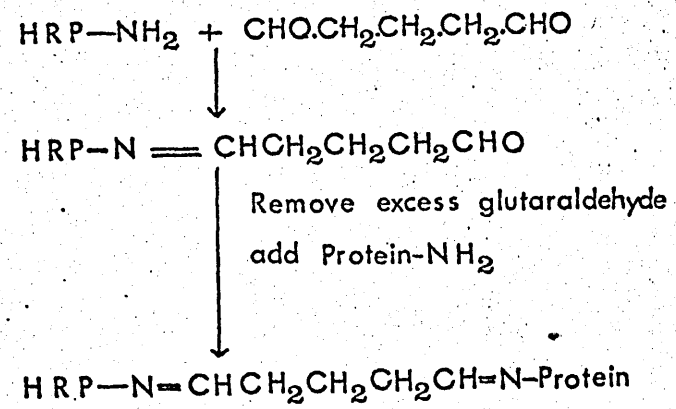

Fig 3 iii Two Step Glutaraldehyde Conjugation Reaction

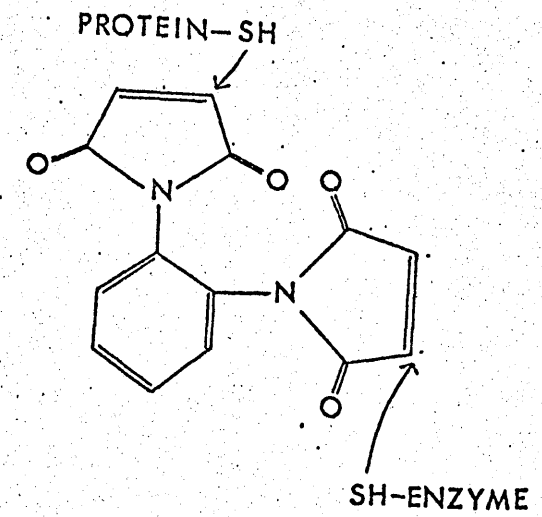

Fig 3 iv Maleimide conjugation reaction

3.3.5 Preparation of Enzyme Labels

A wide variety of chemical methods for preparing antibody-enzyme conjugates have been proposed. Most depend on activation of residues on the antibody/enzyme, or cross linking using a heterobifunctional reagent. The general principles are similar and are shown by reference to two reactions (Figs 3 iii and 3 iv). Each method has points to commend it, but when preparing conjugates of HRP, the most widely used procedure has been that of Avrameas (1969a) - the two step glutaraldehyde procedure. Although the yield is low, the technique is easily performed and provides a stable conjugate with reasonably standard properties from batch to batch.

Following conjugation, separation of the reaction components is effected by techniques such as gel filtration.

Most conjugation procedures give rise to high molecular weight complexes containing several enzyme molecules bound to several antibody molecules. In a solid phase assay only one of the antibody molecules in such a complex will be available to react with an antigen. Thus although the complex may exhibit high specific activity this cannot be realized in practice. The differing nature of these complexes compared with natural antibody does not seem to present problems in ELISA, as results are always obtained by comparing the unknown sample with a standard, which will react identically in a given system.

3.3.6 Detection of Enzyme Labelled Antibody

Detection systems used are largely dictated by the choice of enzyme. Many enzymes may be detected by simple colorimetric procedures and this may have been a reason for the initial rise in ELISA.

Table 3 II

Advantages and Disadvantages of Enzyme Labels

Advantages

1) Sensitive assays result from the enzyme amplification effect.
2) Reagents are relatively cheap and can have a long shelf life.
3) A variety of enzymes can be used as labels. Thus:-
   a) multiple simultaneous assays are theoretically possible
   b) labels may be prepared using a wide variety of conjugation techniques
   c) a number of systems for detecting enzyme activity may be used
4) Equipment can be inexpensive and is widely available.
5) No radiation hazards occur during labelling or disposal of waste.

Disadvantages

1) Measurement of enzyme activity can be more complex than measurement of the activity of some types of radioisotopes.
2) Enzyme activity may be affected by plasma constituents.
3) Solid phase problems.
4) More stages involved than in RIA.
5) Interference by serum components.

Other techniques such as fluorescence (Kato et al 1975), turbidity measurement (Rubenstein et al 1972) and scintillation counting (Van der Waart and Schuurs 1975) have been used.

More novel ideas have included the use of electrode detectors utilizing a thermistor (Mattiasson et al 1978) and recently the advent of luminescence assays (Velan and Halmann 1978) offers the prospect of increased sensitivity. However these methods have their own problems in terms of interfering factors (Tsuji et al 1978).

The particular type of apparatus used is often dictated by the nature of the solid phase. Thus whilst it is possible to aspirate solutions from the wells of a microtitre plate for individual readings in a spectrophotometer, it is easier and quicker to use a microtitre plate Reader, allowing the entire plate to be read in about 60 seconds.

3.3.7 Problems Associated with ELISA

Hosking (1982) has described ELISA as a "paragon of virtue". However, problems do exist. Whilst the use of enzyme labels avoided some of the problems associated with radioactive labels, certain disadvantages were also recognized (Table 3 II).

Leaching of immobilized antibody has been alluded to and non-specific adsorption may occur.

Whilst washing stages avoid many of the problems of interfering factors, difficulties have been experienced with sera containing high concentrations of rheumatoid factor (Maiolini and Masseyeff) 1975) and also with the complement system (Belfield and Macdonald 1979).

It is hoped that the investigations carried out for this thesis will place the assays on a more theoretical basis. This will improve the design and optimization of assays and could lead to an improved laboratory service.

3.4 Enzyme Linked ImmunoSorbent Assay of Pregnancy Specific β_1 Glycoprotein (SP_1)

3.4.1 Introduction and Principle

The assay of this protein was based on the method described by Macdonald et al (1979) following validation and extension of the assay parameters.

Antibodies to SP_1 are bound to the wells of plastic microtitre plates. After removing unbound antibody by washing, antigen is added. After incubation the antigen is removed, and antibody linked to horseradish peroxidase is then incubated in the wells. Following removal of excess antibody enzyme, chromogen is added and colour allowed to develop. The reaction is terminated by addition of acid. The resulting colours are read in a spectrophotometer, the absorbances compared with those for the standards and calculation of the unknown concentrations made.

3.4.2 Materials and Methods

Materials as defined in Appendix I.

Method of Assay Microtitre plates were coated with antiserum to SP_1 (diluted 1/1200 in pH 9.6 carbonate/bicarbonate buffer), overnight at $+4^{o}C$. Prior to use, the plates were emptied and washed three times with wash buffer, following which the plates were shaken dry. 100 µl of standards or test sera (diluted

1/1000 in diluting solution) were pipetted in duplicate into wells of the microtitre plate and incubated for 30 minutes. The well contents were then aspirated, the plate washed 3 times and dried. 100 µl of antibody-peroxidase conjugate diluted 1/1000 in PBS/25% chicken serum with 100 µl of molar sodium citrate per 10 ml of solution, was pipetted into the wells. Incubation was carried out for 30 minutes followed by a further washing cycle. After drying the plate, 100 µl of chromogen were added and colour development allowed to proceed for 30 minutes, at the end of which time, the reaction was stopped by addition of 100 µl of 4 mol /l sulphuric acid.

3.4.3 Investigation of Factors affecting the assay

Investigation of the effect of varying contact time of antigen with antibody, of antibody enzyme with antigen, the effect of the antiserum dilution used for coating and the effect of antibody-enzyme dilution were assessed as described by Macdonald (1979).

Recovery was assessed by spiking a pool of male serum with known volumes of SP_1 standard.

Precision Within batch precision was assessed by replicate assay of 2 serum pools on a single microtitre plate. Overall precision was assessed at two concentrations by assaying serum pools on each plate.

Parallelism of Assay Parallelism was checked using a pool of pregnancy serum. Initial dilutions (neat, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{5}$) were made in pooled male serum. A further dilution of 1/1000 was made in diluting solution. Replicate assays of each dilution were performed.

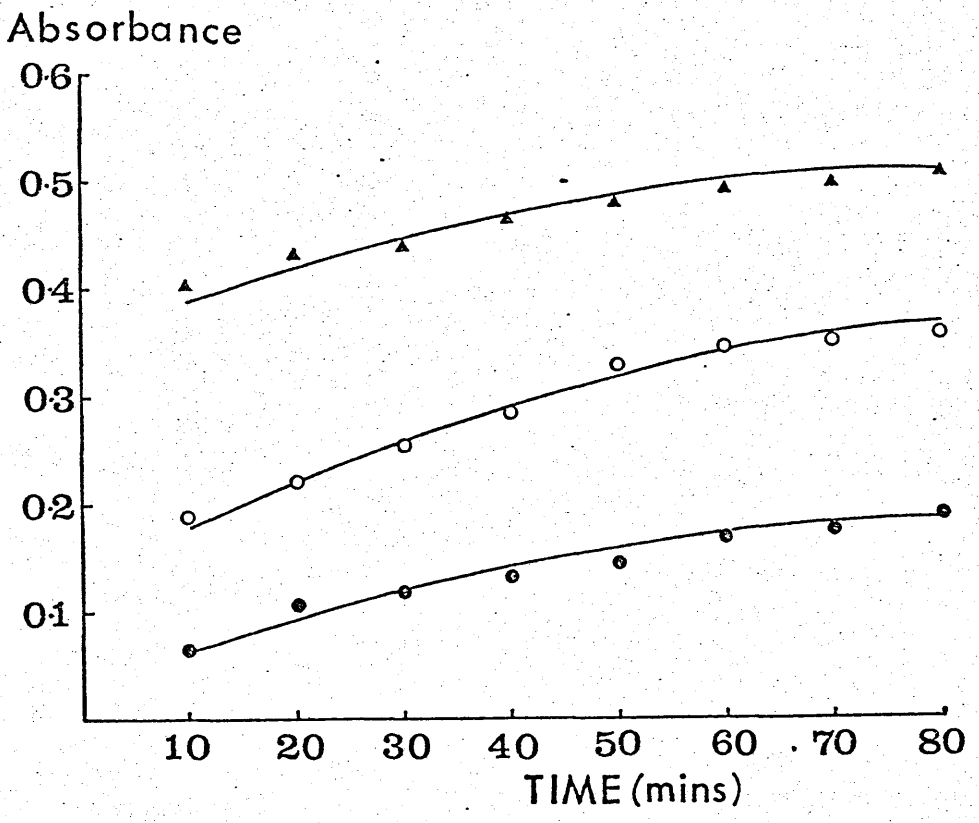

Fig 3 v Time course of SP_1 with immobilized anti SP_1 at 3 concentrations of antigen diluted in PBS containing 1% albumin

(⊙——⊙) 31.25 µg/l

(o——o) 125 "

(▲——▲) 1000 "

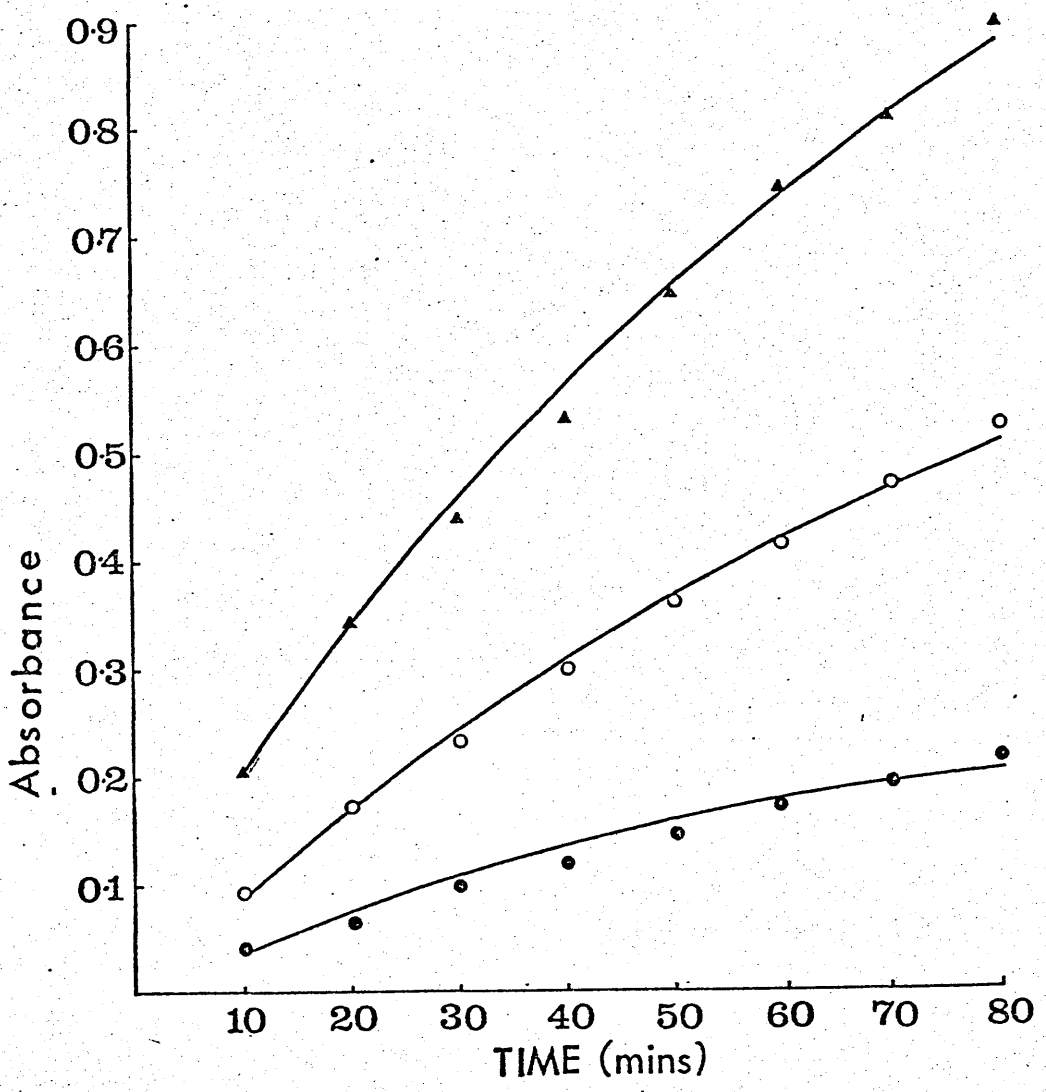

Fig 3 vi Time course of antibody-peroxidase with bound SP_1 at 3 antigen concentrations.

(●——●) 31.25 µg/l

(o——o) 125 "

(▲——▲) 1000 "

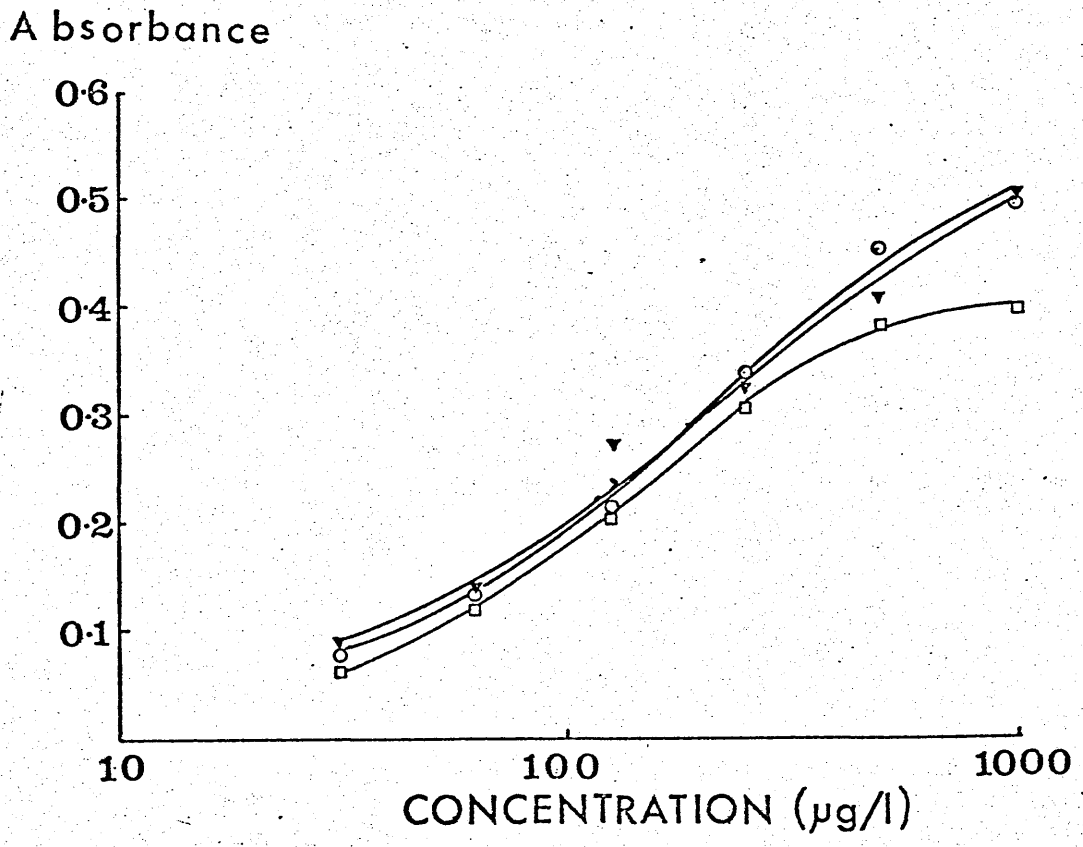

Fig 3 vii The effect of varying coating antibody dilution in the assay of SP_1. Antiserum was diluted (▼——▼) 1/750, (o—o) 1/1200 or (□——□) 1/1500 in coating buffer.

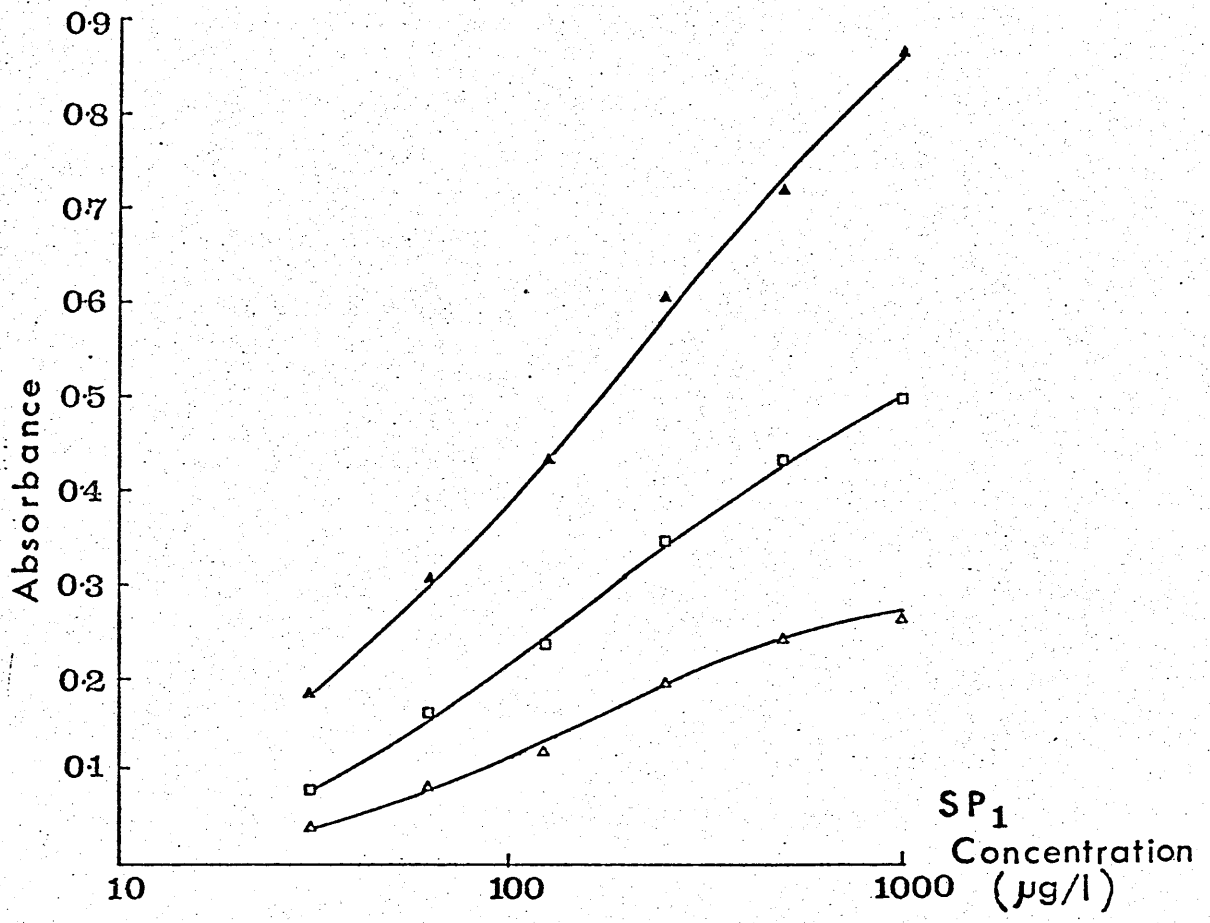

Fig 3 viii The effect of varying antibody-enzyme concentration on the standard curve for SP_1 assay. Antibody-enzyme was diluted (▲——▲) 1/500, (□——□) 1/1000 or (△——△) 1/2000 in PBS containing 25% chicken serum and 10^{-2} mole/l citrate.

Table 3 III

Recovery of SP_1 added to male serum

Theoretical Concentration mg/l	Measured Concentration mg/l	% Recovery
60	58.6	97.5
90	94	104.5
120	126	105

Mean Recovery 100%

Table 3 IV

Precision of SP_1 Assay

a) Within Batch

	Lo	Hi
Mean Concentration (mg/l)	104	198
Std. Deviation	7.2	15.6
CV %	7.0	7.9
n	20	20

b) Overall

	Lo	Hi
Mean Concentration (mg/l)	107	200
Std. Deviation	13.1	32.7
CV %	12.2	16.3
n	60	64

Table 3 V

Parallelism of SP_1 Assay

Dilution	SP_1 Concentration (mg/l) after correction for dilution factor (Mean $\pm$ SD)
Neat	330 $\pm$ 41
$\frac{1}{2}$	322 $\pm$ 42
$\frac{1}{4}$	301 $\pm$ 11
$\frac{1}{5}$	326 $\pm$ 13

From Fig 3 v, 3 vi it can be seen that increased incubation times lead to increased colour development and that at no point is equilibrium reached. Adequate assay sensitivity for the range of SP_1 concentrations seen in pregnancy are achieved using incubation times of 30 minutes.

Optimal antibody dilution for coating the plate (Fig 3 vii) was found to be 1/1200 and adequate assay sensitivity with these factors set was achieved with Ab-E dilutions of 1/1000 (Fig 3 viii). These findings are in agreement with Macdonald's results (1979) except that he used a different Ab-E preparation. Varying Ab-E concentration was however found to affect non specific binding. This is discussed further in chapter 4.

Recovery varied between 97.5% and 105% (Table 3 III) so the method is accurate. It is also acceptably precise (Table 3 IV), CV's being in the range associated with many immunoassays. Parallelism was exhibited over the range of dilutions considered (Table 3 V). This finding is not at variance with the discovery of the heterogeneity of SP_1 (Towler et al 1978, Anthony et al 1980a, Ahmed et al 1982a) as there is no change in the relative concentrations of the two components.

The assay provides an accurate, precise method of quantitating SP_1 in suitably diluted pregnancy serum. Further sensitization would be desirable before its use in detecting SP_1 in patients with neoplasia however (Pledger et al 1981).

3.5 Development of ELISA with Improved Sensitivity for the Assay of SP_1

3.5.1 Introduction

The assay was based on the principles described for the assay

provide increased sensitivity as described. Whilst the ELISA described previously is suitable for assaying SP_1 concentrations commonly found in late pregnancy, increased assay sensitivity is desirable for assay of SP_1 in patients with neoplastic disease.

3.5.2 Method for Removal of endogenous SP_1 from male serum

2.5 g of cyanogen bromide activated-sepharose was swollen and washed for 24 hours in 100 ml of 10^{-3} mol/l hydrochloric acid with 3 changes of wash. 10 ml of sepharose were prepared and to this was coupled 5 mg anti SP_1/ml of sepharose (protein content of antiserum - 25 g/l) as follows.

2 ml of anti SP_1 were equilibrated with 4 ml carbonate/bicarbonate buffer pH 9.6 containing 0.5 mol/l sodium chloride (to prevent protein-protein interaction). This was added to the sepharose gel also equilibrated at pH 9.6 in the same buffer, and mixed for 2 hours at room temperature. The gel volume of 0.2 mol/l Tris buffer pH 8.0 was then added and mixed with the gel at room temperature to block any remaining active sites on the gel. Following centrifugation, the supernatant was discarded and the gel complex washed alternately with acetate buffer (0.1 mol/l, pH 4.0) and borate buffer (0.1 mol/l pH 8.5) each with addition of 1 molar sodium chloride, centrifuged and the supernatant discarded.

Efficiency of Antigen Stripping Initially a study using radioactively labelled SP_1 (kindly donated by Professor K Bagshawe, Department of Medical Oncology, Charing Cross Hospital) was performed to investigate the efficiency of antigen stripping. The experiment was performed either with or without changes of the sepharose-antiserum gel, mixed with a pool of male serum spiked with labelled SP_1. Counting of the remaining

Table 3 VI

Efficiency of Antigen Removal by Sepharose-Anti SP_1 Solid Phase

a) With changes of solid phase (change performed at noted times)

Time	Counts/20 secs	% SP_1 Removed
0 min	12,500	-
60 "	3,060	75
90 "	1,800	88
120 "	1,800	88
16 hrs	960	92

b) No change of solid phase

Time	Counts/20 secs	% SP_1 Removed
0 min	12,500	-
60 "	3,075	75
90 "	3,200	75
120 "	3,100	75
16 hrs	3,100	75

stages to assess the efficiency of removal (Table 3 VI). From the findings of this experiment, the pool of male serum to be used for preparation of standards was mixed with sepharose-antiserum gel for 18 hours at room temperature with 3 changes of gel.

3.5.3 Sensitized SP_1 Assay Method

Anti SP_1 diluted 1/1200 in coating buffer pH 9.6 (100 µl) was pipetted into the wells of microtitre plates and incubated overnight at 4°C. Following washing 3 times with wash buffer and drying, 100 µl of standards, test sera or quality control material was pipetted into the wells in duplicate and incubated at room temperature for 75 minutes, after which the well contents were aspirated and the plates washed and dried again. Ab-E diluted 1/500 in PBS/chicken serum was then added to the wells and incubated for 75 minutes. Following a further washing cycle, chromogen was added (100 µl) to each well and colour development allowed to proceed for 30 minutes. The reaction was stopped by addition of 100 µl of 4 mol/l sulphuric acid to each well, and the developed colour read on a Multiskan plate reader.

3.5.4 Investigation of Factors Affecting the Assay

The effects of increasing incubation times of antigen with antibody and of antigen with antibody enzyme at 3 concentrations of SP_1 were investigated over a prolonged time course in a similar manner to that described previously (Macdonald 1979). The optimal dilution of Ab-E was also investigated as described previously.

The Effect of the Protein Matrix As SP_1 in this sensitized assay was to be assayed in undiluted sera as opposed to highly

used to prepare standards was investigated.

Standards of varying concentration were prepared in 3 different matrices. Behringwerke SP_1 standard was diluted in either PBS/citrate containing 1% BSA, chicken serum or a pool of male serum stripped of endogenous SP_1. A number of sera from male and non pregnant female laboratory staff were also used in these studies.

The standards in PBS/citrate and chicken serum were investigated initially. These standard solutions were assayed according to the routine assay protocol and a graph of absorbance (corrected for blank value) against concentration was made. The sera from laboratory staff were also assayed and also a series of chicken sera containing no SP_1 were assayed.

In a second experiment, standards prepared in chicken serum and SP_1-free male serum were compared. Replicate male serum blanks were also utilized.

3.5.5 Assessment of Assay Sensitivity

Sensitivity was assessed by assaying a series of blanks according to the routine protocol. On 1 plate Ab-E at a dilution of 1/1000 was used, on the second plate Ab-E at a dilution of 1/500 was used. Assay sensitivity was calculated using the formula:-

Sensitivity = OD blank + 2 SD

the SD being calculated from the formula

$$SD = \sqrt{\frac{\Sigma D^2}{2N-1}}$$

where D = difference between duplicate blank ODs

N = Number of duplicates

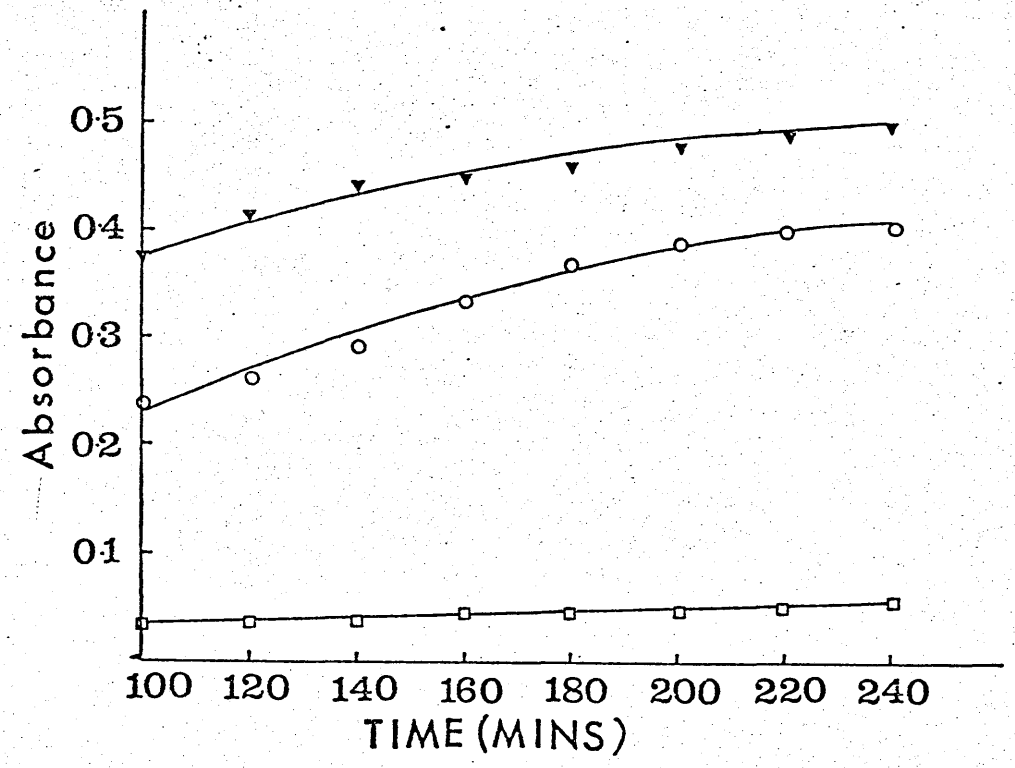

Fig 3 ix Prolonged time course of SP_1 with immobilized anti SP_1 at 3 concentrations of antigen diluted in PBS containing 1% albumin

(□——□) 3.91 µg/l

(o——o) 62.5 "

(▼——▼) 500 "

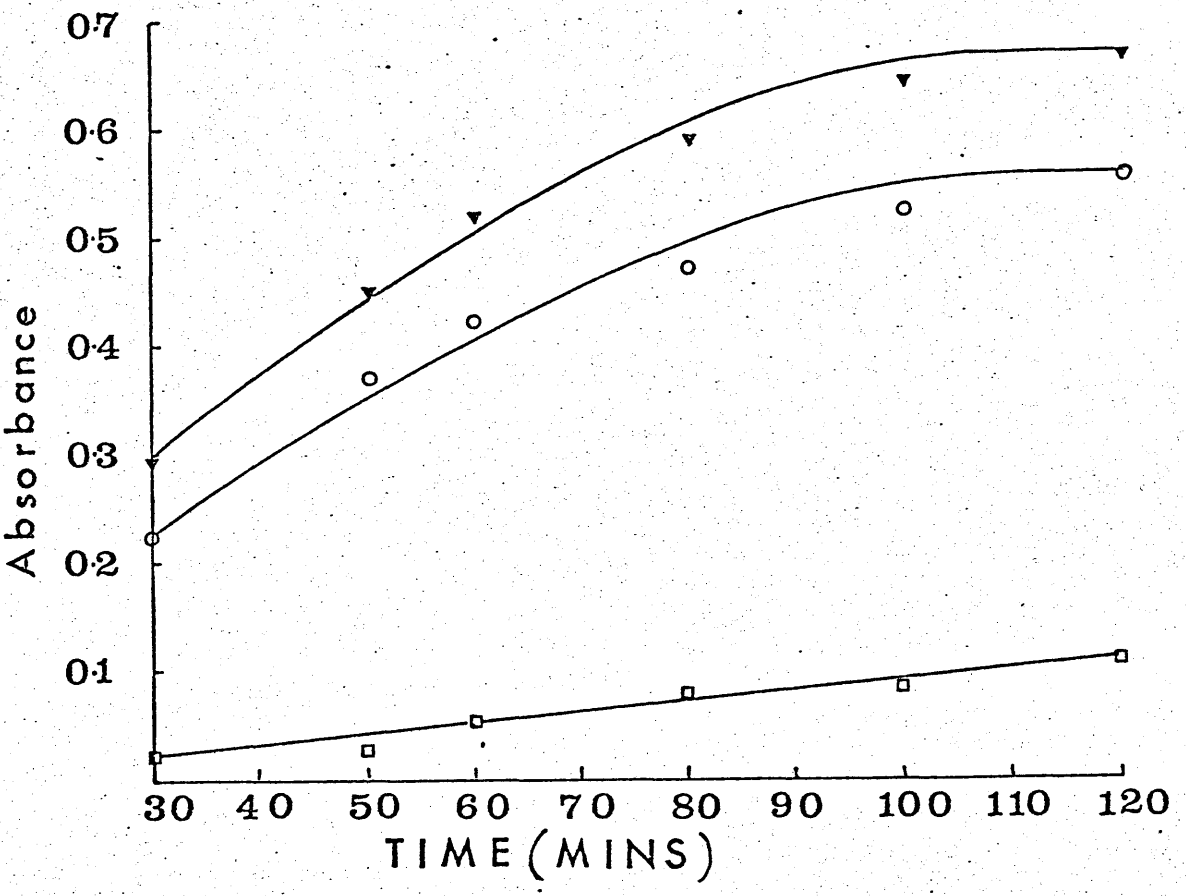

Fig 3 x Prolonged time course of antibody-peroxidase with bound SP_1 at 3 antigen concentrations in PBS/citrate.

(□——□) 3.91 µg/l

(o——o) 62.5 "

(▼——▼) 500 "

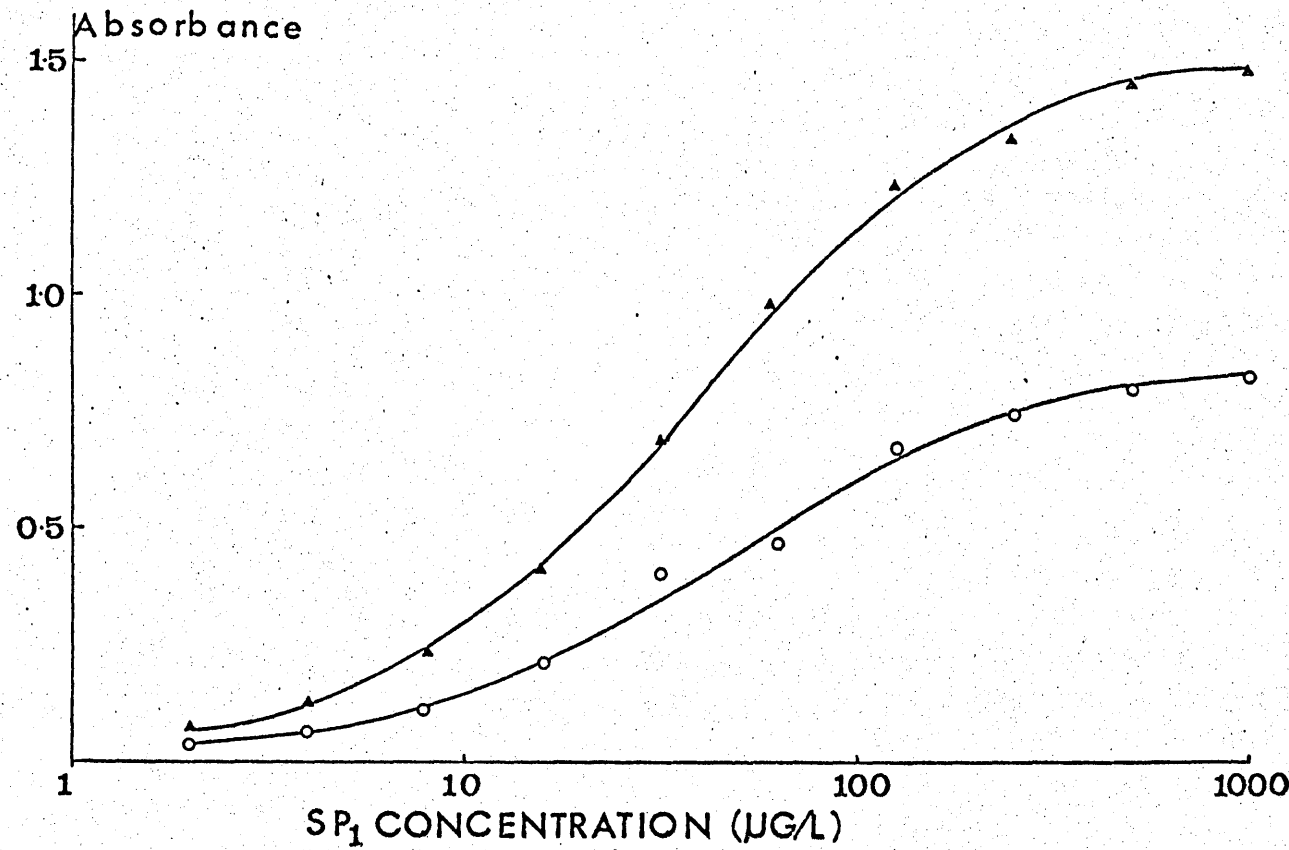

Fig 3 xi The effect of varying antibody-enzyme concentration on the standard curve for sensitive SP_1 assay. Antibody-enzyme was diluted (▲—▲) 1/500 or (o—o) 1/1000 in PBS containing 25% chicken serum and 10^{-2} mole/l citrate.

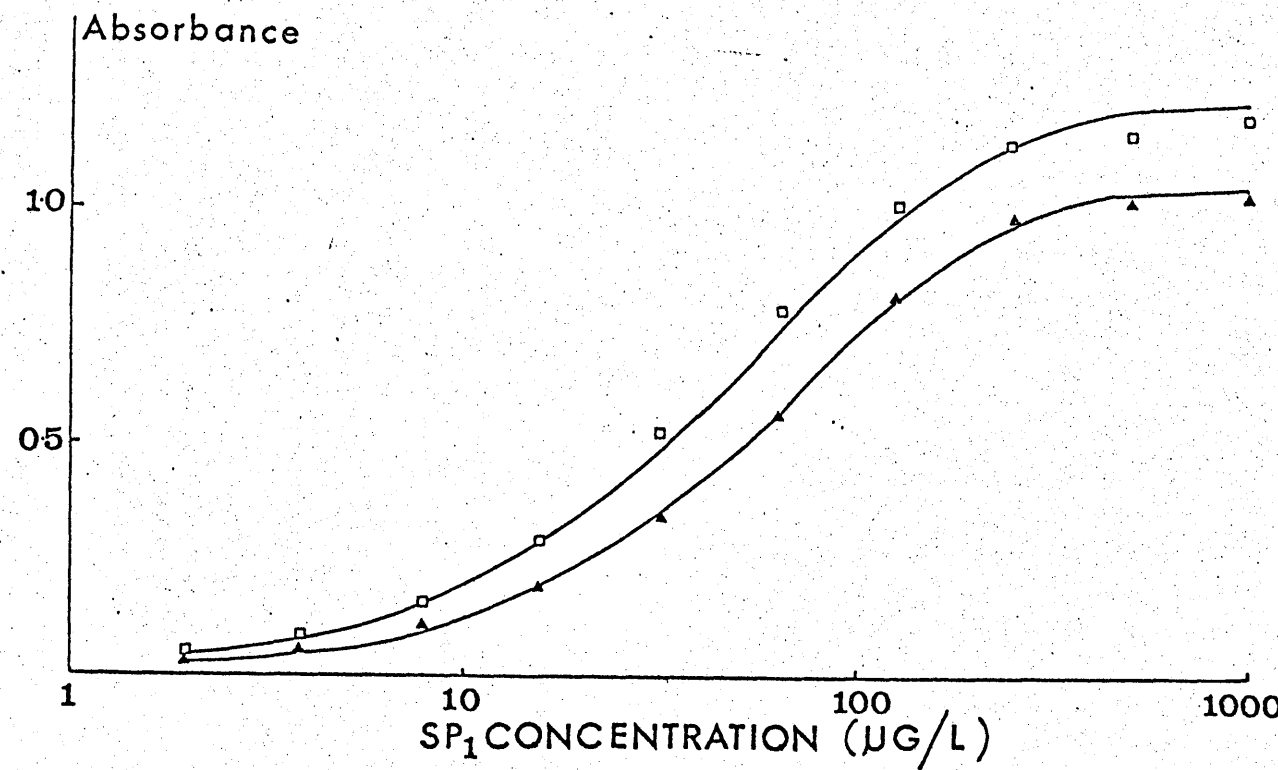

Fig 3 xii The effect of varying protein matrices upon the standard curve for sensitive SP_1 assay. Standards were prepared in (▲—▲) chicken serum or (□—□) PBS containing 1% albumin.

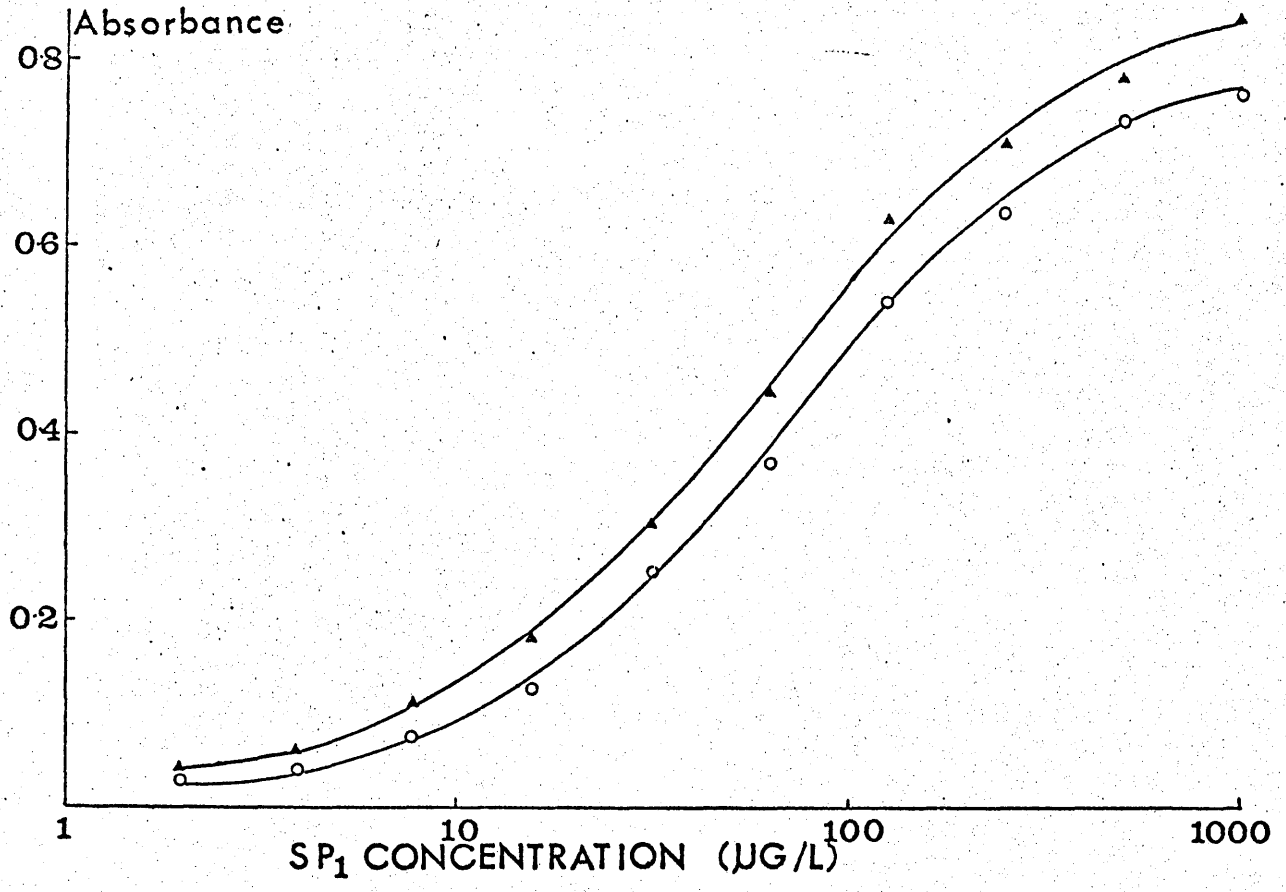

Fig 3 xiii Comparison of standards prepared in (▲——▲) chicken serum or (O—O) male serum stripped of endogenous SP_1.

It had been shown previously that increasing contact time of antigen with antibody and antibody enzyme with antigen led to increased colour development (3.4.4). Figs 3 ix, 3 x show that it is possible to extend these incubation times considerably and thus increase assay sensitivity. However maximal increase in colour is seen over the first 80 minutes at each incubation stage and there is little point in extending incubations beyond this. Fig 3 xi shows that decreased dilutions of Ab-E leads to a more sensitive assay, although NSB increases. A suitable compromise is thus 1/500 dilution.

The assay sensitivity using Ab-E at a dilution of 1/1000 was 4.3 µg/l and using Ab-E at a dilution of 1/500 was 3 µg/l. In practice however, an arbitrary decision must be made in a similar way to that for RIA.

Over a number of assays, the mean practical sensitivity was 6.13 µg/l $\pm$ 0.80 (SD). In view of the findings of Anthony's group (1980a) who found values up to 6 ug/l in the normal population, this practical limit of detection is suitable.

The effect of the protein matrix upon the standard curve is shown in Figs 3 xii, 3 xiii. As undiluted test sera were to be assayed, it is apparent that SP_1 standards must be prepared in a male serum protein matrix or results will not be comparable. Further evidence for this fact is obtained from the fact that a number of normal sera (34%) showed detectable levels of SP_1 when compared against a standard curve prepared in PBS/citrate. Comparing against a standard curve prepared in male serum, only 14% of normal sera were positive. This is close to the number found by Searle (1978) using an assay of comparable sensitivity.

Table 3 VII

Recovery of SP_1 using Radial Immunodiffusion Assay

Expected Concentration (mg/l)	Found Concentration (mg/l)	Recovery %
90	86	98
191	168	88
197	168	85
	Mean	90%

Table 3 VIII

Comparison of SP_1 Concentrations obtained by ELISA with Concentrations obtained by RID

Correlation coefft	0.83 ($p<0.001$)
Gradient	0.32
Y Axis Intercept	66.2
Std Error of Gradient	0.03
Std Error of Intercept	9.0

3.6.1 Materials and Methods

Standards and test sera (5 µl) were pipetted into the wells of the RID plate. After incubation at room temperature for 2 days to allow maximum immunodiffusion, the diameters of the precipitin rings were measured. A standard curve was constructed using a plot of $(\text{diameter})^2$ against antigen concentration.

Recovery was assessed by addition of known volumes of standard SP_1 material to known volumes of pooled male serum. Within batch precision was assessed by replicate analyses of a pool of pregnancy serum on 1 RID plate.

3.6.2 Results and Discussion

Table 3 VII shows the results of the recovery experiments. Within batch precision was 4.0% at a concentration of 164 mg/l. Table 3 VIII shows the comparison of results obtained by RID compared to those obtained by ELISA. Results obtained by ELISA were significantly higher when tested by the paired t test. However, a significant degree of correlation exists between the results obtained.

The finding of a lower recovery using RID may partially account for this and the problem may be further complicated by the molecular heterogeneity of SP_1 (Teisner et al 1978).

Whilst the precision is improved using RID, this is offset by the longer turn-round time to obtain results, the decreased working range, and the unsuitability of RID to cope with large numbers of specimens. ELISA therefore remains the method of choice.

Plasma Protein A (PAPP-A)

3.7.1 Introduction and Principle

This assay was based on an assay in use for the measurement of SP_1 (Macdonald et al 1979). Antibodies to PAPP-A are bound to the wells of plastic microtitre plates. After removing unbound antibody, antigen is added. Following incubation, the antigen is removed and antibody linked to horseradish peroxidase is then incubated in the wells. Following removal of excess antibody-enzyme, chromogen is added and colour allowed to develop. The reaction is terminated by addition of acid. The resulting colours are read in a spectrophotometer, the absorbances compared with those for the standards and calculation of the unknown concentrations made.

3.7.2 Materials

PAPP-A Laboratory Standard - Sera from 50 patients in the 3rd trimester (28-40 weeks gestation) of a normal pregnancy were pooled to give a pool of 250 ml volume. This pool was aliquoted and stored at -20°C until use. A nominal value of 1000 arbitrary units/litre (AU/L) was assigned to this pool until calibration against IRP 78/610 was made. Dilutions of this standard pool were made in PBS/citrate to give a range of standards from 0-20 AU/L.

Anti-PAPPA- Peroxidase was prepared by a two step glutaraldehyde conjugation method (Avrameas and Ternynck 1974) as described (3.7.3). After purification on a Sephacryl S200 column the conjugate was stored at -20°C. For use a 1/20 dilution was made in PBS/chicken serum.

All other materials are as described in Appendix I.

3.7.3 The Method of Avrameas and Ternynck for Antibody Enzyme Conjugation

10 mg of horseradish peroxidase (HRP) were dissolved in 200 µl of 0.1 mol/l phosphate buffer (pH 7.4) containing 1.25% (v/v) glutaraldehyde and mixed for 24 hours at room temperature. The solution was then dialyzed at -4°C for 24 hours against 0.9% saline.

The total protein content of the PAPP-A antiserum was shown to be 15 g/l by the Lowry method for protein assay. 0.3 ml (45 mg) of antiserum were equilibriated in 0.6 ml of carbonate/bicarbonate buffer pH 9.6 added to the HRP solution, and reacted for 24 hours at 4°C with continual mixing.

0.1 ml of a 0.2 mol/l solution of lysine was then added and mixed for 2 hours at 4°C, to inactivate residual aldehyde.

Dialysis was then performed against PBS for 24 hours.

Separation of Ab-E from unreacted components was investigated using Ultragel ACA 34 and Sephacryl S200.

3.7.4 Methods of Separation of Conjugate from Unreacted Components

i) Separation using Ultragel - A column measuring 45 cm x 2.2 cm (total volume 171 ml) was packed with Ultragel ACA 34. The void volume assessed by Dextran Blue was 56.0 ml. The column was equilibrated using a set of protein standards purchased from Pharmacia.

K_{av} values for these standards were estimated using the formula:-

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}$$

where Ve = elution volume
Vo = void volume
Vt = total volume

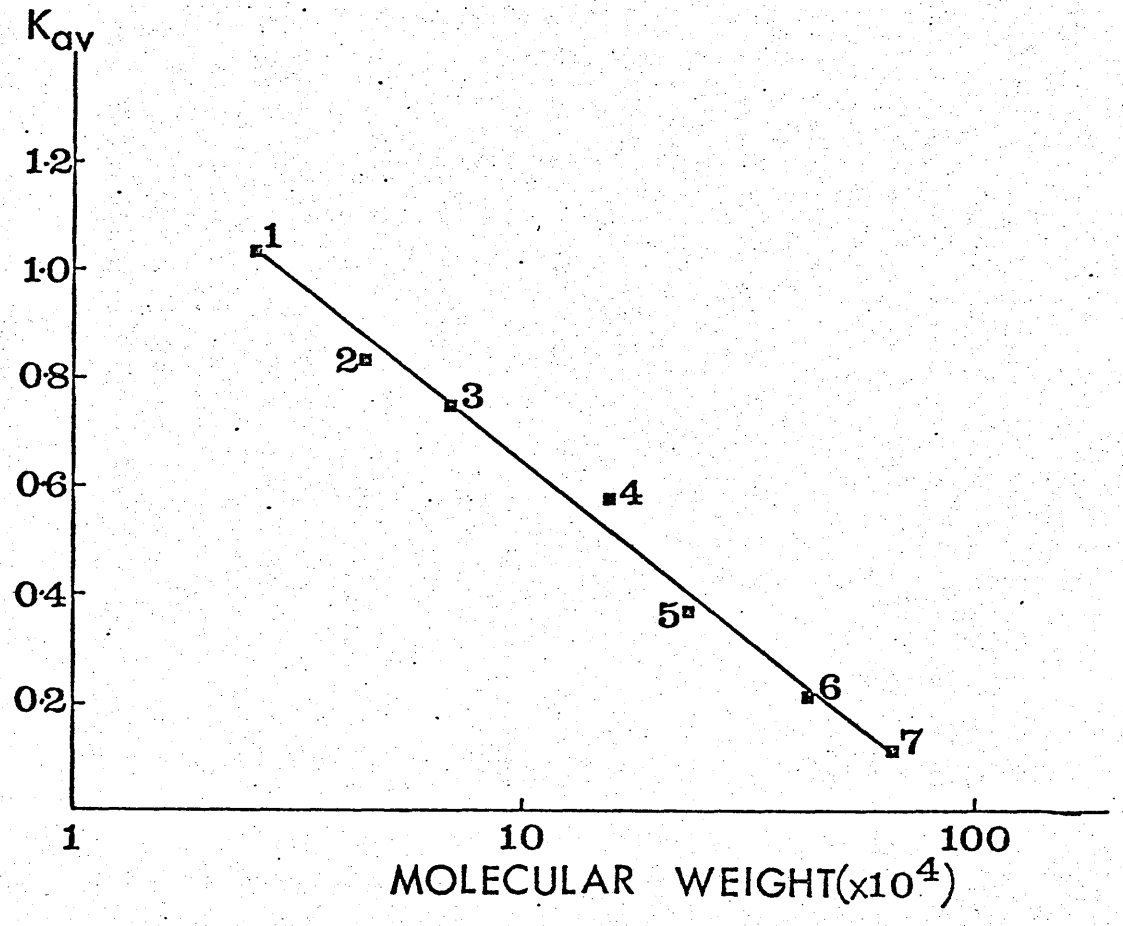

Fig 3 xiv Calibration line for Ultragel Chromatography column. The calibrating proteins used were:-

1)	Chymotrypsinogen A	MW =	25 000
2)	Ovalbumin	" =	43 000
3)	Albumin	" =	67 000
4)	Aldolase	" =	158 000
5)	Catalase	" =	232 000
6)	Ferritin	" =	440 000
7)	Thyroglobulin	" =	669 000

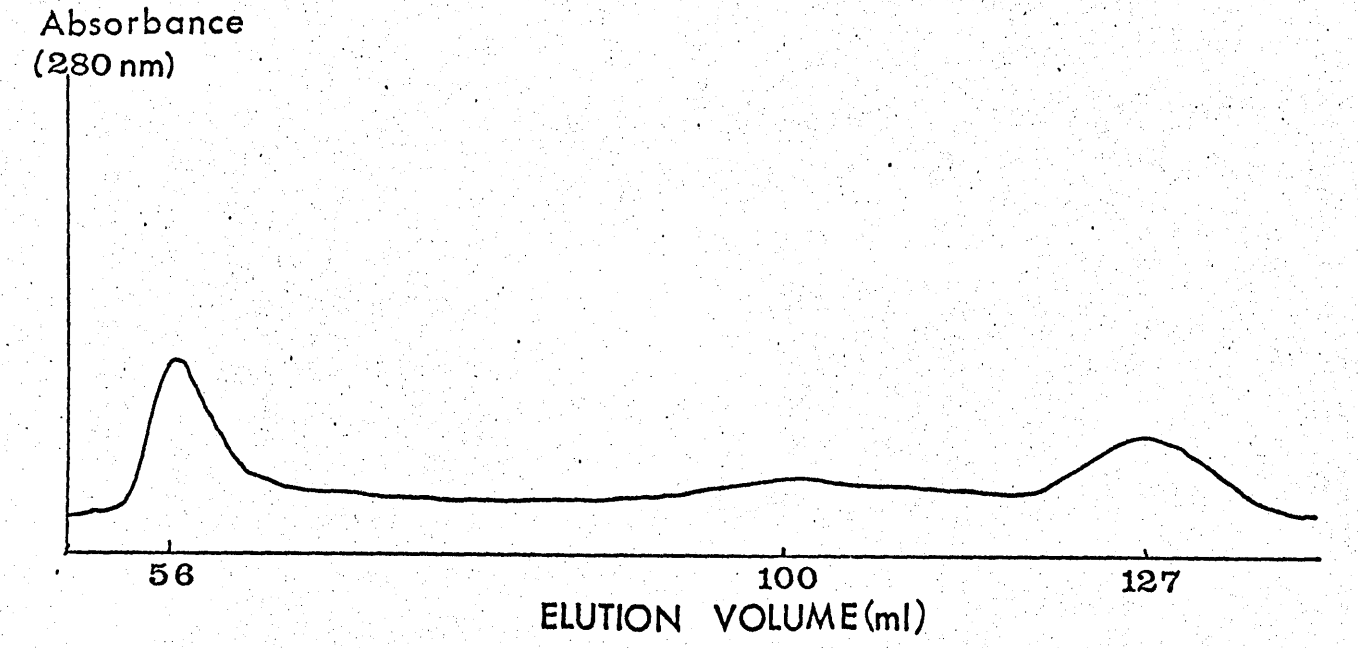

Fig 3 xv The elution profile of Anti PAPP-A-Horse radish peroxidase conjugate on Ultragel ACA34 chromatography column.

Peak I (56 ml) represents Dextran Blue marker

II (100 ml) represents Antibody-enzyme conjugate

III (127 ml) represents Unreacted peroxidase

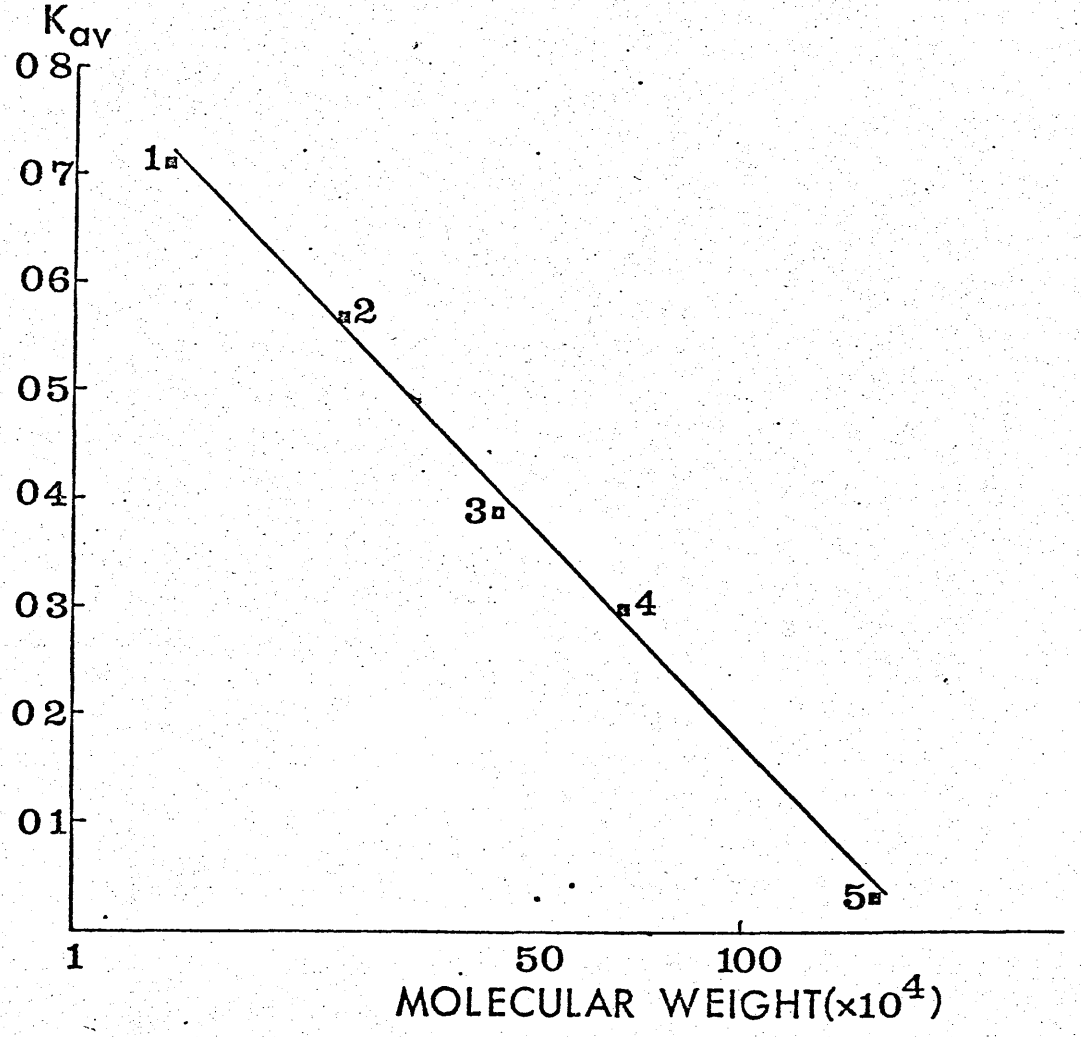

Fig 3 xvi Calibration line for Sephacryl S200 Chromatography column. The calibrating proteins used were:-

1) Ribonuclease A MW 13 700
2) Chymotrypsinogen A " 25 000
3) Ovalbumin " 43 000
4) Albumin " 67 000
5) Aldolase " 158 000

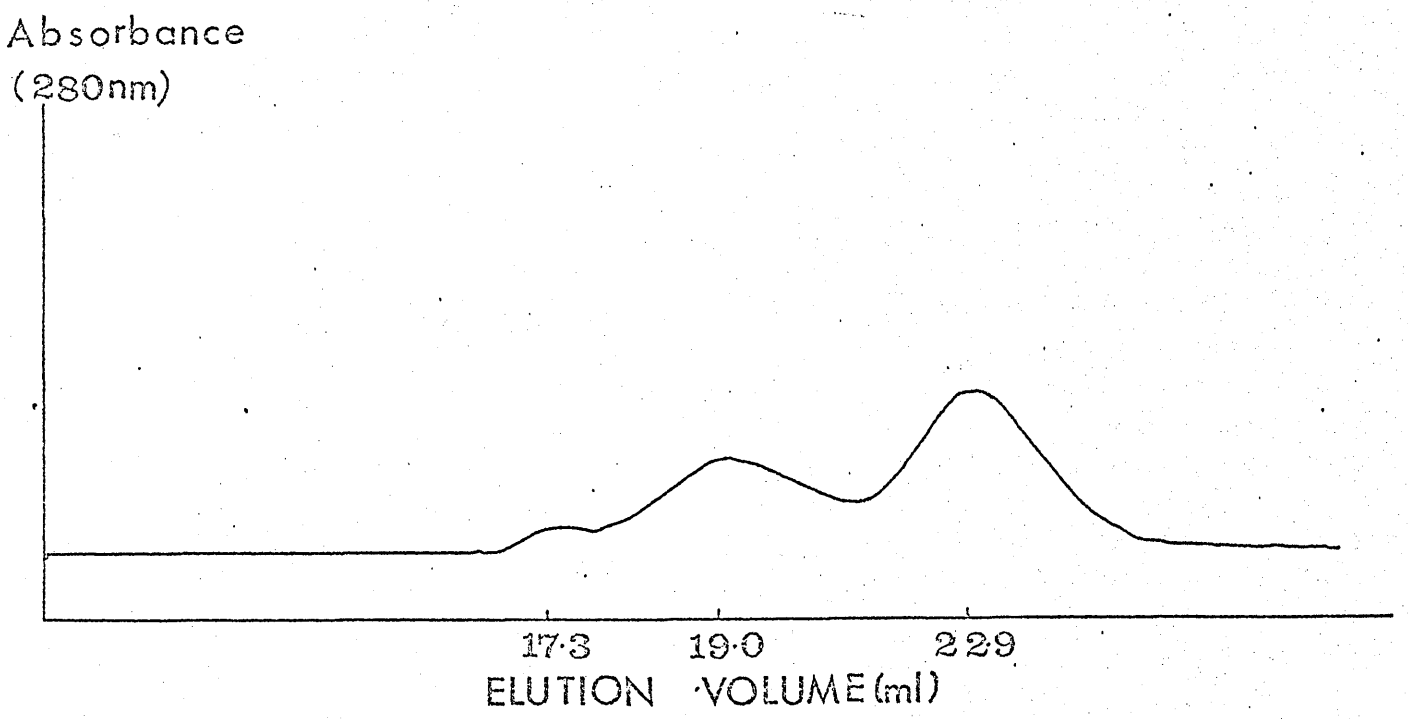

Fig 3 xvii The elution profile of Anti PAPPA Horseradish peroxidase conjugate on Sephacryl S200 chromatography column.

Peak I	(17.3ml)	represents Antibody enzyme conjugate
Peak II	(19.0ml)	represents Unreacted antibody
Peak III	(22.9ml)	represents Unreacted peroxidase

Table 3 IX

Molecular weight estimates of Ab-E conjugate and individual components using Ultragel ACA 34 and Sephacryl S200 columns

a) Ultragel column

Peak Number	Elution Volume (ml)	K_{av}	Molecular Weight
II Anti PAPP-A-HRP	100	0.37	260,000
III Free HRP	127	0.62	110,000

b) Sephacryl S200 column

(i) Conjugate Mixture

Peak Number	Elution Volume (ml)	K_{av}	Molecular Weight
I (Anti PAPP-A-HRP)	Void	-	160,000
II (Free Anti PAPP-A)	19.0	0.08	140,000
III (Free HRP)	22.9	0.27	71,000

(ii) Free Components

Component	Elution Volume (ml)	K_{av}	Molecular Weight
(45 mg) Anti PAPP-A	20.2	0.14	120,000
10 mg HRP	22.5	0.25	76,000

The conjugate mixture was applied to the column and eluted with PBS, collecting 1 ml fractions. Protein elution was monitored at 280 nm and K_{av} values calculated.

A plot of K_{av} against log molecular weight for the standards was made and used to calculate molecular weights for the conjugate mixture proteins.

ii) Separation using Sephacryl S200 - A column measuring 60 cm x 0.9 cm was packed with Sephacryl S200 (total volume 38.2 ml, void volume 17.3 ml). The column was calibrated with protein standards as before.

Ab-E conjugate, unconjugated anti PAPP-A, and HRP were applied in separate chromatographic runs to the column, the elution volumes noted, and molecular weights estimated.

3.7.5 Results

Figures 3 xiv and 3 xv show the calibration line for the Ultragel column and the elution profile of Ab-E conjugate on this column. Figures 3 xvi and 3 xvii show the same details for the Sephacryl S200 column. Table 3 IX shows the molecular weight estimates on the different columns. Initial use of the Ultragel column proved unsatisfactory in its inability to separate the conjugate from unreacted antiserum and further, the estimated molecular weight for HRP using this column was not in accordance with other workers estimates (Morita et al 1980).

Using the Sephacryl column improved resolution of the components was obtained. Molecular weight of the antibody was in agreement with general estimates, although that for HRP was still higher than most estimates. This finding may be due to the

MW estimates by gel filtration unreliable.

It is not possible to assign an exact molecular weight to the Ab-E conjugate as it eluted in the void volume. Avrameas and Ternynck (1974) felt that the molecular weight of the conjugate was not in excess of 200,000 indicating that it consisted of one molecule of HRP linked to 1 molecule of antibody and that the main objective of the two stage procedure had been attained. The major disadvantage of this procedure is its low yield, about 2% of the HRP being conjugated to antibody (Nakane and Kawaoi 1974). Nevertheless experience with the Avrameas technique in this laboratory has shown it to be reproducible in terms of the optimal dilutions of Ab-E required for assay purposes.

Further Anti PAPP-A-HRP conjugates were separated on Sephacryl S200 for use in PAPP-A assay.

3.7.6 Method of Assay

100 µl of antihuman PAPP-A previously diluted 1/1200 in carbonate/bicarbonate buffer pH 9.6 was pipetted into each well of a microtitre plate excluding the outer rows because of previously reported "edge" effects (Burt et al 1979).

The plates were left overnight at +4°C. No special methods were used to enhance antibody binding.

Before use, the plates were emptied by inversion, washed three times with wash buffer and shaken dry.

100 µl of standard materials or 100 µl of test serum diluted 1/100 in PBS/citrate were added in duplicate to the wells at timed intervals. Incubation at room temperature was allowed to proceed for 120 minutes after which the solutions were

aspirated, the plate washed three times with wash buffer and shaken dry.

100 µl of antibody enzyme conjugate (diluted 1/20 in PBS containing 25% chicken serum) was then added to each well and incubated for 120 minutes at room temperature. The solutions were then aspirated and the plate washed and dried as before.

100 µl of chromogen was then added to each well. After 45 minutes of colour development in the dark at room temperature the reaction was stopped by addition of 100 µl of 4 mol/l sulphuric acid to each well.

The developed colours were read on either an Abbott ABA 100 bichromatic analyzer (Abbott Laboratories Ltd, Basingstoke, Hants) or latterly on a Titertek Multiskan (Flow Laboratories).

To enable comparison of results from these 2 instruments readings were converted to absorbance values for a 1 cm path length (A 1 cm).

The Abbott ABA analyzer was used only during initial development stages of this assay. All further work was performed on the Multiskan plate reader giving absorbance values directly.

Calibration curves of absorbance against concentration were plotted on semilogarithmic graph paper. Alternatively a suitable curve fitting procedure may be utilized to allow automation (Chapter 5).

3.7.7 Investigation of Factors Affecting the Assay

i Time course of Antibody Antigen Reaction.

100 µl of standard material containing 1, 2 and 20 AU/L were pipetted in duplicate into wells coated with antiserum as in the routine assay protocol. Preliminary investigation had shown that incubation times of less than 80 mins did not give adequate sensitivity. In

an attempt to increase sensitivity the reaction time course was investigated between 80 and 150 minutes. The plates were then processed as in the routine assay.

ii Time course of antibody-enzyme antigen reaction.

100 µl standard material containing 1, 2 and 20 AU/L were pipetted into the wells of microtitre plates previously coated with antibody and allowed to incubate for 120 minutes. The sera were then aspirated and the plate washed. Diluted Ab-E solution was added to each well and incubated at room temperature for times varying between 80 and 150 minutes. After this incubation the plate was processed according to the routine assay protocol.

iii Variation of incubation time for colour development.

Antigen at concentration of 10, 100, 500 and 1000 AU/L was applied to microtitre plates, followed by Ab-E according to the routine assay protocol. Chromogen was then added (100 µl/well) and incubated for times varying between 10 and 80 minutes.

iv Effect of antiserum dilution used to coat plates.

Antiserum at dilutions varying from 1/500 to 1/2000 in carbonate/bicarbonate buffer was dispensed into the wells of 5 microtitre plates and incubated overnight at $+4^{o}C$.

After washing, standards were added to each well previously coated with antibody and processed according to the routine assay schedule.

colour development.

100 µl of standards were added to wells of a microtitre plate previously coated with anti-PAPP-A at a dilution of 1/1200. After incubating, washing and drying in the normal way, 100 µl Ab-E at dilutions ranging from 1/20 to 1/60 was added to each well and incubated for 120 minutes. Further steps were according to the routine protocol.

3.7.8 The Effect of haemolysis on the assay of PAPP-A

Blood samples were collected from patients undergoing routine antenatal biochemical monitoring, 5 ml blood specimens were collected into lithium heparin containers and mixed well.

On arrival in the laboratory, the specimen was divided into four 1 ml parts and treated as follows:

part (1) immediate separation of plasma from cells (fresh plasma);

part (2) incubation of specimen at room temperature for 24 h before separation of plasma (incubated plasma);

part (3) addition of 0.5 ml deionised water, mixed by inversion, separation of plasma (haemolysed);

part (4) addition of 0.5 ml isotonic saline, mixed by inversion, separation of plasma (non-haemolysed).

Blood samples from male laboratory staff were also collected into heparin and treated as in (3) and (4) above.

All separated plasma specimens were immediately stored at -20°C until assay. PAPP-A and SP_1 concentrations were assessed by enzyme-linked immunoassay described earlier and hPL by RIA.

batch for each analyte. Comparison of results was made by use of the paired t test.

3.7.9 Assay Characteristics

i The standard pool of pregnancy serum prepared in this laboratory was calibrated against International Reference material 78/610 reconstituted according to the schedule supplied. Dilutions of the reconstituted material were made in PBS/citrate to give a range of standards from 0 - 2.0 IU/L.

ii Precision Studies - Within plate precision was calculated for three QC pools prepared by diluting a third trimester pool of pregnancy serum in chicken serum. Past experience in this laboratory has shown that chicken serum provides a suitable protein matrix without interfering factors that may be present in human serum.

Precision was calculated for each pool by running 10 duplicates of each pool on one microtitre plate.

Overall precision was estimated for three QC pools over 45 batches of PAPP-A analyses on separate microtitre plates.

iii Recovery Studies - Dilutions of the standard pool were made in chicken serum to give concentrations of 9.0, 22.5, 45 and 90 IU/L. Aliquots of a male serum pool were spiked with the above material and recoveries estimated. The male pool used showed no evidence of containing PAPP-A.

iv Limit of Detection - The sensitivity was assessed by running 20 duplicates of 0AU/L standards (blanks) on

the microtitre plate. Sensitivity was defined as that concentration in an undiluted specimen which correspond to an OD of the zero standard in the standard curve + 2 standard deviations (SD) where:

$$SD = \sqrt{\frac{D^2}{2N - 1}}$$

D = Difference in duplicate blanks

N = Number of duplicates

v Specificity of Antiserum - Possible cross reactions with three other placental proteins were investigated at comcentrations which may be present in pregnancy. These proteins were human placental lactogen (10 MU/L), human chorionic gonadotrophin (44,000 IU/L) and pregnancy specific β_1 glycoprotein (705 mg/l) prepared as follows.

7.3 µg iodination grade hPL (obtained from NIBSC) was dissolved in 7.2 ml of diluting solution and used neat in cross reaction studies. The hPL concentration was thus 10 times that routinely encountered in the PAPP-A assay using diluted serum.

1 vial of hCG (Calbiochem) was dissolved in 0.5 ml of diluting fluid and used neat in these studies.

SP_1 extracted from placentae was diluted 1/100 in diluting solution to give a concentration of 7050 µg/l. This is 20 times greater than that routinely encountered in the PAPP-A assay after dilution of the test serum.

3.7.10 PAPP-A Levels in Non Pregnant Subjects

PAPP-A levels were measured in serum from laboratory staff (29 males and 31 non pregnant females).

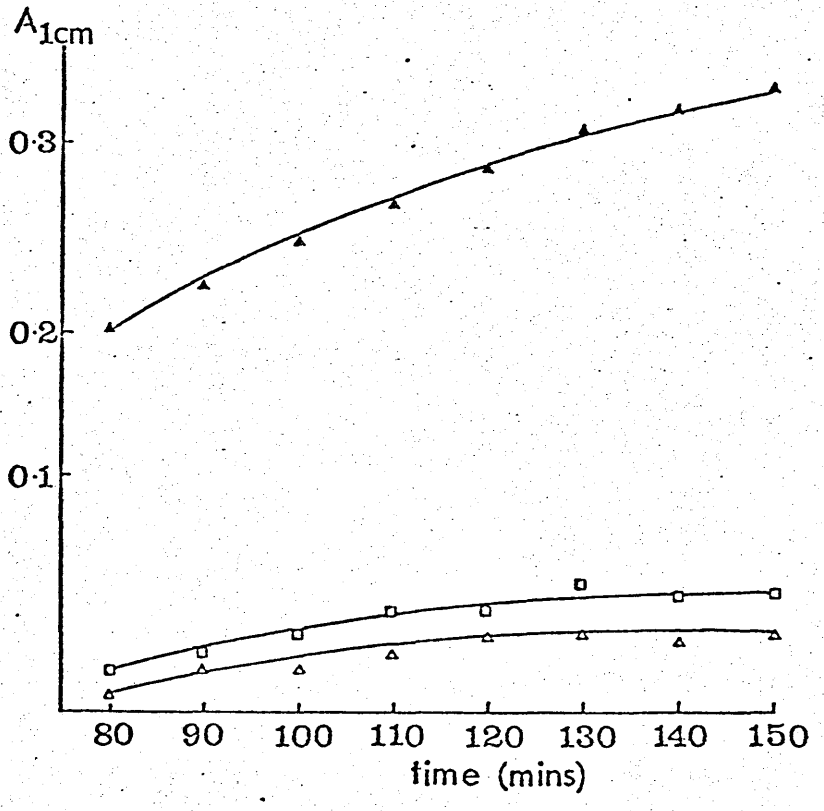

Fig 3 xviii Time course of PAPP-A with immobilized anti PAPP-A at 3 concentrations of antigen

(△——△) 1 AU/L

(□——□) 2 "

(▲——▲) 20 "

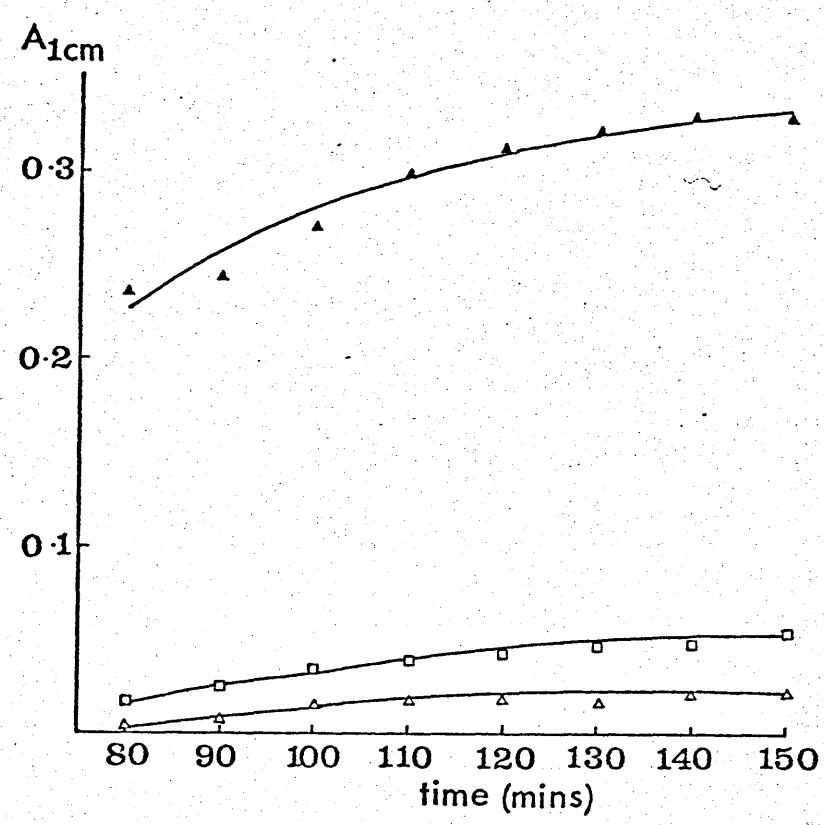

Fig 3 xix Time course of antibody-peroxidase with bound PAPP-A at 3 antigen concentrations

(△——△) 1 AU/L

(□——□) 2 "

(▲——▲) 20 "

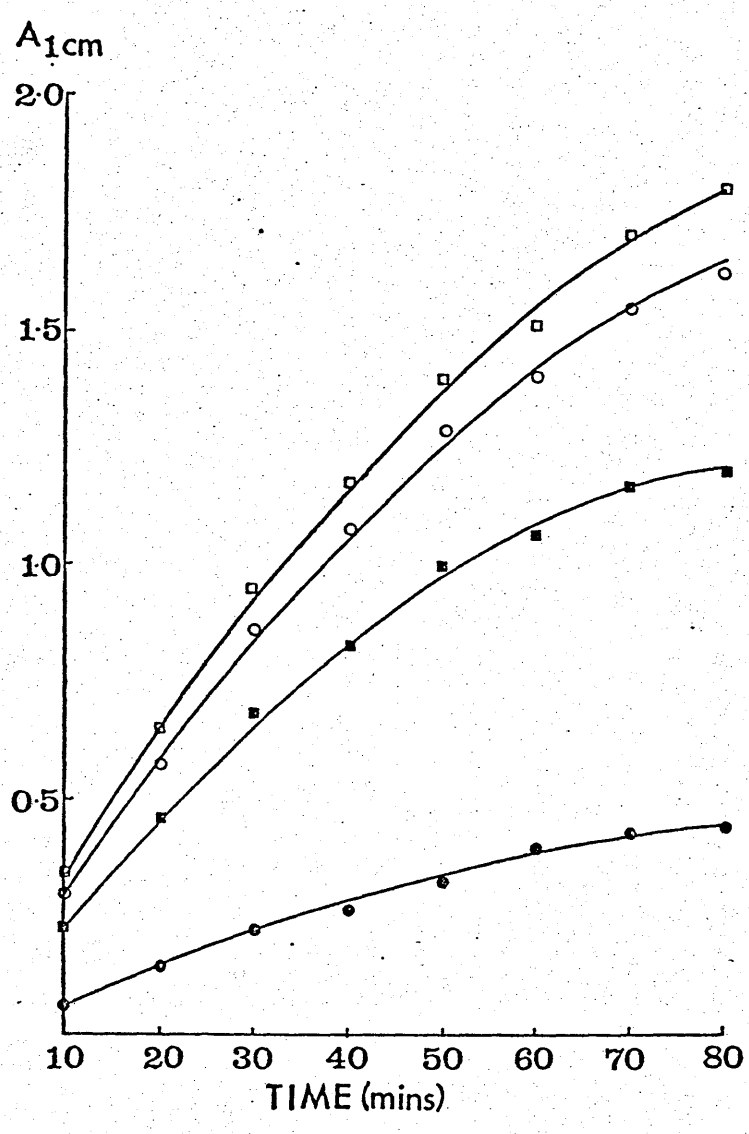

Fig 3 xx Time course of chromogen reaction for PAPP-A assay at 4 concentrations of antigen.

(●—●) 10 AU/L

(■—■) 100 "

(○—○) 500 "

(□—□) 1000 "

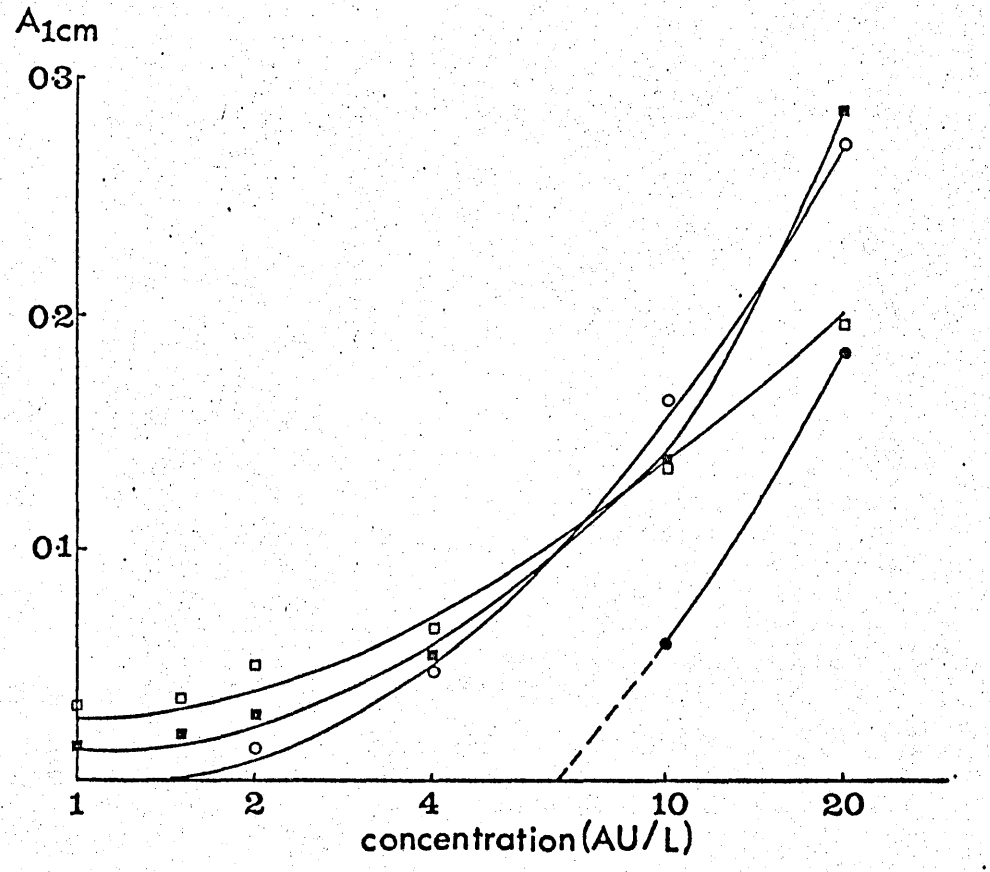

Fig 3 xxi The effect of varying coating antibody dilution in the assay of PAPP-A. Antiserum was diluted (●—●) 1/500, (○—○) 1/750, (■—■) 1/1250 or (□—□) 1/1500 in coating buffer.

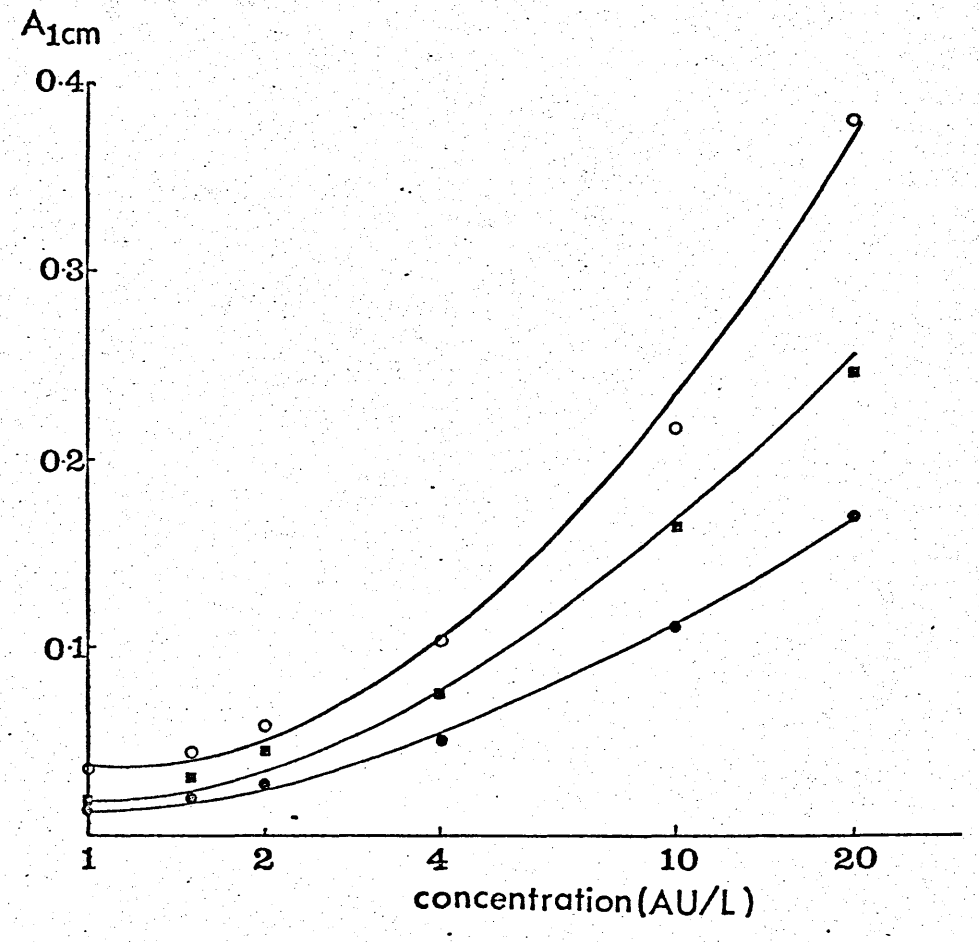

Fig 3 xxii The effect of varying antibody-enzyme concentration on the standard curve for PAPP-A assay. Antibody enzyme was diluted (o—o) 1/20, (▪—▪) 1/30, or (●—●) 1/50 in PBS containing 25% chicken serum and 10^{-2} mole/l citrate.

Figs 3 xviii - xx show the effect of increasing contact time of antigen with antibody, antibody enzyme with antigen and chromogen with antibody enzyme respectively. Over the time course considered, equilibrium was not achieved at any stage of the assay.

Adequate assay sensitivity was achieved by allowing contact of antigen with antibody for 120 minutes and antibody enzyme with antigen for 120 minutes. With these parameters set, a suitable colour development time was found to be 45 minutes, thus allowing the assay to be completed within the working day.

Fig 3 xxi shows the effect of varying antibody dilutions used to coat the microtitre plates. The best sensitivity was achieved using an antibody dilution of 1/1250.

The influence of antibody-enzyme dilutions upon final colour development is shown in Fig 3 xxii. A 1/20 dilution was found necessary to achieve adequate sensitivity within the time courses previously set.

Fig 3 xxiii shows the curves obtained for standards prepared from the laboratory serum standard pool and International Reference Preparation 78/610. Parallelism was exhibited from 1 - 20 AU/L this being the range routinely used for analysis. It is thus valid to assign values to the laboratory standard pool based on IRP 78/610. From these curves, a regression line was plotted - IRP 78/610 has an assigned value of 100 IU/L (Bohn et al 1980_b) and based on this value, the undiluted laboratory pool is equivalent to 90 IU/L (Fig 3 xxiv).

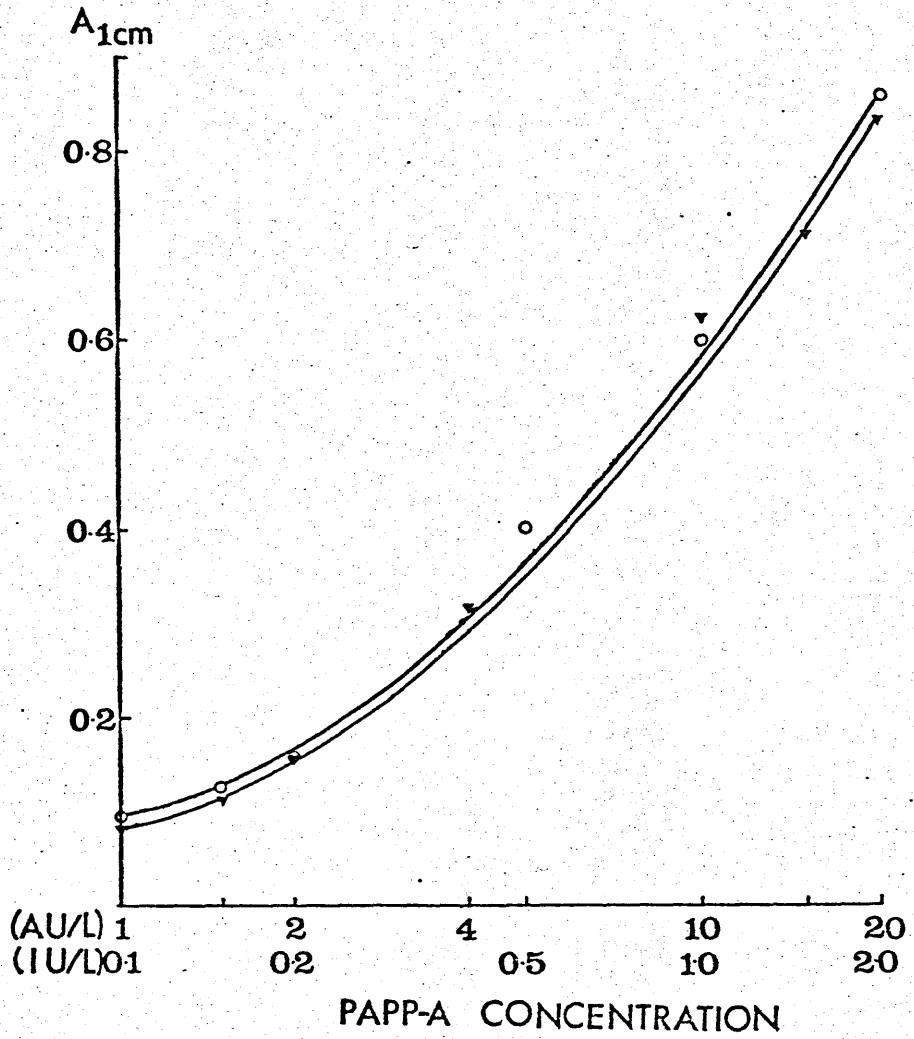

Fig 3 xxiii Standardisation of laboratory standard for PAPP-A (▼——▼) against International Reference Preparation 78/610 (○——○).

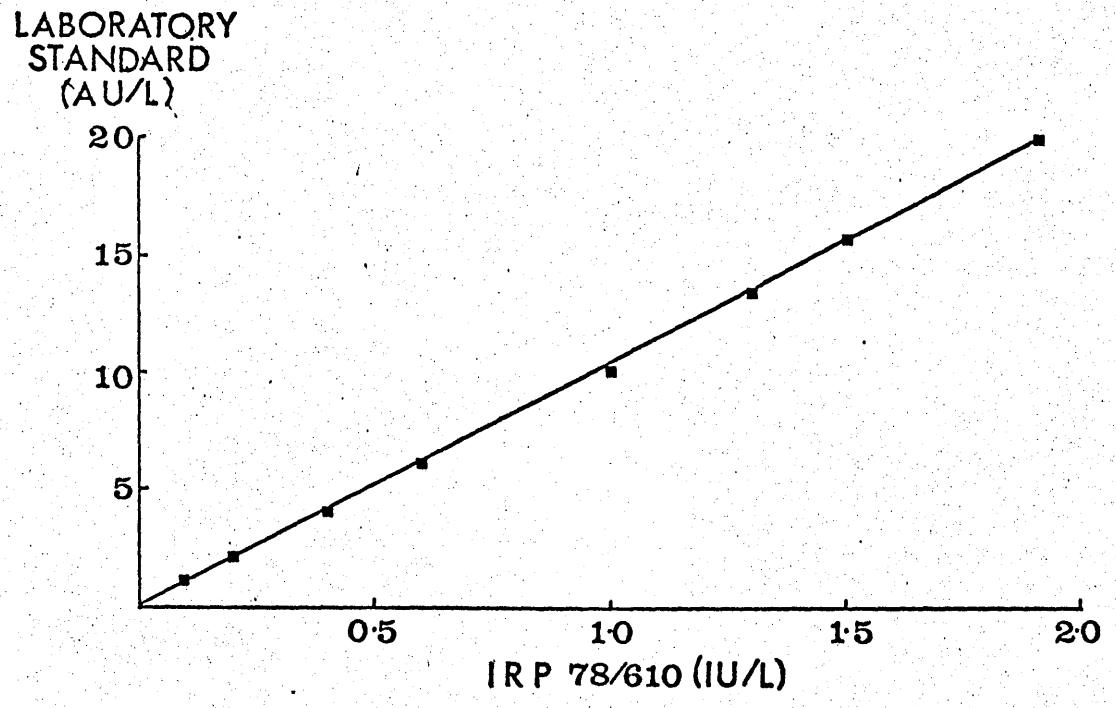

Fig 3 xxiv Regression line of laboratory PAPP-A standard against IRP 78/610.

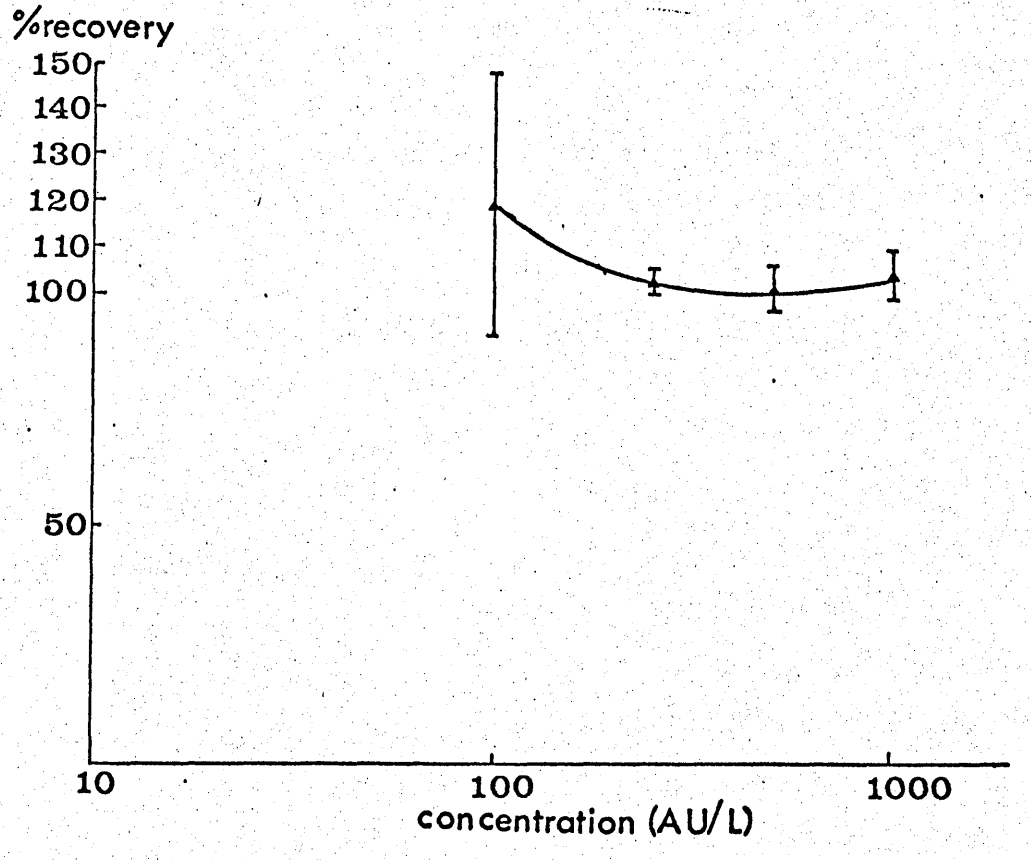

Fig 3 xxv Recovery profile of PAPP-A assay. The points represent mean ± 2 SE.

Table 3 X

Precision of PAPP-A Assay

A) Within batch

	I (IU/L)	II (IU/L)	III (IU/L)
Mean	13.2	16.7	35.8
SD	1.19	1.19	1.49
CV%	9	7.1	4.2
n	10	10	10

B) Between batch (overall)

	I (IU/L)	II (IU/L)	III (IU/L)
Mean	22.1	29.6	70.5
SD	2.30	2.62	7.62
CV%	10.2	8.9	10.8
n	38	45	44

Table 3 XI

PAPP-A concentrations (in terms of IRP 78/610)

	Fresh plasma	Incubated plasma	Haemolysed	Non-haemolysed
Mean concentration (IU/L)	42.3	48.5	49.6	33.0
Standard deviation	31.3	30.5	33.5	24.2
Number of samples	13	13	31	31
	Paired t = 2.03 $p > 0.05$		Paired t = 4.3 $p < 0.01$	

Table 3 XII

hPL concentrations (in terms of IRP 73/545)

	Fresh plasma	Incubated plasma	Haemolysed	Non-haemolysed
Mean concentration (mU/l)	7.6	7.8	4.21	4.15
Standard deviation	2.1	2.1	2.7	2.5
Number of samples	13	13	30	30
	Paired t = 1.71 $p > 0.1$		Paired t = 0.76 $p > 0.1$	

Table 3 XIII

SP_1 concentrations (in terms of Behringwerke SP_1 standard)

	Fresh plasma	Incubated plasma	Haemolysed	Non-haemolysed
Mean concentration (mg/l)	454	423	144	140
Standard deviation	303	262	116	109
Number of samples	13	13	28	28
	Paired t = 1.8 $p > 0.05$		Paired t = 1.1 $p > 0.1$	

Table 3 XIV

Absorbances obtained in ELISA for PAPP-A content of male plasma

	Haemolysed	Non-haemolysed
Mean absorbance	0.144	0.078
Standard deviation	38.6	8.9
Number of samples	12	12

paired $t = 3.6$; $p < 0.01$

No evidence of cross reaction of the antiserum with the three other pregnancy proteins tested was observed, all the protein solutions having ODs less than the zero PAPP-A standard.

From Fig 3 xxv it can be seen that recovery varied between 120% and 102%. Over the range 200 to 1000 AU/L of undiluted serum, recovery did not vary significantly from 100%.

The assay sensitivity was shown to be 7.6 IU/L in an undiluted specimen 0.076 IU/L per well after 1/100 dilution of test material.

Comparison of assay sensitivity is difficult until a universally acceptable reference preparation is available. Thus Sinosich reported an assay sensitivity of 2.9 µg/l (of pure protein) (1982) Bischof reported 32.5 ng/ml (1981a), or by Laurell rocket technique 10 u/ml (1979b). It is not possible at present to relate our sensitivity in terms of IRP 78/610 to the above findings.

In a further immuno-enzymatic assay (Bersinger 1983) an equivalent sensitivity was however achieved.

Table 3 X shows that the assay precision is satisfactory over the range of concentrations considered.

Results obtained in the haemolysis studies are shown in Tables 3 XI - 3 XIV. Only in the case of PAPP-A was a significant difference in concentration between haemolysed and non haemolysed specimens observed ($t = 4.3$ $p < 0.001$).

A slight increase in PAPP-A concentration in the incubated specimens was observed but this difference was not significant ($t = 2.03$ $p < 0.05$).

Table 3 XIV shows the effect of haemolysis on the male blood specimens. Since the concentrations of PAPP-A found in the

it was necessary to compare absorbances obtained in ELISA rather than concentrations. The absorbances with haemolysed male plasma were significantly greater than for unhaemolysed specimens ($t = 3.6$; $p < 0.01$).

Toop and Klopper (1983) and Sutcliffe (1982) have previously alluded to the conditions under which specimens for pregnancy associated proteins are collected. The apparent PAPP-A concentration in deliberately haemolysed specimens was significantly higher than in non haemolysed specimens.

Since the unhaemolysed male specimens showed no evidence of containing PAPP-A, the apparent increase in PAPP-A concentration is probably due to a red cell component binding the PAPP-A antibody non-specifically.

It appears for the proteins under consideration here, that this effect is specific to PAPP-A. It is unlikely that this finding is due to the method of assay (ELISA), since no significant difference could be shown for SP_1 concentrations in the same haemolysed and non-haemolysed pregnancy specimen pairs using a similar ELISA technique.

Similarly, no difference was observed for hPL using radioimmunoassay. The suggestion that anti-PAPP-A binds a component present in red blood cells would account both for the small increase in concentration found in plasma unseparated for 24 h and the considerable elevation in concentration upon haemolysis.

Of the normal sera from laboratory staff (male and non pregnant female), 28% of the male sera and 32% of female sera showed PAPP-A concentrations above the limit of detection. This is

perhaps not surprising with the discovery of PAPP-A production by the endometrium (Bischof et al 1982a) and the PAPP-A content of seminal plasma (Bischof et al 1983).

Previously published assays for this protein have been either radioimmunoassays , or Laurell "rocket" techniques : measurement of PAPP-A by ELISA allows results to be obtained within one working day which is of advantage in the clinical context envisaged.

The advantage of this assay may lie in possible improvement in sensitivity by prolonging incubation times. The assay may then find use in the investigation of PAPP-A production in neoplasia and in early pregnancy.

3.8 Carcino Placental Alkaline Phosphatase (CPAP)

3.8.1 Introduction

This enzyme was measured by an immunoenzymatic assay developed in this department (Mabon et al 1984).

Principle - CPAP is specifically extracted from serum using an immobilized antibody raised against placental alkaline phosphatase. The antibody bound CPAP is then detected using intrinsic hydrolytic activity to release the yellow chromophore p-nitrophenol from p-nitrophenyl phosphate.

3.8.2 Alkaline Phosphatase Preparations and Antisera

Placental alkaline phosphatase was obtained commercially (Miles Laboratories). This preparation was assayed at 37°C in accordance with the recommendations of the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry (1974) and used to standardize the assay. Bone, liver and intestinal preparations were made using a butanol extraction procedure (Pledger et al 1982).

Antiserum to CPAP - New Zealand rabbits were injected with 500 µg of placental alkaline phosphatase (Miles Laboratories) in Freund's Complete Adjuvant and boosted at monthly intervals with 20 µg. Animals were bled about 2 weeks after booster dose.

3.8.3 Method of Assay

Antiserum was diluted 1 in 1000 in carbonate-bicarbonate buffer and 200 µl dispensed into each of the wells of a microtitre plate (Nunc Certified Grade I). After refrigeration overnight at 4°C the antiserum was removed and the wells washed with TBS. Standards and test sera were diluted, where necessary, to lie within the range 0 to 8 u/l and incubated at room temperature (18-20°C) overnight in covered microtitre plates. The volume of standards and of serum used in each was 200 µl and estimations were performed in triplicate. Following incubation the sera were removed, the wells washed and 200 µl of chromogen dispensed into each well.

One hour was allowed for colour development at room temperature after which the absorbance of the chromophore at 405 nm was measured using a Titertek Multiskan plate reader interfaced to a Commodore PET 4016 micro-computer.

3.8.4 Investigation of factors affecting the assay

Dilution of antiserum - A range of dilutions ranging from 1 in 10 to 1 in 100 000 was used to coat the wells of the microtitre plates. Pooled sera from pregnant women was used as a convenient source of antigen and the same volume was added to each well and assayed according to the procedure described above.

Specificity of antiserum - Bone, liver and intestinal alkaline phosphatases were diluted in TBS containing albumin and assayed.

Time of reaction - Pooled pregnancy sera diluted to three different activities was incubated with immobilised antibody for times ranging from two to 24 h. In each case colour development was allowed for 60 min.

Time of colour reaction - Standard sera were processed as usual but extra measurements of the optical density of the chromophore were made within and beyond the normal time allowed for colour development.

Concentration of albumin in the diluent - The concentration of albumin necessary to prevent non-specific adsorption onto the wells of the microtitre plate was determined by studying calibration curves from assays using TBS containing 10, 50, 70 and 100 g/l human albumin respectively.

Detection limit - Forty-two replicate blank determinations were carried out and the precision of the blank determined.

Precision - Replicate measurements were made at two CPAP levels of activity and both within-batch and overall precision calculated.

3.8.5 Comparison with a chemical inhibition method

Because the primary concern was to develop a sensitive assay to be used in the investigation of cancer, direct comparisons with the less sensitive chemical inhibition method using samples from patients with cancer was not possible. Instead, 30 sera from pregnant women were assayed for placental alkaline phosphatase , after suitable dilution, by the immunoenzymatic assay described above.

Normal Range - Sera from 27 male and 29 female apparently healthy laboratory workers were obtained and assayed for CPAP.

Table 3 XV

Cross reactivity of three alkaline phosphatases with anti-CPAP

Alkaline phosphatase	Activity (u/l)	Measured activity in immunoenzymatic assay system (u/l)
Bone	550	1.6
Liver	630	0.6
Gut	550	3.3

3.8.6 Results and discussion

Dilution of antiserum - Maximum colour development occurred at a dilution of 1 in 1000 and this dilution was therefore used routinely, being similar to that used in other similar systems (Macdonald et al 1979, Pledger et al 1982).

Specificity of antiserum - The commercial preparation of placental alkaline phosphatase was not further purified before it was used to immunize the rabbits as antibodies raised to contaminants would not bind alkaline phosphatase, which is eventually detected by its intrinsic enzyme activity; thus only CPAP should be detected unless all isoenzymes have a common structure recognized by the antiserum. In their method for CPAP, Haije et al first heated their specimens to 65°C for 10 min to destroy any intestinal alkaline phosphatase which cross-reacted with their antiserum. We chose not to pre-heat samples because the extent of the cross-reactivity which we found (Table 3 XV) was insufficient to cause interference within the range of alkaline phosphatase activation encountered in normal subjects or at significantly elevated activities.

Concentration of albumin in the diluent - Increasing the albumin added to TBS from 10 to 50 g/l reduced non-specific adsorption slightly but between 50 and 100 g/l no additional reduction occurred. Routinely 75 g/l was used.

Detection limit - The detection limit was calculated as twice the standard deviation of the blank value absorbance, which was referred to the standard curve and a detection limit of 0.12 u/l found. This is quite adequate representing about 5% of the normal range (as defined below).

Table 3 XVI

		Number	Mean	Standard deviation	Coefficient of variation (%)
Within-batch precision	Sample 1	11	1.02	0.07	6.6
	Sample 2	10	3.06	0.17	5.6
Overall precision	Sample 1	21	1.05	0.16	15.5
during one month	Sample 2	22	3.39	0.50	14.8

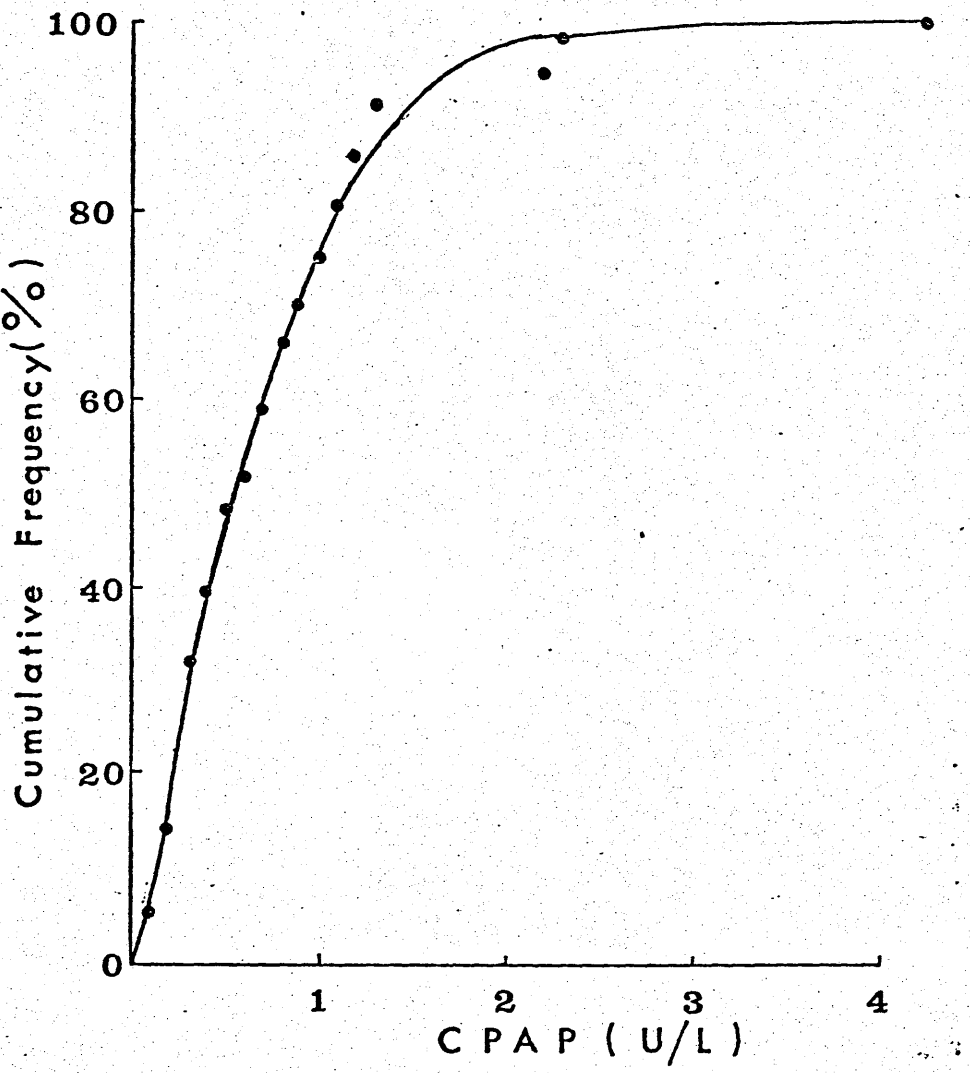

Fig 3 xxvi Cumulative Frequency Curve of CPAP concentrations in a normal population.

coefficient of variation of 10% obtained by Haije et al (1979), at the upper limit of their normal range. (NB These workers also used triplicate measurements).

Comparison with a chemical inhibition method - Mean activity of the 30 samples was 102 u/l with a standard deviation of 66 u/l, when measured by a chemical inhibition method. After allowance for dilution the mean activity by immunoenzymatic assay was 99 u/l with a standard deviation of 60 u/l. The paired t test revealed no significant difference between the results obtained by the two methods.

Normal range - A positively skewed distribition was obtained for male and female subjects. As there was no apparent sex difference the results were pooled, a cummulative frequency curve constructed and a median value of 0.5 u/l and a 97.5th centile value of 2.0 u/l obtained (Fig 3 xxvi). Haije et al (1979) also failed to detect any significant sex difference but the upper limit of their normal range was lower than that which we obtained. Comparisons with results obtained by other workers (Kellen et al 1976, Millan and Stigbrand 1981) are difficult because of extensive methodological differences.

The assay provides a sensitive specific method for the estimation of carcino-placental alkaline phosphatase in patients with neoplastic disease.

CHAPTER 4

THE ASSESSMENT OF CRITICAL FACTORS WHICH INFLUENCE THE PERFORMANCE OF ELISA

During the last two decades many of the physico-chemical factors which influence radio-immunoassay have been thoroughly investigated and the design of new radioimmunoassays is based on established scientific principles. Radioimmunoassay suffers several disadvantages inherent in the use of radioisotopes - short shelf life, health hazard, etc. These disadvantages do not apply to enzyme labels and for this reason the new methods employed in this thesis were based on enzyme immunoassay.

Several versions of ELISA exist, but the one used here, which is best suited to the assay of proteins and peptides is a two site assay and is not strictly comparable with RIA. Several factors were found which influenced the performance of ELISA in a manner not encountered in RIA.

4.2 Characterization of Microtitre Plates used in ELISA

4.2.1 Investigation of Optical Uniformity of Microtitre Plates

Three types of microtitre plate were investigated:-

a) Cooke Flexible Microtitre Plates (M29A) obtained from Dynatech Laboratories, Billingshurst, Sussex.

b) Immulon Micro Elisa Plates (M129A) obtained from Dynatech Laboratories, Billingshurst, Sussex.

c) Nunc Micro Elisa Plates (Grade I certified) obtained from Gibco Europe, Paisley, Scotland.

Absorbance values for the empty wells were read using a Multiskan Plate Reader (Flow Laboratories, Irvine, Scotland) and normal distribution statistics calculated.

	1	2	3	4	5	6	7	8	9	10	11	12
A	001	007	005	004	006	003	009	009	010	011	010	013
B	003	005	001	004	007	005	010	004	010	011	016	017
C	001	006	009	002	007	006	007	000	008	005	010	008
D	003	007	002	005	001	008	005	009	011	011	011	014
E	003	000	007	007	005	001	002	008	012	011	010	017
F	002	014	006	010	012	013	012	011	016	012	D11	017
G	002	009	006	001	005	008	008	008	009	013	010	012
H	002	001	004	013	010	003	010	010	010	005	008	009

Fig 4 i Absorbance values for empty wells of M29A Flexible Microtitre Plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	005	005	006	005	006	008	005	007	006	007	009	006
B	004	004	004	005	005	004	007	006	005	006	007	006
C	004	003	004	006	005	002	006	004	006	004	007	005
D	005	007	007	007	008	007	007	008	008	008	008	011
E	004	005	005	005	005	006	006	006	005	006	006	007
F	004	004	004	004	006	007	005	006	006	005	006	008
G	004	007	006	006	005	005	006	005	004	005	005	005
H	004	002	003	002	003	003	003	002	004	004	003	003

Fig 4 ii Absorbance values for empty wells of M129A Immulon Microtitre Plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	001	003	002	003	004	004	002	003	004	005	007	003
B	001	000	001	002	000	001	002	009	003	004	006	002
C	001	000	000	000	002	002	008	007	002	003	000	000
D	000	000	001	000	001	001	000	000	001	001	001	001
E	002	004	000	000	007	002	003	001	002	000	001	002
F	001	005	001	004	005	004	001	003	005	002	004	004
G	001	001	001	002	003	002	002	002	002	004	003	004
H	001	002	001	001	003	001	001	001	001	003	002	012

Fig 4 iii Absorbance values for empty wells of Nunc Immuno Plate Grade I.

Table 4 I

The Optical Uniformity of 3 Types of Microtitre Plates for use in ELISA

	Nunc Immuno I	Cooke Flexible M29A	Dynatech Microelisa M129A
Mean OD	.0053	.0076	.0023
Std Deviation	.0015	.0043	.0022
n	96	96	96
99% Confidence Limits (3SD)	± .0045	± .0129	± .0066

Table 4 II

Ad Readings for Standard Curves and Backgrounds for Three Different Types of Microtitre Plate.
(Readings corrected for O standard)

SP_1 Concentration (µg/l)	Nunc Certificated I	Cooke Flexible M29A	Immulon Rigid M129A
31.25	0.067	0.009	0.06
62.5	0.269	0.151	0.039
125	0.466	0.277	0.060
250	0.773	0.567	0.256
500	1.043	0.911	0.445
1000	1.252	0.918	0.507
Replicate blanks	0.118	0.150	0.159
	.102	.168	.135
	.113	.151	.138
	.103	.134	.150
	.088	.156	.139
	.098	.152	.140
	.101	.156	.143
	.103	.157	.138
	.113	.152	.154
	.112	.149	.151
	.119	.158	.151
	.097	.150	.143
	.099	.201	.122
	.104	.142	.142
	.101	.134	.129
	.104	.163	.141
	.115	.158	.135
	.109	.160	.134
	.105	.153	.141
	.093	.160	.142
	-	.174	.129
	-	.179	.193
	-	.172	-
Mean Background	0.105	0.158	0.143
n	20	23	22
Std Deviation	0.008	0.015	0.014
CV %	7.9	9.5	9.8

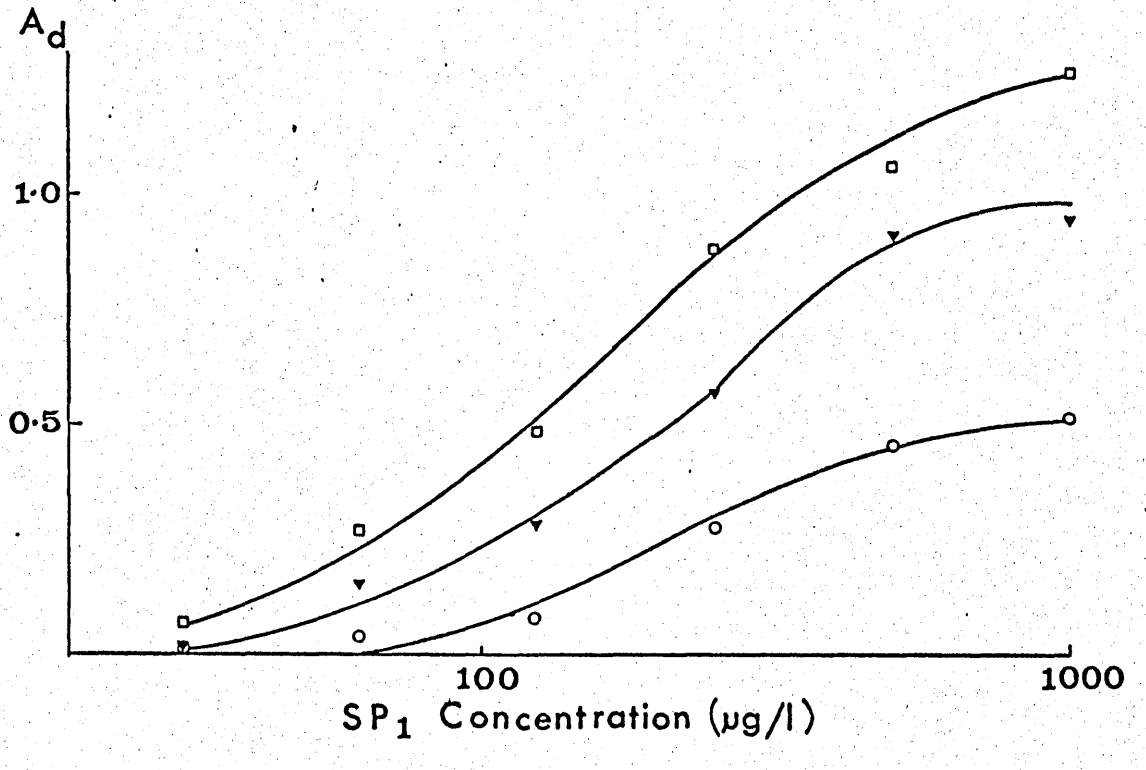

Fig 4 iv Standard curves obtained for ELISA of SP_1 on three types of microtitre plate:-

(□——□) Nunc Certified I

(▼——▼) M29A Flexible

(○——○) M129A Immulon Rigid

Table 4 III

SP_1 Assay Precision on Various Microtitre Plates

a) Low Pool

	Nunc Certified I	M129A Immulon Rigid	M29A PVC Flexible
Mean Concentration	113.2	130.5	109.3
Standard Deviation	9.00	13.5	21.8
n	11	12	12
CV %	8.0	10.4	20.0

b) High Pool

	Nunc Certified I	M129A Immulon Rigid	M29A PVC Flexible
Mean Concentration	210.7	207.7	194.5
Standard Deviation	21.8	18.2	45
n	14	14	13
CV %	10.4	8.8	23.0

4.2.2 Investigation of Assay Sensitivity and Non Specific Binding using Various Microtitre Plates

Microtitre plates (as detailed in 4.2.1) were coated overnight at 4°C with antibody diluted 1/1200 in coating buffer (Appendix I). Before use the plates were emptied and washed three times with wash buffer. 100 µl of standard SP_1 solutions and a series of zero standards were pipetted into the wells and incubated for 30 minutes at the end of which, the well contents were aspirated and a further washing cycle performed. Antibody enzyme conjugate (100 µl) diluted 1/1000 in PBS containing 25% chicken serum and 0.1% citrate was then added to each well and incubated for 30 minutes followed by a further aspiration/washing cycle. 100 µl of chromogen was then added to each well and after colour development for 30 minutes, the reaction was terminated by the addition of 100 µl sulphuric acid. The developed colours were read on an Abbott ABA 100 bichromatic analyzer and the Absorbance Difference (Ad) obtained.

Assay precision was assessed by assaying two pools of pregnancy serum in replicate on each type of plate.

4.2.3 Results and Discussion

Figs 4 i - iii show the optical densities of empty wells for the three types of plate considered. The Nunc plates showed least variation and the 99% confidence limits were within the range quoted by the manufacturer (Table 4 I). Table 4 II and Figure 4 iv show the standard curves and background readings obtained for each type of plate. As the variances between the backgrounds for each plate were not equal, comparison was made by means of the Wilcoxon U statistic. Nunc plates gave significantly lower backgrounds than either Immulon plates or Flexible plates ($p < 0.01$). Furthermore the standard curve obtained with the

Precision of the assays is shown in Table 4 III . Nunc plates and Immulon Plates gave comparable precision whilst precision using the Flexible plates was decreased.

These initial studies suggest that either Nunc or Dynatech Immulon Microtitre Plates would provide a suitable basis for ELISA.

4.3 Antibody Binding Characteristics of Plastic Microtitre Plates, Polystyrene Beads and Nylon Beads

4.3.1 Method

Anti-TSH was coupled to cellulose, the unbound antibodies eluted and the antibody complex iodinated using a Chloramine T method. After iodination, the complex was washed extensively with 10^{-3} mol/l HCl, pH 3.0. Iodinated anti TSH was eluted from the column using 10^{-2} mol/l HCl pH 2.0, followed by 0.1 mol/l sodium bicarbonate pH 10. The pH 2 and pH 10 fractions were pooled and further purified on Sepharose 6B, collecting the iodinated antiserum peak.

Iodinated anti-TSH was diluted 1/10 with coating buffer. Individual wells cut from the three types of microtitre plates listed in 4.2.1 were coated using 100 µl of diluted antibody overnight at $+4^{o}C$.

Polystyrene (6.4, 4.8, 3.2 mm diameter) and nylon beads (6.4, 3.2 mm diameter) were coated by immersion in the diluted antibody overnight at $+4^{o}C$. The following day the antibody solution was aspirated from the wells and beads and both were washed 3 times with wash buffer, the wells dried by shaking and the beads by blotting on absorbent paper.

Individual wells and beads were then counted on a γ counter for 75 seconds.

Table 4 IV

The Binding of Iodinated Anti-TSH to Polystyrene Beads

Bead Diameter	3.2 mm	4.8 mm	6.4 mm
Number of Beads	12	11	12
Mean % binding	0.88	1.36	2.36
Std Deviation	0.07	0.24	0.33
Coefft. of Variation	8.28	17.75	13.94
			Following additional 48 hr incubation Mean % binding = 2.6

Table 4 V

The Binding of Iodinated Anti-TSH to Nylon Beads

Bead Diameter	3.2 mm	6.4 mm
Mean % Binding	0.43	1.01
SD	0.09	0.28
CV	21%	27%
n	12	12

Table 4 VI

The Binding of Iodinated Anti TSH to Microtitre Plates

	Nunc Immuno I	Dynatech Microelisa	Cooke Flexible
Number of Wells	12	12	12
Mean % binding	17.2	19.4	23.2
Variance	2.7	15.2	0.3
Standard Deviation	1.6	3.9	0.5
Standard Error	0.5	1.1	0.2
Coefft of Variation	9.6	20.1	2.3

antibody once more and incubated for a further 48 hours, followed by washing and counting as before.

The total counts for the microtitre plates experiment were assessed by counting duplicate 100 µl aliquots of the diluted antiserum and taking the mean value.

For the beads, total counts were assessed by counting duplicate 100 µl aliquots of the diluted antiserum and multiplying the counts by a factor to allow for the total volume in which the beads were suspended.

4.3.2 Results

Tables 4 IV - 4 VI show the mean % labelled antibody bound to the various solid phases. Comparison of the differences were made by the t test. The mean binding on Dynatech Micro Elisa plates was significantly lower than binding to the Cooke Flexible Plates ($t = 3.5$ $p < 0.01$). Binding to Nunc microtitre plates was not significantly lower than binding to Dynatech plates ($t = 2.1$ $p > 0.05$). There was a significantly lower binding to Nunc plates compared with the Cooke Flexible plates ($t = 11.4$ $p < 0.01$).

4.3.3 Discussion

For all 3 microtitre plates investigated, the capacity to bind antibody is far in excess of the capacity of the polystyrene or nylon beads.

The low binding observed for nylon beads is perhaps not surprising in view of the inert nature of nylon. Nonetheless, nylon rings have been used in ELISA systems without prior chemical activation (Lehtonen and Viljanen 1980). Hendry however (1980) found it necessary to carry out chemical activation of nylon

higher amount of protein could be bound using this technique and the binding was stable, a further stage of reagent preparation is encountered.

The improved binding capacity of polystyrene microtitre plates compared with polystyrene beads was unexpected. The surface areas of a polished 4.8 mm bead (72 mm^2) and a microtitre plate well (60 mm^2) are comparable. However the beads used were matt finished and thus the practical surface area is far in excess of the calculated area.

The answer may lie in different structural forms of polystyrene used in manufacture. It has been pointed out (Ratcliffe 1974) that there is considerable variation in suitability of polystyrene tubes from different sources and indeed between different batches of tubes from the same source.

A further possibility is that the leakage of bound antibody from the plates and beads during washing varies. Proteins are bound to plastic polymer surfaces by weak physical forces (van Oss and Singer 1966) and the stability of this binding is uncertain. Thus the amount of antibody apparently bound might also vary.

It was noted in 4.2.2 that there was a gradation of NSB (background) values according to the type of plate used. It is interesting that those plates exhibiting the highest NSB (Cooke PVC plates) are those exhibiting the maximal binding capacity. Conversely plates with lower binding capacity (Nune Immuno I) had the lowest NSB and also showed the steepest standard curve.

Extending this concept further, the most sensitive assays might be expected with polystyrene beads. However other problems

further in 4.4.2.

4.4 ELISA using Polystyrene Beads as a Solid Phase

4.4.1 Materials and Method

Polystyrene beads as detailed in 4.3.1. Other ELISA reagents as detailed in Appendix I.

Method - Antiserum to SP_1 was diluted 1/1200 in coating buffer and the beads (3.2, 4.8, 6.4 mm diameter) coated overnight at $+4^{\circ}C$ by immersion in the diluted antiserum.

Before use, the beads were removed from the coating mixture, blotted on absorbent paper and washed three times in wash buffer with gentle stirring. The beads were then dried and placed into individual test tubes.

300 µl of standard SP_1 solutions were added in triplicate at timed intervals, the tubes covered and incubated for 30 minutes with gentle agitation at room temperature. After incubation, the standard solutions were aspirated, and each bead washed 3 times in its tube with wash buffer, using a vortex mixer to ensure adequate washing.

After drying the bead, 300 µl of antibody enzyme diluted 1/1000 in PBS/25% chicken serum were added to each tube and incubated for 30 minutes with gentle agitation at room temperature.

The tube contents were then aspirated and the beads washed as before. 300 µl of chromogen were then added to each tube and colour allowed to develop for 30 minutes after which the reaction was stopped by addition of 300 µl of 4 mol/l sulphuric acid.

A parallel assay was performed using Nunc Immuno Plates Grade I.

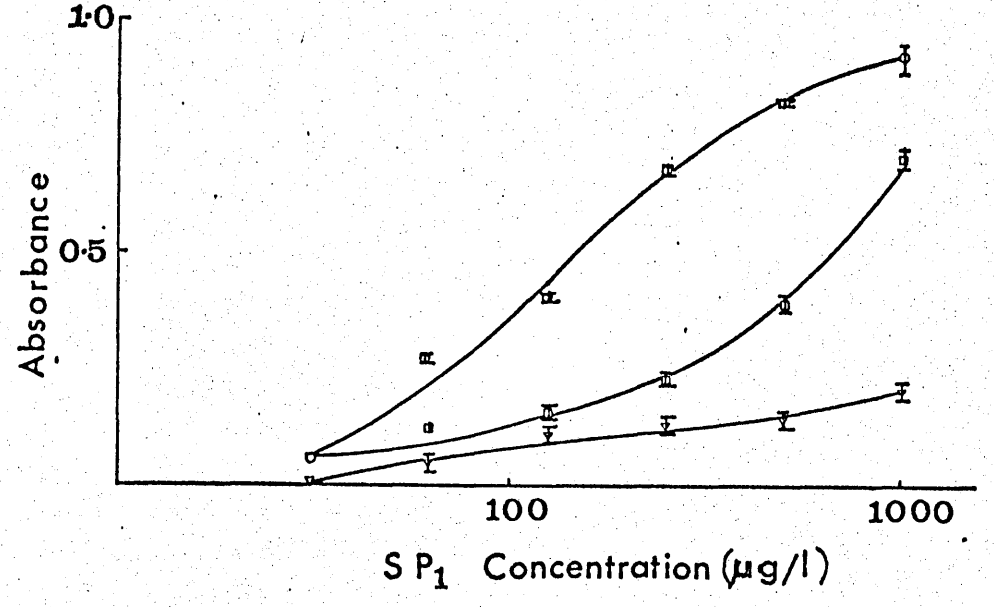

Fig 4 v Standard curves obtained for ELISA of SP_1 using three types of solid phase:-

(o——o) Nunc I Microtitre Plates

(□——□) 6.4 mm polystyrene bead

(▽——▽) 4.8 mm " "

The colours were read on an Abbott ABA 100 bichromatic analyzer and results converted to absorbance for a 1 cm path length.

After correction for blanks, a plot of mean absorbance value against SP_1 concentration was made.

4.4.2 Results and Discussion

The standard curves obtained are shown in Fig 4 v. All assays yielded increasing colour with increasing antigen concentration. The assay carried out on the microtitre plate yielded a sigmoid curve whilst those assays utilizing the beads gave parabolic curves. The curves obtained in ELISA assays are discussed further in Chapter 5.

Although it would be possible to use polystyrene beads as a solid phase, it appears that the microtitre plates provide a more sensitive assay as evidenced by the steeper standard curve. This might be expected on theoretical grounds as discussed in 4.3.3.

Furthermore considerable difficulty was experienced in handling the beads. This, together with the less precise coating of antibody on the beads (4.3.2) may account for the greater standard errors observed for this solid phase. ELISA based on beads would require specifically designed handling techniques.

By contrast, microtitre plates provide a convenient format for multiple tests. The plates are easily handled and plate readers are available which obviate the problem of reading individual tests.

Two commercial systems utilizing beads are now available - the Quantum System (Abbott Diagnostics, Queensborough, Kent) and a combined assay module/spectrophotometer (Kone Instruments, Stockport, Cheshire). It seems likely however that "in house" assays will continue to be based on the microtitre plate format.

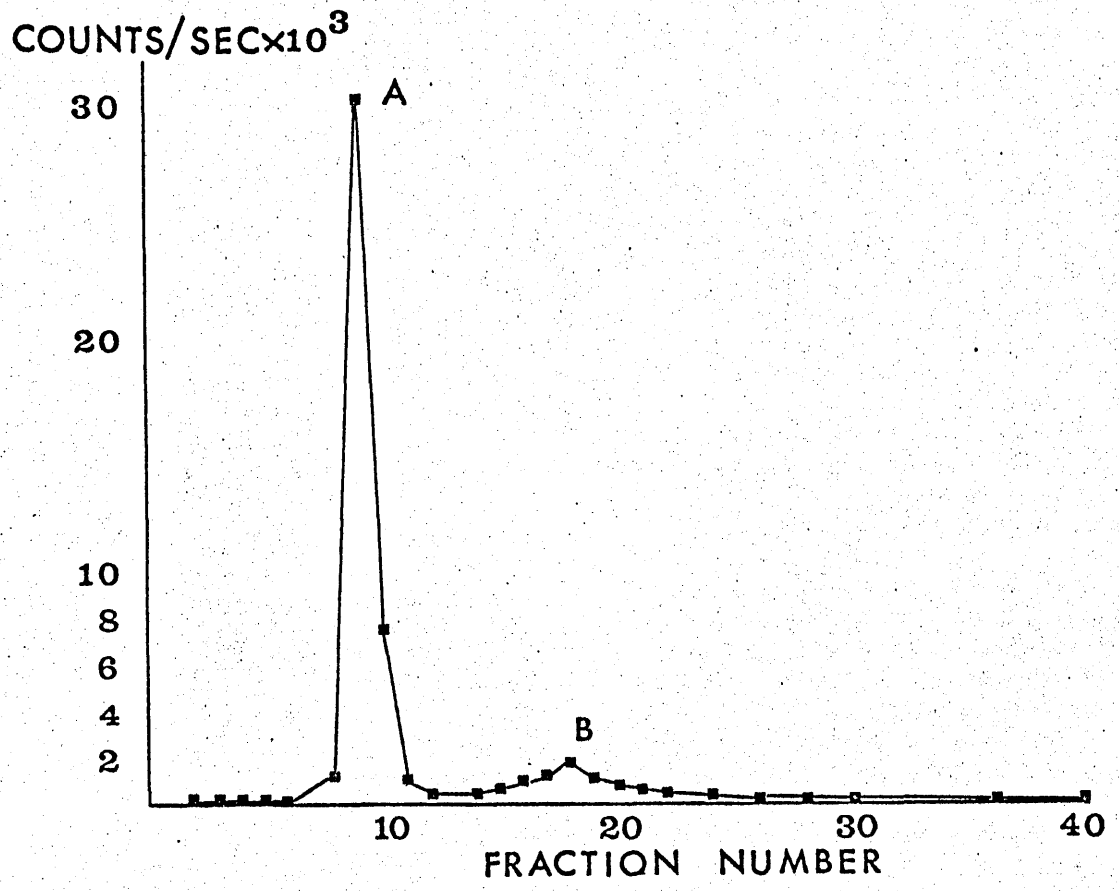

Fig 4 vi 'Typical column profile for separation of iodinated anti-SP_1 (peak A) from unreacted iodine (peak B) on Sephadex G25. Fraction volume was ≃ 0.5 ml and the eluting buffer was PBS.

Table 4 VII

Antibody Binding to Nunc Immuno Plates I for a Variety of Antisera

	Anti SP_1	Anti hCG	Anti PAPP-A	Anti TSH
Mean % Binding	55.2	56.8	56.8	17.2
SD	2.72	2.66	3.04	1.77
CV %	4.9	4.7	5.4	10.3

of Antibodies to Microtitre Plates

4.5.1 Method

Anti SP_1, anti PAPP-A and anti-hCG were desalted on G25 Sephadex column and iodinated by a Chloramine T technique. Separation of the iodinated immunoglobulins from free iodine was achieved on a Sephadex G25 column (Fig 4 vi). 10 drop fractions were collected and counted in a γ counter to identify the iodinated protein peak.

Antiserum to TSH was bound to cellulose and iodinated as previously described.

After iodination, a 1/10 dilution of each iodinated antiserum was made in carbonate/bicarbonate buffer pH 9.6. Twelve wells cut from the microtitre plate (Nunc Grade I) were used for each antibody investigated. 100 µl of the diluted antisera were pipetted into each well and incubated overnight at $4^{o}C$. The following day the well contents were aspirated, the wells washed 3 times with wash buffer and dried. The iodinated antibody bound to the wells was counted in a γ counter and compared with the counts given by 100 µl of the diluted antisera used to coat the plate.

4.5.2 Results and Discussion

The % bindings achieved for each antiserum are shown in Table 4 VII. There was no significant difference in binding between anti-SP_1, anti-hCG or anti-PAPP-A ($p > 0.05$). Similarly there was no difference in binding between anti hCG and anti PAPP-A. The ability of various proteins to bind to polystyrene solid phases remains open to question. Lee et al (1974) showed that adsorption was dependent on the protein and polymer used.

suggested that the binding rate was not protein dependent and that all proteins bound equally well. More recently Cantarero et al (1980) have adduced further evidence in favour of Lee's findings.

The data presented here may be interpreted as evidence that binding is dependent on the particular antibody only when there is a difference in purity. The commercial antisera were iodinated without prior purification whereas the anti-TSH was initially bound to cellulose before iodination.

Protein assay on the diluted antisera used to coat the wells showed that all wells received the same order of magnitude of protein (250-500 µg). Cantarero discussed a "region of independence" where the proportion of protein bound was independent of the amount added (1980). It is not possible to challenge these findings on the basis of the data obtained here.

Further, it is shown that the optimal antibody dilution for the ELISA systems seems dependent on the solid phase rather than the antibody. This is further evidence in favour of non-specific antibody binding ie all antibodies bind equally well when they are of equivalent immunopurity.

4.6 Investigation of Loss of Antibody from Wells of Microtitre Plates during Enzyme Immunosorbent Assay

4.6.1 Method of Assessing Optimal Dilution for Iodinated Antibody

In order to optimize the assay system to approach as nearly as possible the antibody dilution used in routine assay, a series of ELISA standard curves were run with varying iodinated antibody dilutions.

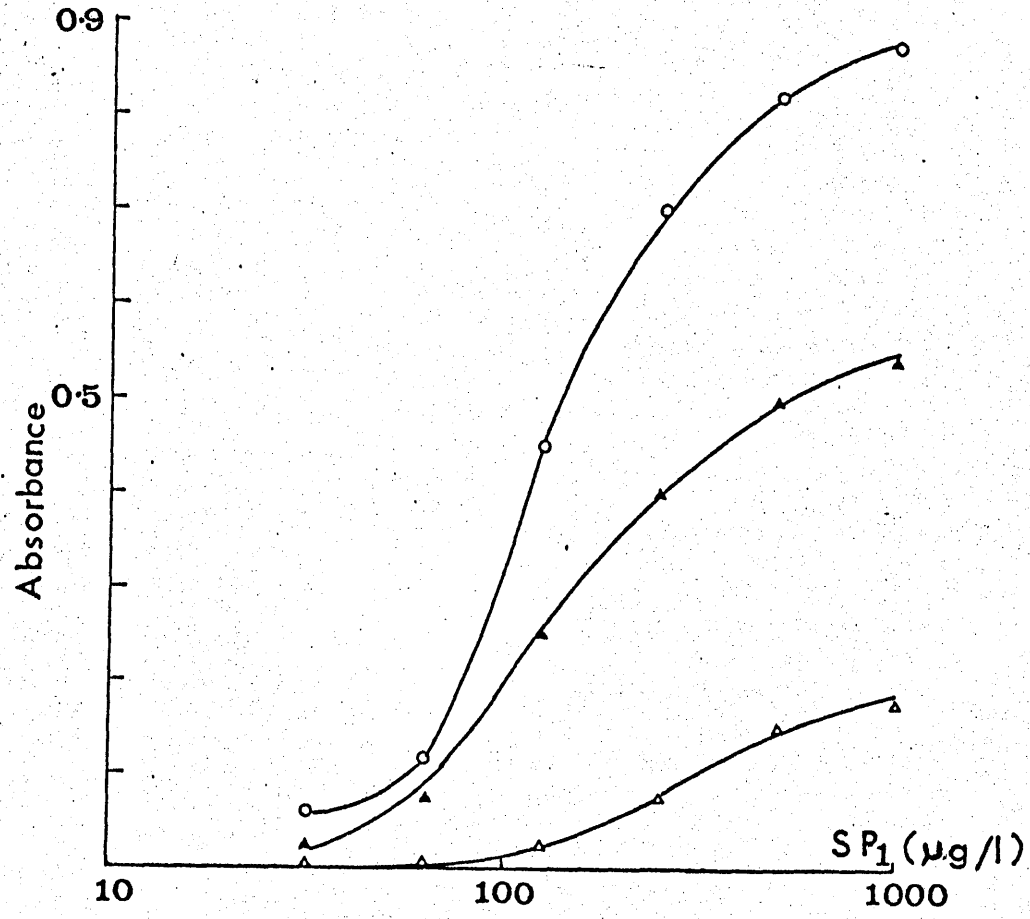

Fig 4 vii The Optimal Dilution of iodinated anti-SP_1 for use in Leaching Experiments. Iodinated antiserum was diluted (O——O) 1/2, (▲——▲) 1/3 or (△——△) 1/5 in PBS/25% chicken serum containing 10^{-2} mol/l citrate.

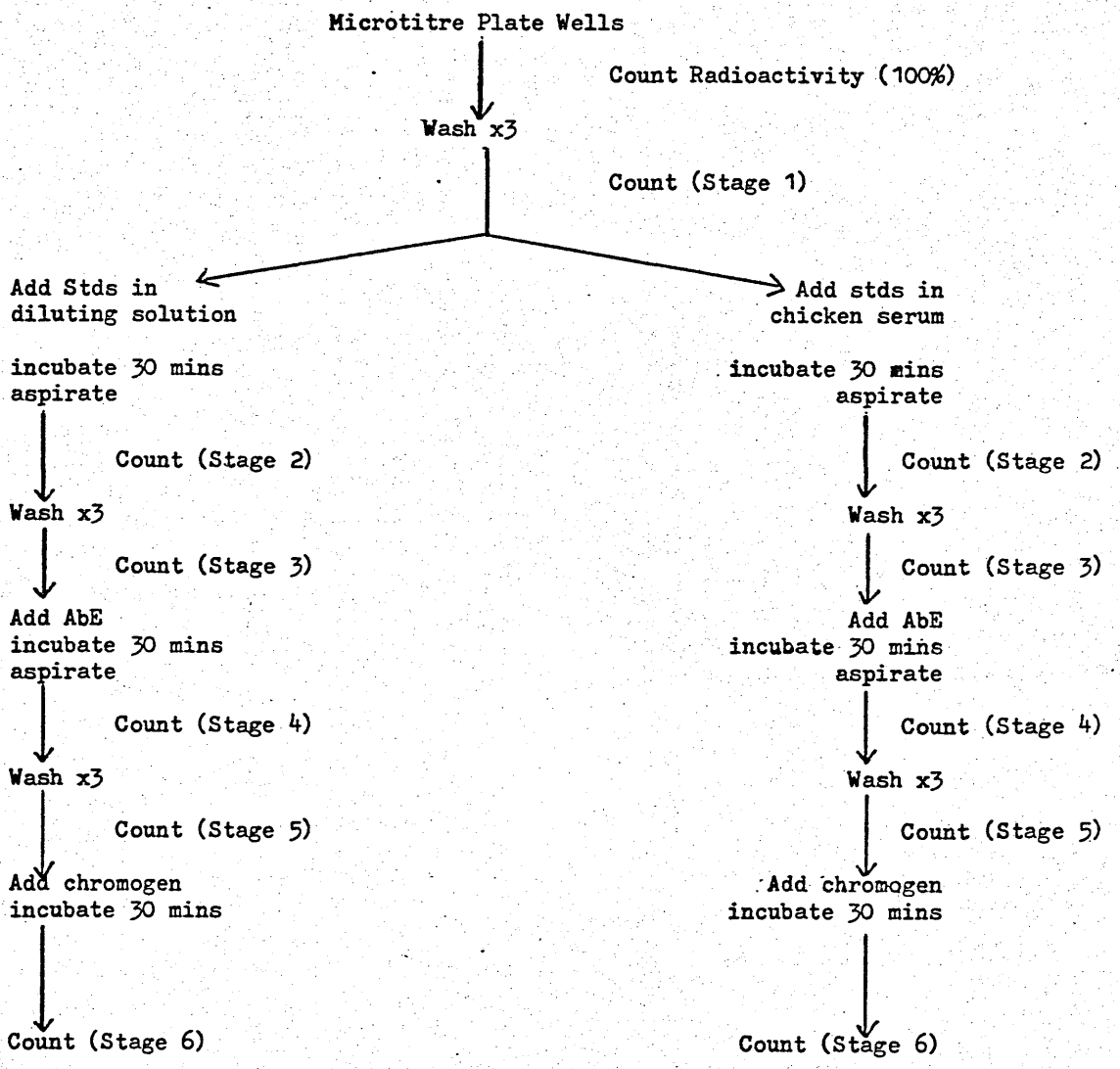

Fig 4 viii Flow Chart for Loss of Antibody Experiment.

Table 4 VIII

% Antibody loss from the wells of microtitre plates during ELISA

a) Antigen diluted in PBS citrate

Antigen Conc^{n} µg/l	Assay Stage 1	2	3	4	5	6	Overall % Loss
0	5.9	1.2	1.3	1.6	2.9	1.1	14.0
31.25	6.4	0.7	0.6	2.5	2.1	1.3	14.6
62.5	5.4	2.0	1.0	1.9	2.9	1.2	14.4
125	4.8	1.8	0.8	2.9	0.7	2.6	13.6
250	5.4	2.0	1.0	1.9	2.9	1.0	14.2
500	5.4	1.4	0.7	2.5	2.3	2.5	14.8
1000	6.2	1.6	1.3	2.9	2.0	0.9	14.9
Mean loss (%)	5.6	1.5	1.0	2.3	2.3	1.5	14.4

Table 4 IX

% Antibody loss from the wells of microtitre plates during ELISA

b) Antigen diluted in raw chicken serum

Antigen Conc µg/l	Assay Stage 1	2	3	4	5	6	Overall % Loss
0	5.7	3.8	4.4	1.4	4.1	0.9	20.3
31.25	5.8	4.4	5.4	1.4	2.7	1.4	21.1
62.5	6.2	3.5	2.9	3.5	1.6	2.7	20.4
125	7.0	4.4	2.3	2.2	2.9	0.7	19.5
250	6.2	4.9	2.4	1.5	1.8	2.9	19.7
500	4.9	4.1	2.5	2.8	2.8	1.1	18.3
1000	5.2	4.4	3.5	1.5	2.8	1.1	18.2
Mean loss (%)	5.7	4.2	3.3	2.0	2.7	1.5	19.6

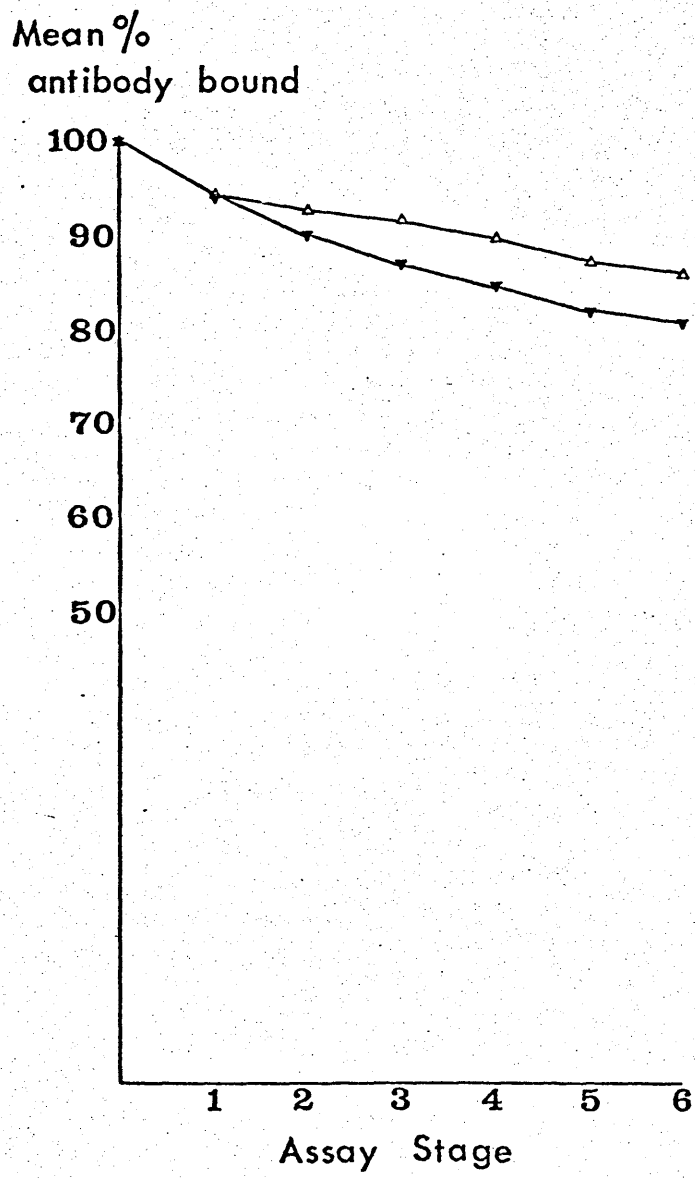

Fig 4 ix Loss of bound iodinated antibody at various stages of ELISA. Antigen was prepared in (△——△) PBS/citrate or (▼——▼) raw chicken serum.

Iodinated anti-SP_1 was diluted in coating buffer pH 9.6 to give a range of dilutions 1/2 to 1/10. Nunc microtitre plate wells were coated with 100 µl of these dilutions overnight at 4°C. Standards were then assayed following the routine assay protocol and graphs of absorbance against log concentration prepared.

4.6.2 Results

The standard curves obtained are shown in Figure 4 vii. Dilutions of greater than 1/4 resulted in absorbances too low to be measured reliably. A dilution of 1/2 was selected for further experiments.

4.6.3 Method of Assessing Loss of Antibody

Nunc microtitre plates (Grade I) were used. Other ELISA reagents as detailed in Appendix I.

A range of SP_1 standards were prepared in diluting solution or chicken serum.

Microtitre plate wells (Nunc Grade I) were incubated with 100 µl of a 1/2 dilution of anti-SP_1 in coating buffer overnight at 4°C. The remaining steps of the experiment are shown in Fig 4 viii.

4.6.4 Results and Discussion

Tables 4 VIII, IX and Fig 4 ix show the loss of antibody compared with the initial amount bound taken as 100%, for each standard preparation at each stage of the assay. Antibody was lost at each stage of the assay, the overall loss amounting to 14.4% for antigen diluted in a non-protein matrix and nearly 20% for antigen diluted in chicken serum.

The loss of immobilized protein from the solid phase has been the subject of a number of investigations. Engvall and Perlmann (1972) first noted a "hook" effect which they suggested was due to loss of the complex from the solid phase and also reported a total leakage of 20-30% of bound material. Similarly Lehtonen and Viljanen (1980) reported a loss of I^{125} labelled BSA from the walls of polystyrene cuvettes. Antigen detachment was shown to occur to a considerable degree for up to 6 washes in normal saline containing 0.05% Tween 20. During a complete ELISA, this group also found a loss of 3-8% of the initial bound antigen during serum incubation and a loss of 7-10% at the first wash stage. Total leakage over the assay varied between 20-29%. Similarly Hendry and Herrmann (1979) reported a loss of viral antigen of up to 68% during an ELISA.

However, Cantarero et al (1980) were unable to show loss of complex from the solid phase, although their data did exhibit a "hook" effect which they suggested was due to insufficient reagents to develop the system.

The results obtained here favour the former concept ie there is a loss of antibody/complex from the solid phase. No antigen concentration dependence was shown however. As the plates were coated at only a single concentration of antibody, it was not possible to show the reported difference in loss with varying protein uptake (Lehtonen and Viljanen 1980). The major loss of antibody occurred at the initial washing stage (5.6%). Thereafter, for antigen diluted in PBS/citrate, loss at each stage was of the order of only 1-2%, with a mean overall loss of 14.4%.

In contrast however, when antigen was diluted in a protein matrix (chicken serum) the results obtained differed. Whilst the initial loss during the wash stage did not differ, during antigen incubation a further 4.2% antibody was lost. This was significantly greater than the loss occuring with PBS/citrate diluted antigen as assessed by the t test ($p < 0.001$). Similarly during the washing cycle succeeding this stage, a significantly greater loss of antibody was observed than at the same stage with PBS/citrate diluted standards ($p < 0.001$). At further stages however no difference was shown for antibody loss between the two sets of antigens.

It appears therefore that a high protein concentration leads to weakening of the antibody-solid phase bond resulting in greater loss of antibody at this incubation stage and the following wash stage.

Previously, Hendry had pointed out (Hendry and Herrmann 1980) that to prevent non specific adsorption, it is necessary to add ....."protein solutions plus a non ionic detergent, but that these inhibitors caused desorption of both adsorbed antigen and antibody". The results obtained here are in agreement with this finding.

These findings may have important consequences. Ideally sera should be diluted sufficiently in a non-protein containing matrix to cut down this leaching. This however is at variance with reducing NSB. Further,for work simplification, the exclusion of a diluting step is advantageous.

The difficulty is to ensure that the leached antibody does not set up a competitive effect with the incubated antigen, which will lead to decreased assay sensitivity and precision. Investigation of the time course of all reactions remains an important

Table 4 X

Loss of Anti-SP_1 from the Walls of Microtitre Plates with Pregnancy Pooled Serum and Chicken Serum

	Pregnancy Pooled Serum			Raw Chicken Serum		
Time mins	Initial Counts	Final Counts	Mean % remaining	Initial Counts	Final Counts	Mean % remaining
10	46576 45871	41827 40335	88.8	43882 46486	39207 40786	88.5
20	48802 43287	41049 39566	87.8	45798 47711	41031 41726	88.5
30	47355 46657	41229 40938	87.4	47939 46229	42220 38785	86.0
40	48118 48034	42425 41774	87.1	49289 47315	42093 40363	85.4
50	49826 49380	41039 41319	83.1	45773 46591	41125 39030	83.8
60	47070 47703	41111 41199	86.8	48305 46822	40543 40481	85.2
70	48459 47515	40519 40774	84.7	45607 46314	39464 39842	86.2
80	46107 48169	38074 41068	83.9	45261 47378	41465 40975	86.5
90	47093 47603	40337 40124	83.5	47654 45648	38395 38961	83.0
100	47598 45466	38873 39127	83.9	45782 47050	39119 39193	84.4
110	46174 47397	38571 39041	83.0	43308 45010	34894 37980	82.5
120	48182 46784	40232 39786	84.2	41772 -	34226 -	82.0

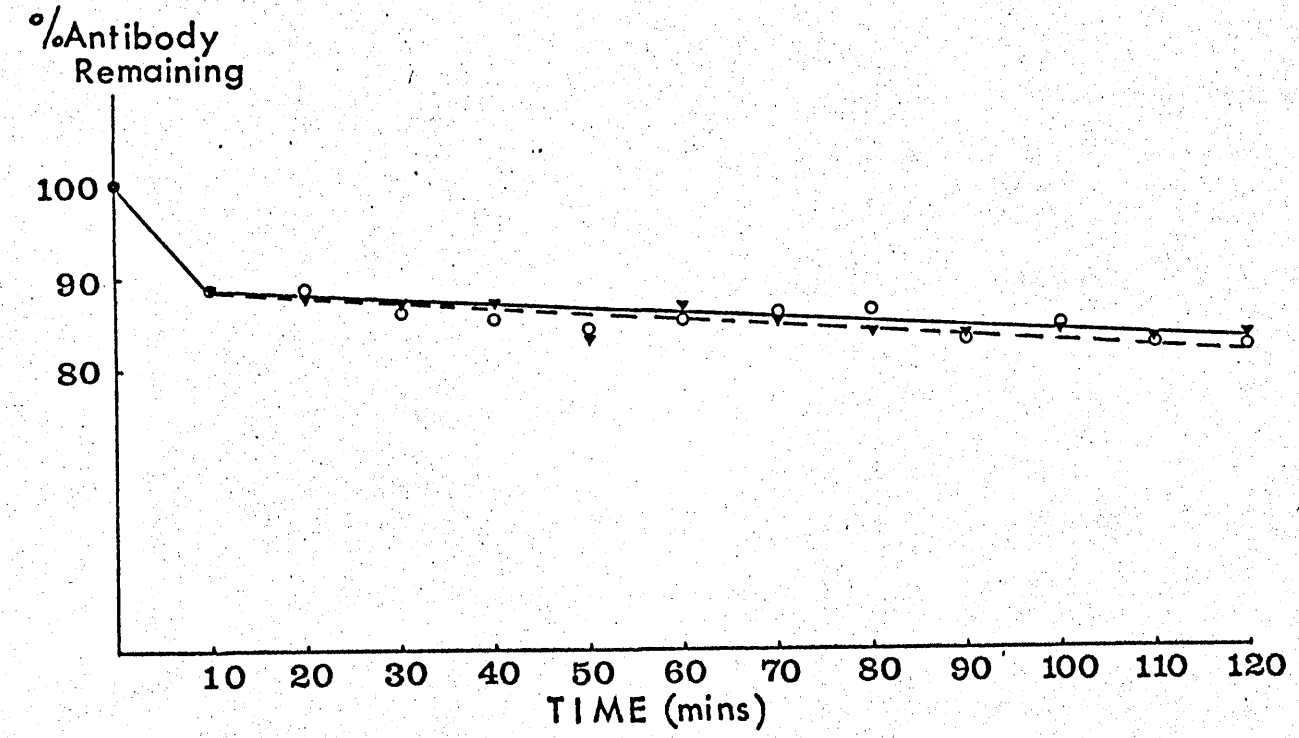

Fig 4 x Time course for loss of iodinated antibody from microtitre plate wells during Antigen Incubation. Antibody was incubated with a protein matrix containing no antigen (raw chicken serum) (o——o) or pooled pregnancy serum (▼——▼).

4.7 Investigation of Time Course of Reaction Between Bound labelled Antibody and Antigen

4.7.1 Materials and Method

A pool of sera from women in the third trimester of pregnancy was prepared. All other reagents were as given in Appendix I.

Wells of Nunc Microtitre I plates were coated overnight with I^{125} labelled anti-SP_1. The following morning, the antibody solution was aspirated, the plates washed 3 times with PBS, dried and counted (initial counts).

Duplicate aliquots (100 µl) of either the pooled pregnancy serum or raw chicken serum were added to the wells and incubated for times varying between 10 and 120 minutes. At the end of each reaction time the well contents were aspirated and the remaining labelled antibody counted (Final Counts). A graph of % labelled antibody remaining against time was drawn.

4.7.2 Results and Discussion

Table 4 X shows the counts and % bound antibody at various incubation times, and Fig 4 x represents these results graphically. A paired t test was performed on the mean % antibody remaining. No significant difference in the remaining antibody was demonstrated ($t = 0.44$ $p > 0.1$) between the wells containing pregnancy serum and those containing chicken serum.

The problem of antibody loss from the solid phase in relation to antigen content remains open to debate. Thus Lehtonen and Viljanen (1980) found detachment of coated antigen to be independent of the presence of specific antibodies. This finding was previously observed by Zollinger et al (1976).

Lehtonen also found that almost half the total leakage of antigen had taken place within 15 minutes and that after 2 hours incubation, almost three quarters of the total leaking antigen was detached (1980).

Engvall's original finding of the hook phenomenon is often interpreted as evidence for loss of complex from the adsorbing surface. It seems more probable however that this effect relates to protein concentration during coating of the solid phase, a view reinforced by Cantarero et al (1980).

From the results obtained here there seems no reason to disagree with the previous findings by other groups of workers. No difference in loss of antibody over the 2 hour incubation period could be observed in those wells with a high antigen content compared to those with no antigen. Thus the loss of antibody from the solid phase appears from this experiment to be independent of antigen concentration.

A greater loss of antibody was observed during antigen incubation compared with the previous experiment. This may be due to different batches of plates being used. Of a total loss of 14-16%, 4 to 6% occurred in the first 10 minutes of assay. Lehtonen however found that more than 50% of the total loss during antigen incubation occurred during the first 10 minutes. It is possible that a difference in characteristics of the polystyrene solid phase could account for this.

It is apparent however that loss of antibody does occur from the solid phase throughout ELISA and that ideally improved solid phases must be developed in order to maximise the potential of ELISA.

Table 4 XI

The Effect of Varying Ab-E concentration on NSB

Ab-E Dil^{n}	1/2000	1/1000	1/500	1/100
Mean OD	0.037	0.048	0.057	0.133
SD	0.006	0.006	0.007	0.008
n	11	11	11	11

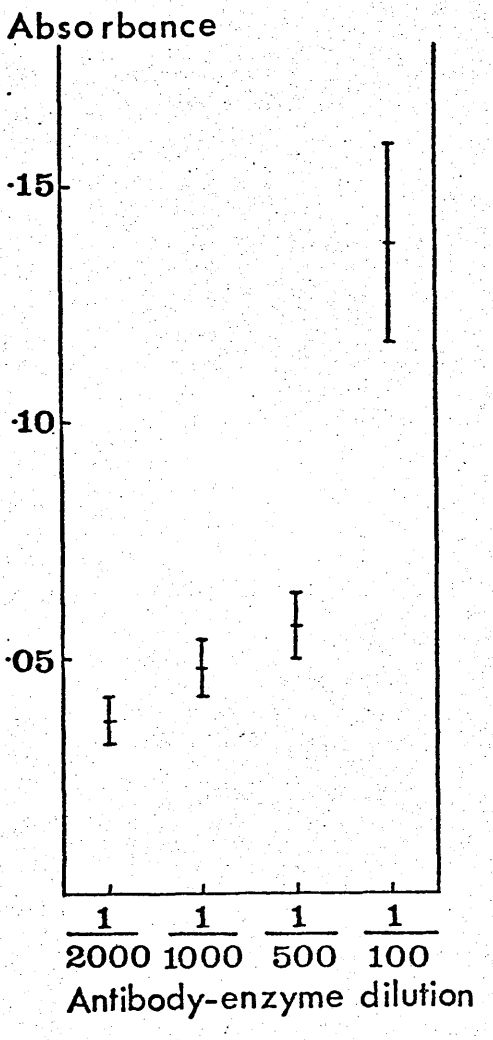

Fig 4 xi The effect of antibody enzyme concentration on Non Specific Binding. Ab-E was diluted as shown in PBS/25% chicken serum containing 10^{-2} mol/l citrate.

4.8.1 Investigation of the effect of varying antibody enzyme concentration

Materials were as given in Appendix I for ELISA. Four series of zero standards (SP_1) were assayed according to the routine protocol for SP_1 assay except that Ab-E was added at different dilutions to each series of zero standards. Following colour development, the mean and SD for the blank standards, for each dilution of Ab-E were estimated.

4.8.2 Investigation of the Use of Albumin and Tween-20 in the reduction of NSB

The routine protocol for the assay of SP_1 was followed (3.4.2) reagents as specified in Appendix I with the following exceptions. 0 µg/l standards were prepared in either diluting solution (PBS/citrate) or in PBS containing 1% human serum albumin.

Microtitre plates (Nunc Grade I) were washed with either PBS saline or with PBS containing 0.1 ml Tween 20/litre of buffer (wash buffer).

The effect of Tween in the antibody enzyme stage of the assay was investigated by incorporating 5 µl Tween 20 into 50 ml of antibody enzyme conjugate prepared according to Appendix I.

Plates were processed according to the routine protocol incorporating the variations above as necessary.

4.8.3 Results and Discussion

Reduction in Ab-E concentration leads to decreased NSB values (Table 4 XI and Fig 4 xi). There is a significant increase in NSB at Ab-E dilutions of 1/1000, 1/500, 1/100 compared to

Table 4 XII

Reduction of NSB in ELISA by use of albumin and Tween 20. Results represent the optical densities obtained for replicate background specimens. Optical densities for the standard curve are after blank subtraction.

Well	PBS Wash PBS	PBS + albumin Wash PBS	PBS Wash PBS/Tween	PBS + albumin Wash PBS/Tween
1	.122	.106	.120	.107
2	.122	.130	.107	.103
3	.120	.108	.108	.097
4	.115	.103	.101	.104
5	.125	.102	.098	.098
6	.116	.094	.115	.128
7	.121	.095	.107	.121
8	.123	.112	.121	.097
9	.136	.122	.134	.099
10	.172	.106	.122	.119
11	.174	.099	.108	.113
12	.186	.100	.113	.101
13	.177	.111	.119	.095
14	.176	.089	.120	.094
15	.190	.096	.099	.102
16	.155	.094	.105	.103
17	.189	.097	.107	.092
18	.142	.099	.110	.087
19	.137	.118	.123	.095
20	.108	.095	.130	.102
Mean	0.145	0.104	0.113	0.103
SD	0.029	0.011	0.010	0.010
CV %	19.7%	10.1%	8.8%	10.0%

STD Curve SP_1 µg/l		
31.25	0.120	0.113
62.5	0.327	0.330
125	0.473	0.460
250	0.563	0.572
500	0.593	0.590
1000	0.624	0.617

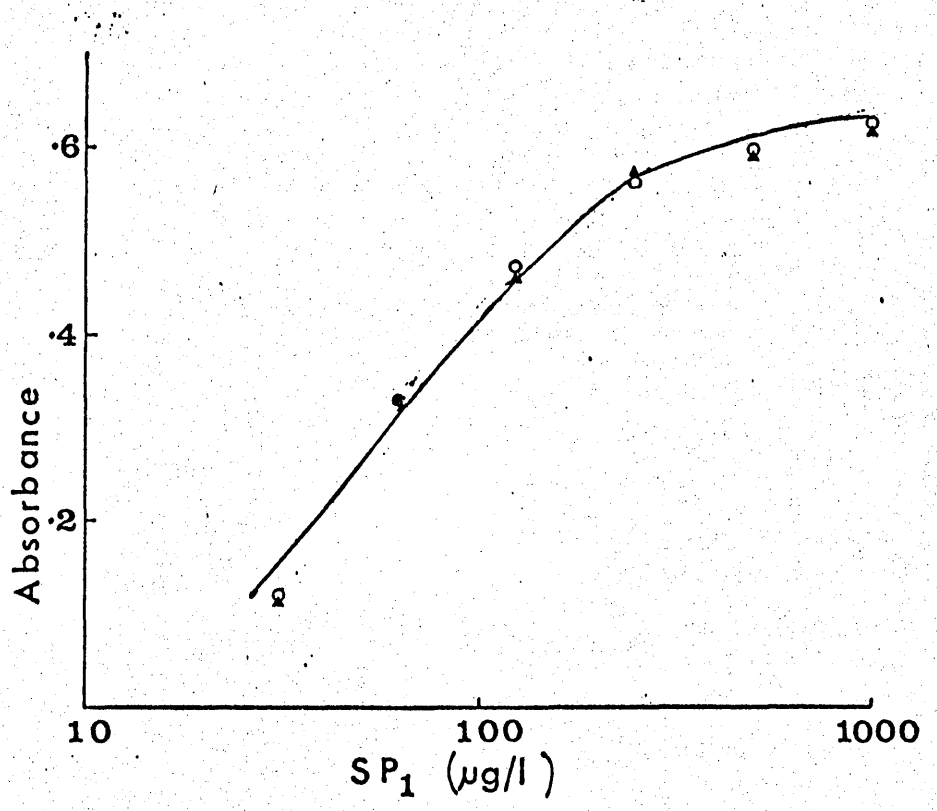

Fig 4 xii The effect on the standard curve of adding 1% albumin to standard preparations:-

(o——o) standards in PBS

(▲——▲) standards in PBS + 1% albumin

Table 4 XIII

Effect of Tween 20 in antibody-enzyme reagent on reduction of NSB. Results are absorbance values for standard curve (after blank subtraction) and replicate blank specimens.

$SP_1{}^n$ Conc^n µg/l	Ab-E in PBS/chicken serum	Ab-E in PBS/chicken serum + Tween 20
31.25	.110	.117
62.5	.300	.307
125	.443	.449
250	.537	.517
500	.573	.563
1000	.581	.582
0	.113	.097
0	.112	.103
0	.097	.103
0	.105	.121
0	.097	.117
0	.098	.118
0	.117	.098
0	.120	.104
0	.108	.106
0	.110	.110
0	.103	.097
0	.102	.105
Mean blank OD	0.107	0.107
SD	7.8	8.3
CV %	7.3	7.8

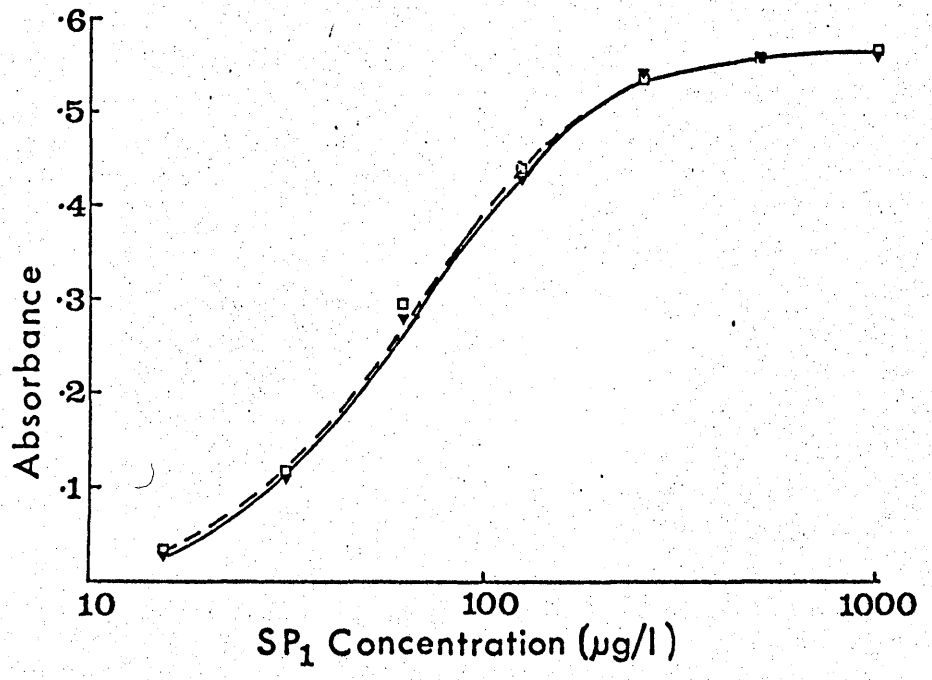

Fig 4 xiii The effect on the standard curve of Adding Tween 20 to diluted antibody enzyme:-

(□---□) AbE + tween 20 (5 µl/50 ml diluted antibody enzyme)

(▼——▼) AbE no tween

dilution of 1/2000 ($t = 33.7$, 7.7 and 5.1 $p < 0.01$). The reduction in blank values with increasing dilution is probably due to decreased non specific binding of Ab-E to the polystyrene. The effect of 25% chicken serum at this stage was shown to be effective in reducing NSB (Macdonald 1979) but does not completely negate the effect. Thus a low concentration of Ab-E must be used. This however offsets gains in sensitivity possible with increased Ab-E concentrations (3.5.4) and a compromise is thus necessary.

Table 4 XII and Figure 4 xii shows the results of absorbances for background specimens (0 µg/l SP_1) with and without albumin added and also the effect of washing plates in wash buffer as opposed to PBS alone.

Table 4 XIII and Figure 4 xiii shows the effect on absorbance of adding Tween 20 to the antibody enzyme conjugate reagent.

Incorporation of albumin into the diluent resulted in a significant decrease of NSB compared with PBS diluent alone ($t = 6.1$ $p < 0.001$). Similarly incorporation of Tween 20 into the wash buffer led to a reduction of NSB compared with PBS as a diluent ($t = 4.7$ $p < 0.001$) but not when PBS-albumin was used as a diluent ($t = 0.22$ $p > 0.05$).

The addition of 1% albumin to diluent caused no effect on the standard curve position and NSB was reduced. Similarly addition of Tween 20 to the antibody enzyme reagent caused no shift in the standard curve, but neither was there any reduction in non specific binding ($t = .07$ $p > 0.05$).

Most authors agree that some means of reducing non specific binding and interference in ELISA systems is necessary. Hendry noted "that to prevent non specific adsorption of immunoreagents it is necessary to use inhibitors such as protein

solutions plus a non-ionic detergent" (Hendry and Herrmann 1980). The stage at which these are used and indeed whether both protein and detergents are necessary has however varied greatly. Engvall and Perlmann (1972) used only Tween 20 both in their sample diluent and conjugate reagent, as did Hevey et al (Hevey et al 1976) in an assay for AFP for their serum diluent, but added human serum albumin and Tween to their conjugate reagent. Other workers however have used albumin in their sample diluent and both albumin and Tween in their wash buffer (Linpisarn et al 1981). Little study seems to have been carried out on the exact requirements for reduction of NSB and as this can often be a problem in ELISA, it seems central to the concept that an awareness of these conditions is necessary. Bullock and Walls (1977) investigated the use of Tween 20 and BSA and concluded that the addition of Tween had little effect in reducing NSB when incubation in BSA were used. When BSA incubation was omitted however, Tween 20 in saline or water was as efficient in NSB reduction. With neither BSA or Tween 20, a weakly positive test became completely positive.

The results presented here are not at variance with these findings. Incorporation of albumin into the sample diluent resulted in a decrease of NSB as did use of Tween 20 when PBS alone was used as sample diluent. Use of Tween in the wash buffer conferred no extra advantage when the sample diluent contained albumin however.

Use of albumin in the sample diluent caused no shift of the standard curve compared to PBS diluent after allowance for blank values. Incorporation of albumin into the sample diluent thus provides an effective means of reducing NSB. It is possible that this may only be required where protein concentrations

are low and the practice of diluting samples and standards in a serum matrix stripped of the antigen being assayed has much to commend it. This is particularly true if linearity problems are observed for samples.

This suggestion may be more pertinent with the finding by Samake et al (1983) that different proteins may affect an ELISA in different ways both on the maintenance of enzymatic activity and on the hapten-antibody reaction.

In the results obtained here the source of protein was human serum albumin. Although a relatively expensive source of protein, the low concentration (1 g/l) at which it is used makes this problem of less importance. The variation of assay results with different proteins has not been investigated as there was no evidence that HSA had a deterious effect on any stage of the assay system.

Taking these findings into account, it is perhaps not surprising that use of Tween in the antibody enzyme reagent had no effect in further reducing NSB, since the conjugate was diluted in a 25% chicken serum matrix. This concentration of chicken serum was taken from the literature (Macdonald et al 1979) and further investigation of the ideal chicken serum concentration was not performed as assay systems functioned well with this concentration.

The use of chicken serum in the sample diluent in place of albumin has yet to be investigated and may provide a cheaper alternative to HSA.

Although investigated in an enzyme immunoassay system these findings must also be taken into account in solid phase assays utilizing radioactively labelled antibodies based on the same principles (IRMA).

4.9.1 Materials and Methods

A horseradish peroxidase conjugate of antiserum to PAPP-A was prepared by the method of Avrameas and Ternynck (1974) (3.7.3). Other materials as given in Appendix I for ELISA systems.

The time course of the antigen/antibody reaction for these 3 oncodevelopmental proteins was investigated (the antigens chosen to give a range of molecular weights - hCG 39,000 (Morgan et al 1974), SP_1 90,000 (Engvall 1980) and PAPP-A 750,000 (Lin et al 1974). Pooled pregnancy serum was used as the source of antigens.

Binding of antigens was measured after 10-80 minutes incubation as described elsewhere for investigating the time courses of ELISA systems (3.7.7). Neat serum and several dilutions in either diluting solution, chicken serum or horse serum (Wellcome III) were utilized,

100 µl of antibody diluted 1/1200 in coating buffer was added to the wells of microtitre plates (Nunc Grade I) and incubated overnight at 4°C. After washing the plate with wash buffer and drying, 100 µl of pregnancy serum at various dilutions was added and stood at room temperature for times ranging from 10-80 minutes. The serum was then aspirated, the plates washed and dried as before. 100 µl of Ab-E (suitably diluted in PBS/25% chicken serum containing 10 mmol/l citrate) was then added to the wells and incubated at room temperature for 50 minutes. Following a further aspiration/washing cycle, 100 µl of chromogen was added to the wells and incubated for 60 minutes at room temperature, after which, 100 µl of 4 mol/l sulphuric acid was added to stop the reaction. The absorbance

was measured at 492 nm using a Titertek Multiskan Plate Reader (Flow Laboratories, Irvine).

A series of experiments were also carried out adjusting the above procedure as follows.

4.9.2 Investigation of serum protein effects on the system

Dilutions of raw (unheated) pregnancy serum were made in raw (unheated) chicken serum to investigate the effect of other serum proteins on the system.

4.9.3 Investigation of the effect of varying albumin content

Investigation of the effect of decreasing albumin content with increasing dilution of the pregnancy serum was made by preparing a 1/100 dilution of serum in PBS/citrate and adding to aliquots of this, sufficient human serum albumin to give concentrations of 17 g/l and 35 g/l These concentrations correspond to the albumin concentrations in a 1/2 dilution of the pregnancy serum and undiluted pregnancy serum.

4.9.4 Investigation of the effect of varying calcium concentration

Investigation of the effect of decreasing calcium concentration with increasing dilution was made in a similar way to that for albumin (b). A 1/4 and 1/10 dilution of BDH Calcium Standard for Atomic Absorption (10 mmol/l) was prepared in PBS to give calcium concentrations of 2.5 mmol/l and 1 mmol/l respectively. A 1/100 dilution of pregnancy serum was then prepared in these solutions. These calcium concentrations correspond to those in a neat and 1/2 dilution of the pooled serum.

4.9.5 Investigation of possible interference by the complement system

Investigation of interference by the complement system was

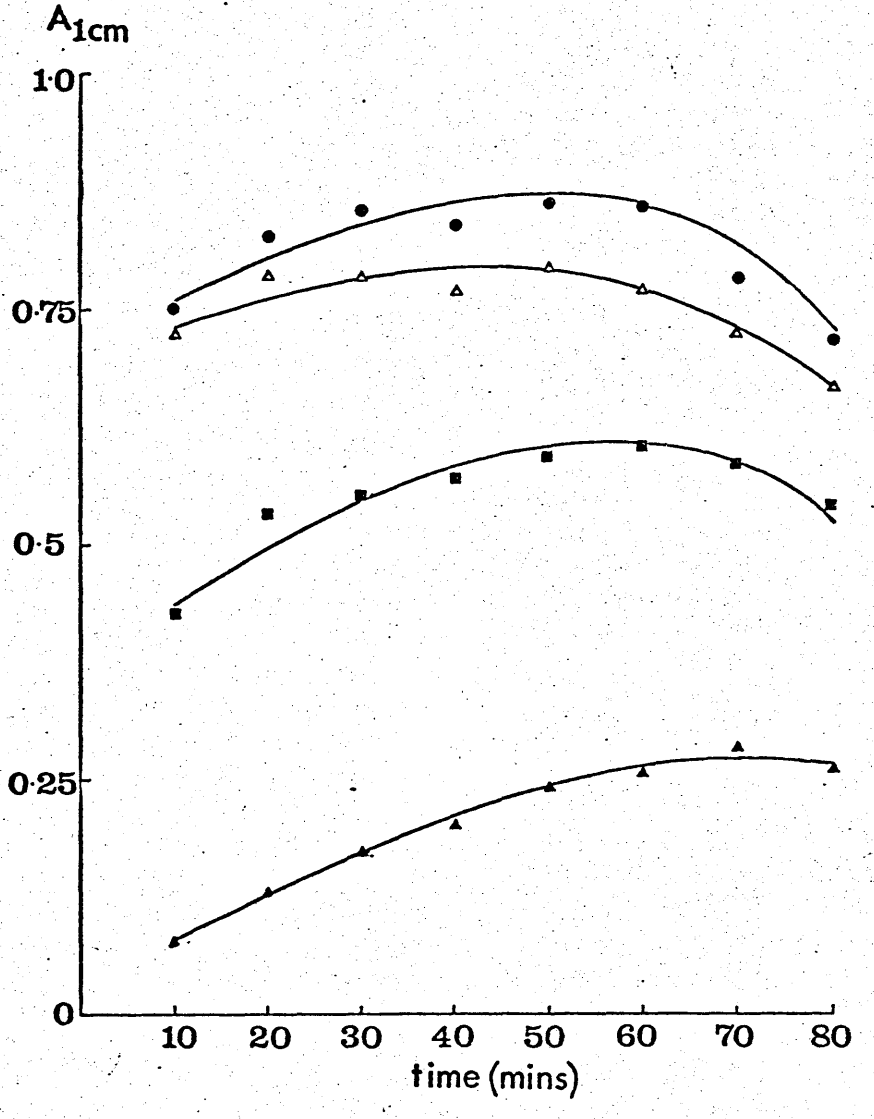

Fig 4 xiv The effect of Increasing concentration of PAPP-A upon the time course of the antigen-antibody reaction.
Pooled pregnancy serum was used (●—●) neat, or diluted (△—△) 1/2, (■—■) 1/10, (▲—▲) 1/100 in PBS citrate.

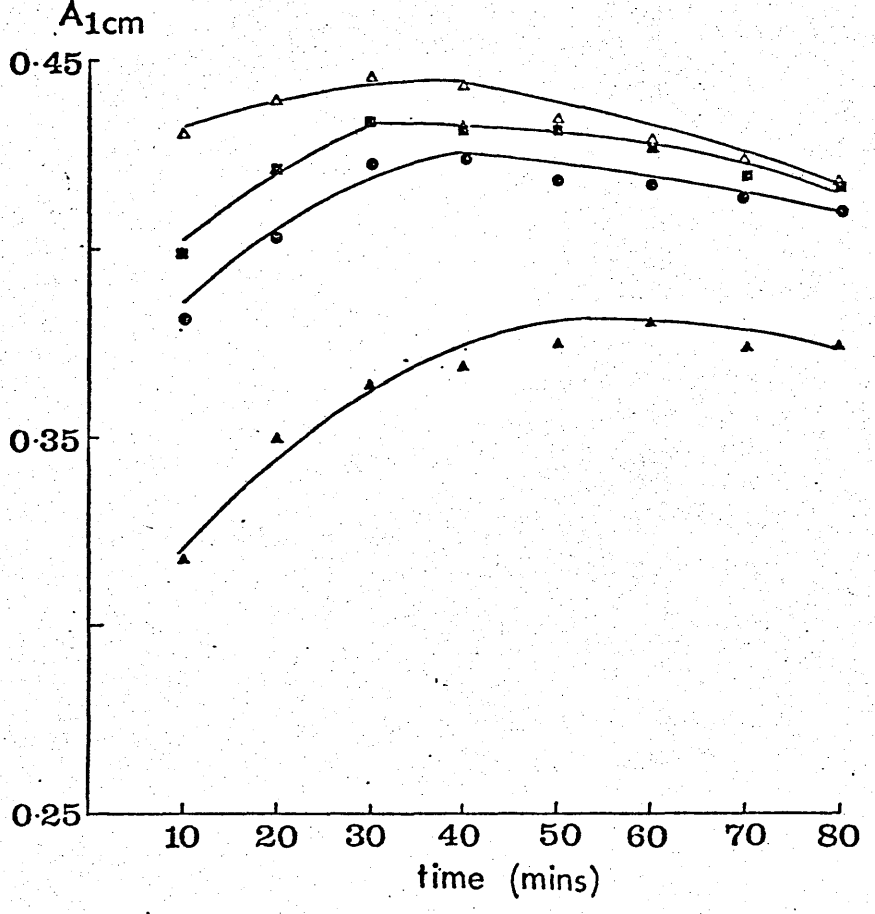

Fig 4 xv The effect of increasing concentration of SP_1 upon the time course of the antigen-antibody reaction.
Serum dilutions as in Fig 4 xiv.

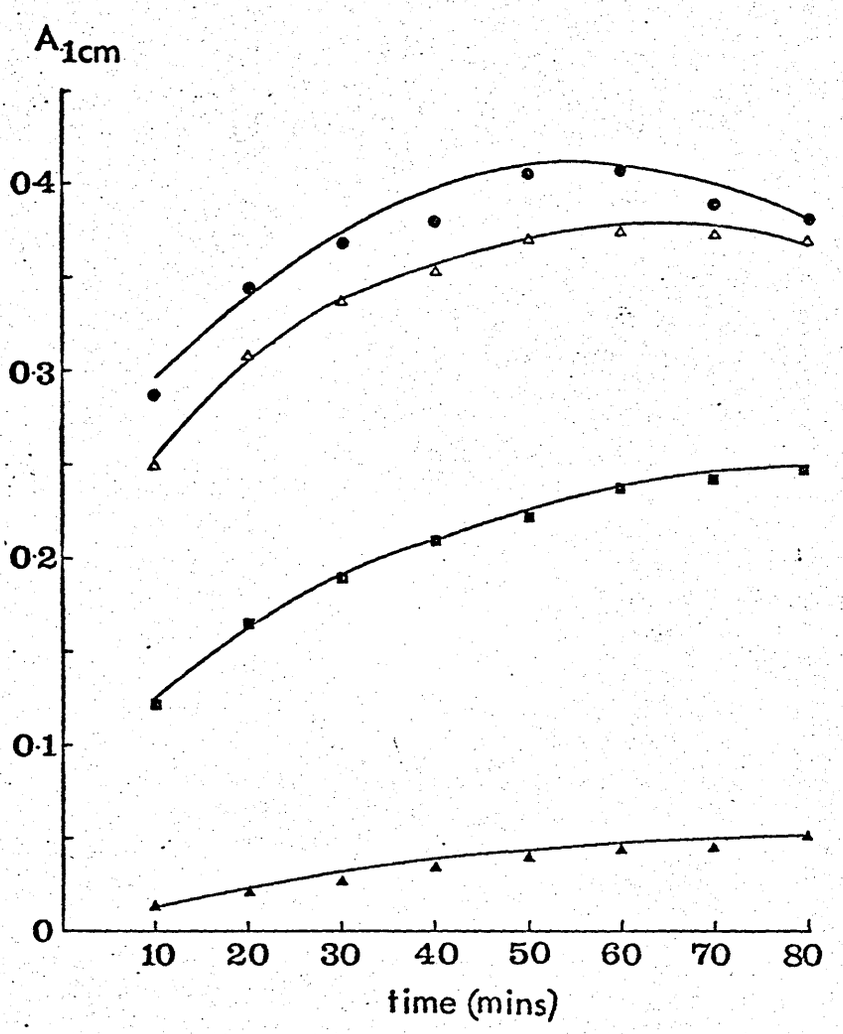

Fig 4 xvi The effect of increasing concentration of SP_1 upon the time course of the antigen-antibody reaction.
Serum dilutions as in Fig 4 xiv.

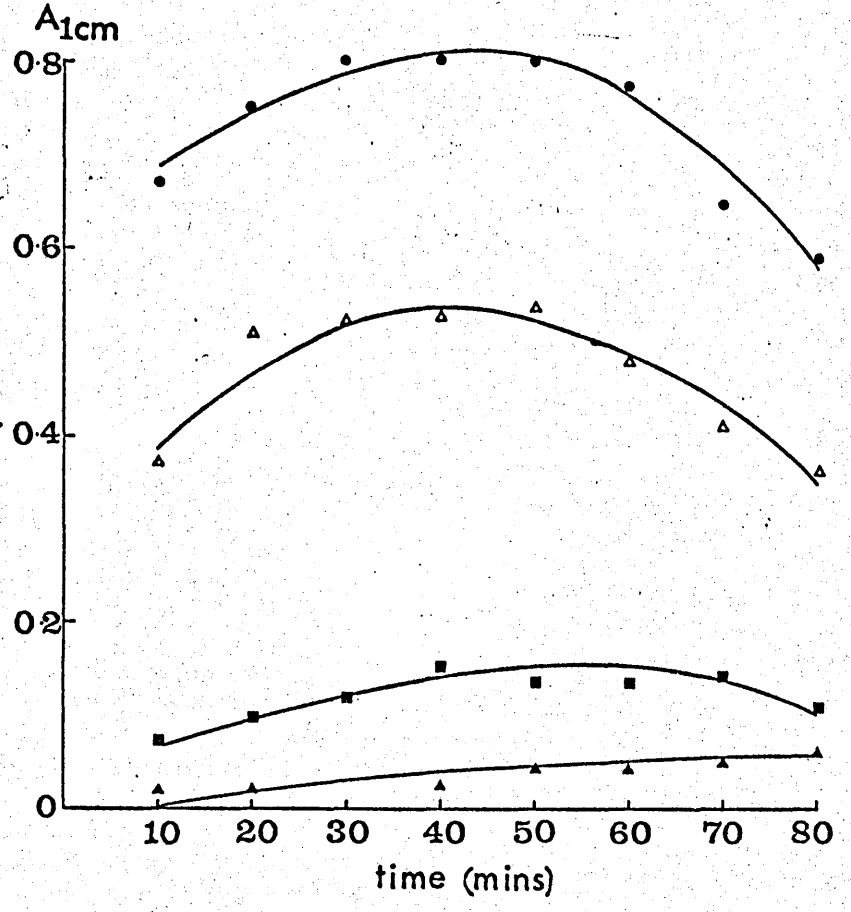

Fig 4 xvii The effect of Increasing concentrations of PAPP-A on the time course of the antigen-antibody reactions.
Serum dilutions as given in Fig 4 xiv but dilutions were made in raw chicken serum.

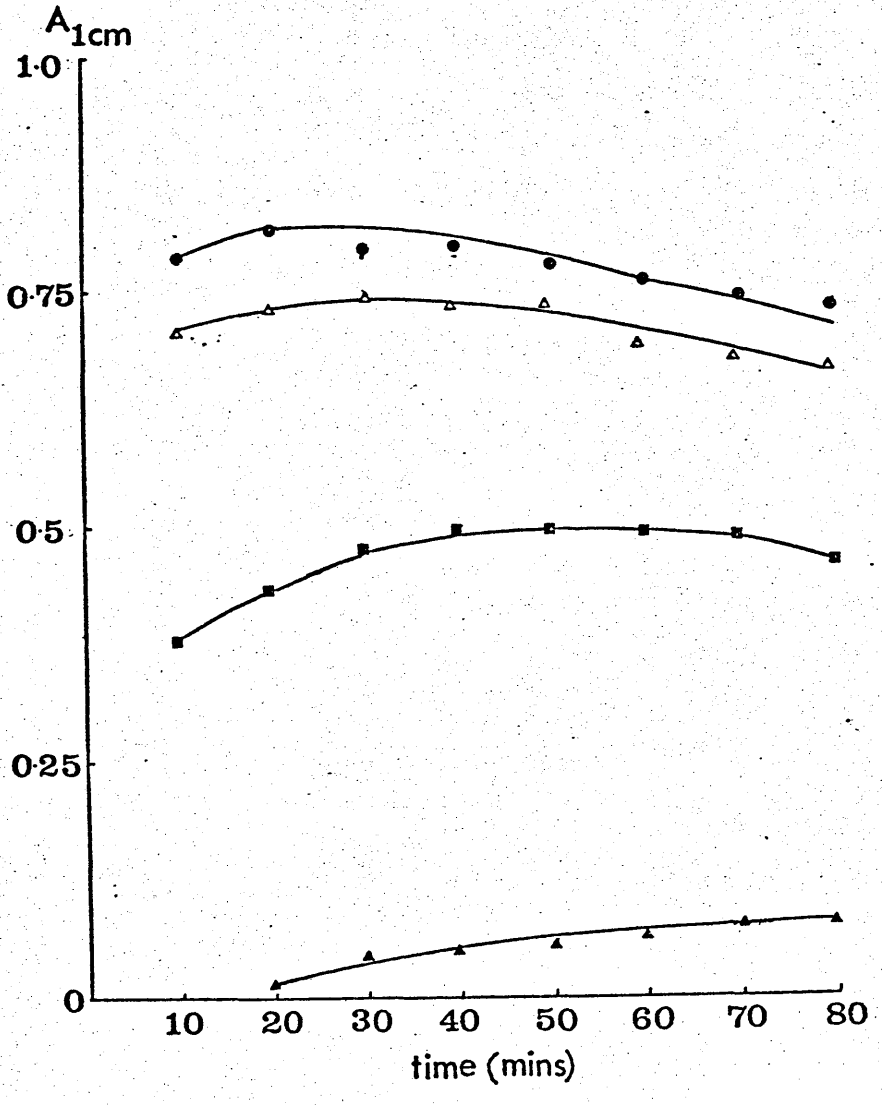

Fig 4 xviii The effect of increasing concentration of PAPP-A on the time course of the antigen-antibody reaction.
Serum dilutions as given in Fig 4 xiv but diluted in heat inactivated horse serum.

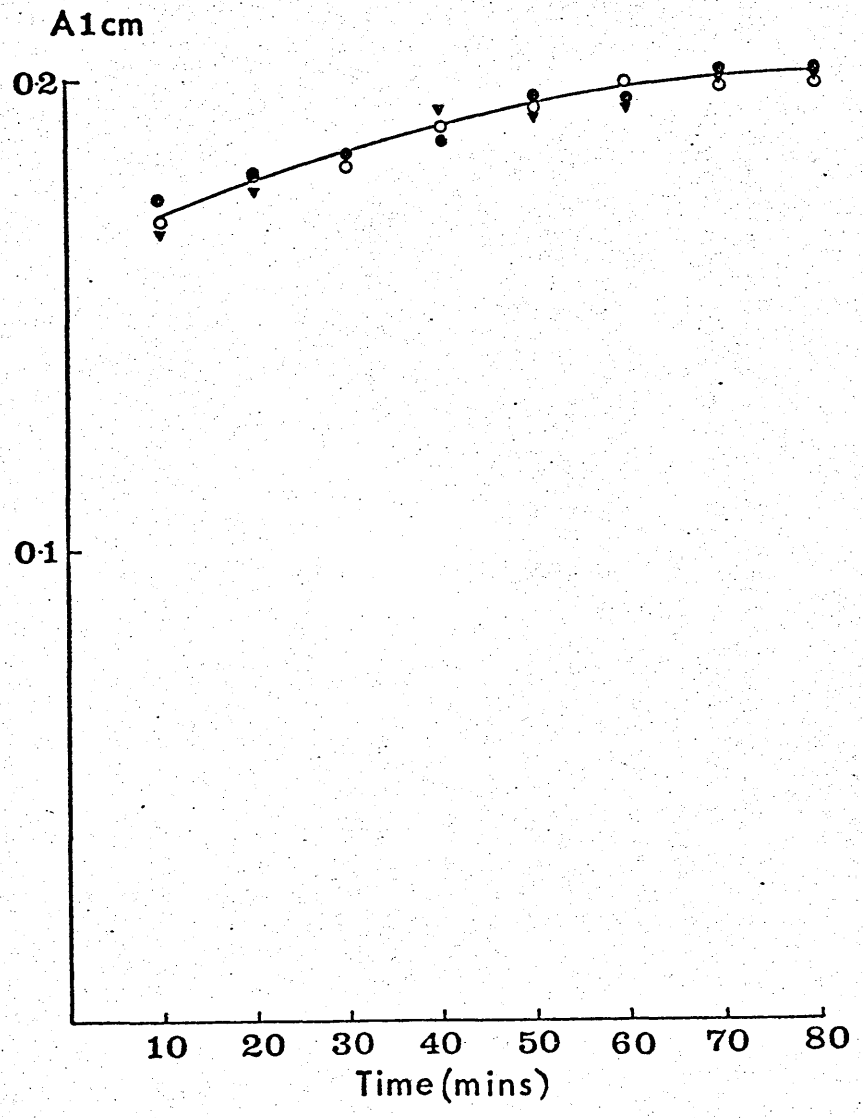

Fig 4 xix The effect of varying calcium concentrations on the time course of antigen (PAPP-A) with antibody. Pooled pregnancy serum was diluted 1/100 in PBS/citrate (O——O) and calcium added at 1 mmol/l (●——●) or 2.5 mmol/l (▼——▼).

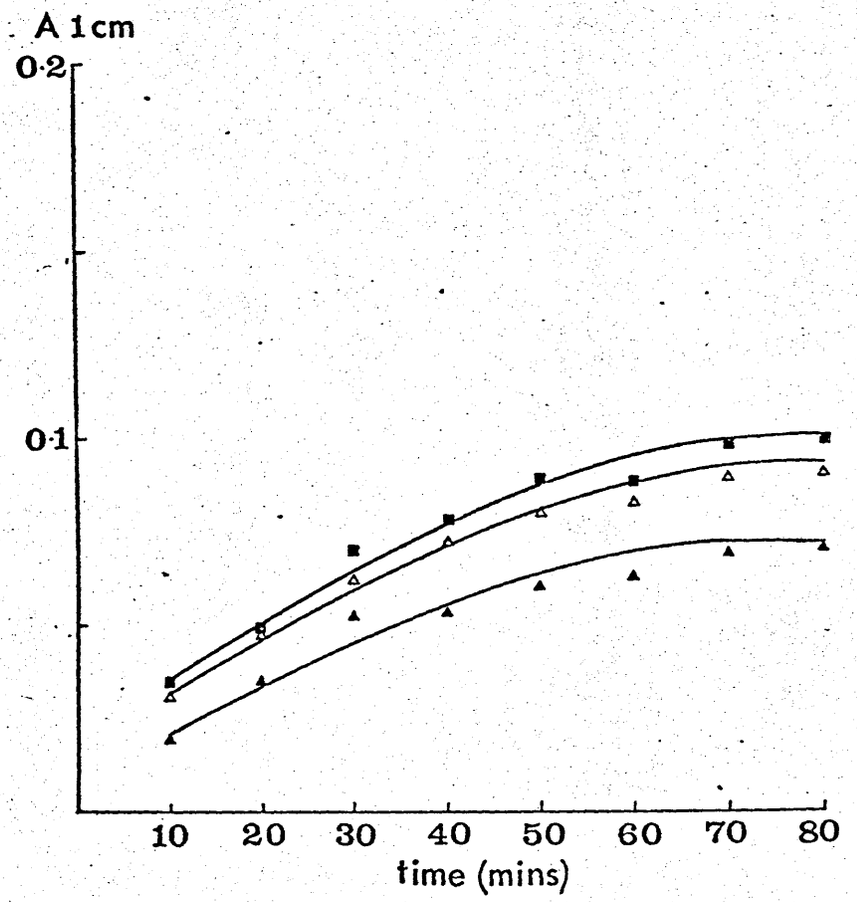

Fig 4 xx The effect of varying albumin concentrations on the time course of antigen (PAPP-A) with antibody. Pooled pregnancy serum was diluted 1/100 in PBS/citrate (▲——▲) and albumin added at 17 g/l (△——△) or 35 g/l (■——■).

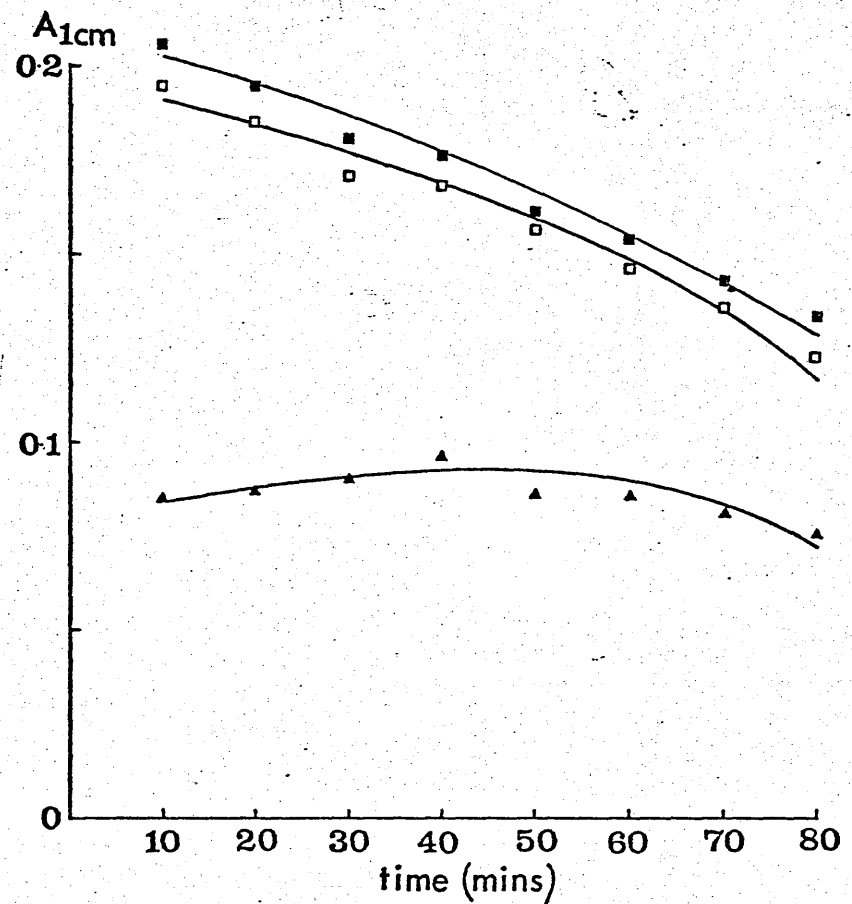

Fig 4 xxi The effect of reducing coating antibody concentration on the time course of antigen (PAPP-A) with antibody. The antiserum concentration used was 1/5000 instead of 1/1250 used previously.

Antigen was used neat (■—■), diluted 1/2 (□—□) or 1/10 (▲—▲) in PBS/citrate.

carried out by heat inactivating both pooled pregnancy serum and horse serum at 56°C for 1 hour. Dilutions of the pregnancy pool were then made in the horse serum.

4.9.6 Investigation of the effect of varying antibody concentration

This was investigated by increasing the dilution of antibody used to coat the plates to 1/5000.

4.9.7 Results

All 3 antigens in undiluted serum showed enhanced binding with increasing length of incubation up to a maximum value, followed by a decrease. When the serum was diluted in PBS/citrate, maximum binding appeared later than with undiluted serum and in some instances at the highest dilutions there was no subsequent decrease in the apparent bindings (Fig 4 xiv - xvi).

Similar results were obtained for PAPP-A with dilutions of pregnancy serum made in raw chicken serum (Fig 4 xvii) or with preheated pregnancy serum diluted in preheated horse serum (Fig 4 xviii).

Increasing the concentration of either calcium or albumin in the 1/100 dilution of pregnancy serum did not cause decreased colour development at any time. Conversely enhanced colour development with increasing albumin concentration was observed (Fig 4 xix and xx).

When the dilution of antiserum used to coat the microtitre plates was increased from 1/1200 to 1/5000, maximum binding occurred much earlier than at the low dilution (Fig 4 xxi).

4.9.8 Discussion

The diminution of the measured antigen binding may be due

either to inhibition of the reaction of the bound antigen with the antibody enzyme or to detachment of immobilized antibody from the solid phase. The simplest form of inhibition envisaged is some type of steric hindrance which prevents the antibody-enzyme conjugate gaining access to the bound antigen, so that the amount of antigen bound is underestimated.

If the steric hindrance is due to antigen bound so closely as to prevent the antibody enzyme conjugate gaining access to immobilized antigen sites, the effect should be more pronounced with large antigen molecules. This does not seem to be the case with the antigens under investigation here, for which there was a 19 fold difference in size between the smallest and largest molecules.

An alternative cause of steric hindrance may be interactions with the C_1 component of the complement system (Belfield and Macdonald 1979). This heat-labile macromolecule is composed of subunits held together by calcium ions, and in vivo is able to attach itself to the F_C portions of IgM and IgG. The immobilized antibodies in ELISA may have a conformation appropriate for reaction with C_1 and if this takes place, the C_1 which has a molecular weight of more than 500,000 daltons may prevent antigen gaining access to the immobilized antibody. However this reaction depends on calcium ions. The inclusion of citrate in both sample diluent and antibody enzyme conjugate should avoid this problem by chelation of calcium. The fact that no diminution in antigen binding could be achieved by addition of calcium to a diluted serum sample is further evidence that this effect is not related to the complement system.

Moreover C_1 is heat labile and the experiments in Figure 4 xxii

unlikely that the complement system is involved.

It is also possible that displacement of immobilized antibody may occur by relatively large amounts of serum protein. If this was so, increasing dilution of the sample in a non protein matrix (PBS/citrate) would be expected to show a lessening of this effect. However dilution in a protein matrix (chicken serum) should cause persistence of this effect due to the relatively constant protein concentration. Dilutions in chicken serum however did not show accentuation of the effect.

Similarly when albumin at two different concentrations was added to the diluted serum specimen in PBS/citrate, no diminution in antigen binding was observed. Indeed the eventual colour developed at all stages of incubation was enhanced by this process. This is possibly due to the reduced NSB's observed previously on addition of albumin to the sample diluent (4.8).

Lehtonen and Viljanen (1980) reported leakage of antigen from the solid phase (polystyrene and nylon) at all stages of an ELISA for serum antibodies but particularly during the sample incubation stage. This finding was confirmed in the experiments in section 4.6 and 4.7. In contrast with the findings presented here however, their loss was less marked at high serum concentrations. They concluded that serum was not an essential factor in this leakage and that binding of antibody to antigen was not responsible for this detachment.

The findings presented here are not in agreement with this. Since the decrease in binding was observed only at high serum concentrations and hence relatively high antigen concentrations and was more rapid when the antibody used to coat the solid

phase was more dilute, it is concluded that it is the reaction between antigen and antibody which is responsible for apparent decrease in bound antigen.

Many years ago Dean and Webb (1926) demonstrated that an optimal antigen-antibody ratio exists for the precipitation of antigens in the presence of antibodies. Their studies demonstrated that for a constant dilution of antiserum, addition of antigen in increasing dilutions showed at first a clear mixture, becoming opalescent as antigen dilution increased and finally becoming clear at high antigen dilutions ("prozone effect"). In an analagous manner, prolonged incubation with large amounts of antigen may solubilize the antigen-antibody complex which is bound to the solid phase by weak physical forces (Marrack et al 1951).

However it was shown in section 4.7 that using a direct measure of bound antibody viz a radiolabelled antibody, no difference in antibody loss could be shown for wells incubated with serum containing no antigen to those containing high antigen concentrations. Taking into account the fact that the wells were counted in a γ counter for short periods of time, it is possible that no significant difference in loss was observed. However the amplification effect of the enzyme system in ELISA may unmask the effect.

Several conclusions follow from these findings. When new assays are being established, investigation of the antigen-antibody time course is essential in order to ensure that an optimal antigen-antibody ratio is achieved. Improved solid phases may need to be developed with higher antibody binding capacity. To achieve this increased binding capacity it may

prove necessary to develop simple chemical methods of binding antibodies to the solid phase.

4.10 The Effect of Molecular Heterogeneity on the measurement of SP_1 by ELISA

4.10.1 Introduction

The initial discovery of SP_1 reported molecule of molecular weight about 100,000. It was later shown however that SP_1 existed as heterogeneous forms (designated $SP_1\alpha$ and $SP_1\beta$) (Teisner et al 1978). This was followed by the observation that reaction of the component antigens with antisera differed (Towler et al 1978, Schultz-Larsen et al 1979, Ho and Jones 1980). These problems make necessary an investigation into the characteristics of SP_1 assay by ELISA. It is first necessary to obtain $SP_1\alpha$ free from $SP_1\beta$ and vice versa.

4.10.2 Separation of $SP_1\alpha$ and $SP_1\beta$ by gel filtration

Materials and Methods - Preliminary attempts to separate the two components were made using gel filtration utilizing two different packings.

Chromatography columns were prepared using either Ultragel ACA34 or Sephacryl S200. After equilibration with phosphate buffered saline, the columns were calibrated with proteins of known molecular weight. Pooled pregnancy serum was then applied to the column and eluted with PBS. The eluant fractions were collected and assayed by ELISA for SP_1 content.

Several runs on each column were performed; fractions containing SP_1 were pooled and concentrated against PEG 6000 for further experimentation.

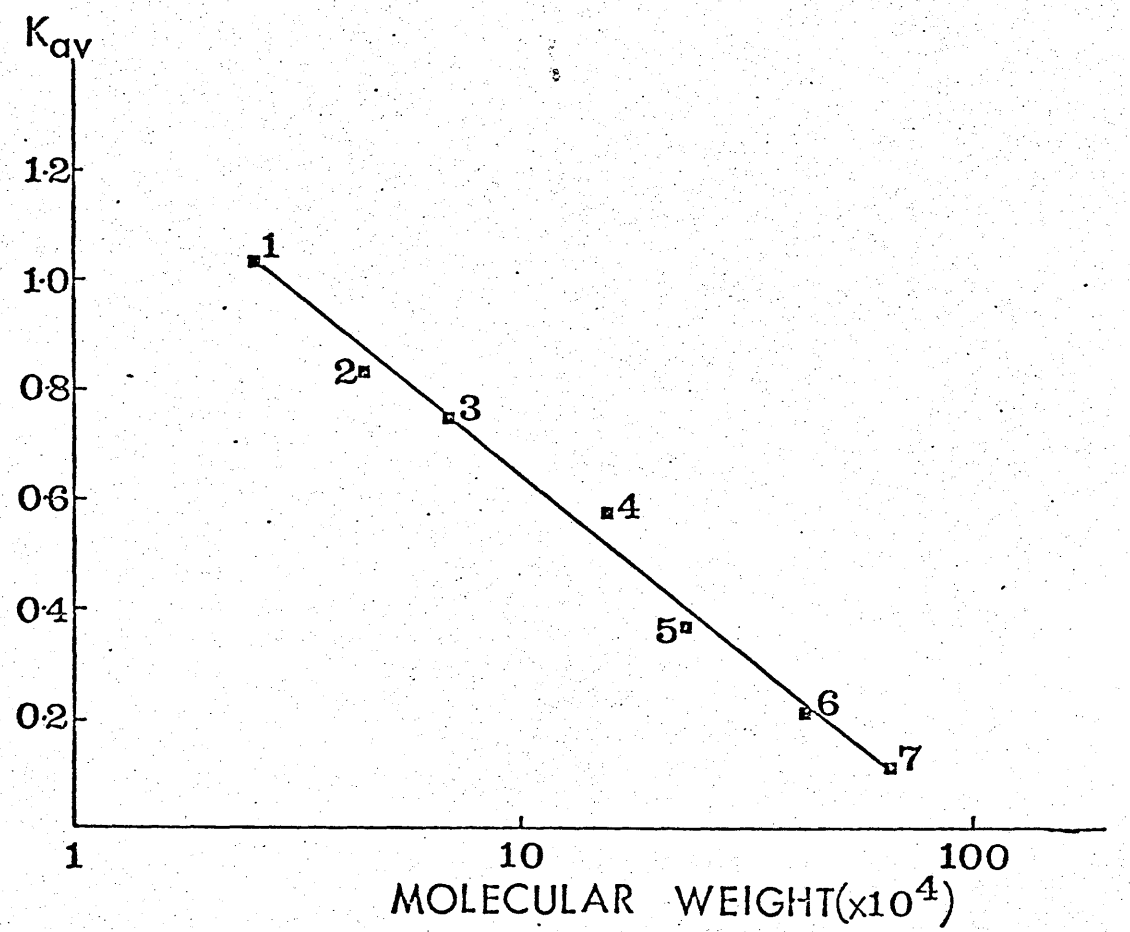

Fig 4 xxii Calibration line for Ultragel Chromatography column. The calibrating proteins used were:-

1)	Chymotrypsinogen A	MW =	25 000
2)	Ovalbumin	" =	43 000
3)	Albumin	" =	67 000
4)	Aldolase	" =	158 000
5)	Catalase	" =	232 000
6)	Ferritin	" =	440 000
7)	Thyroglobulin	" =	669 000

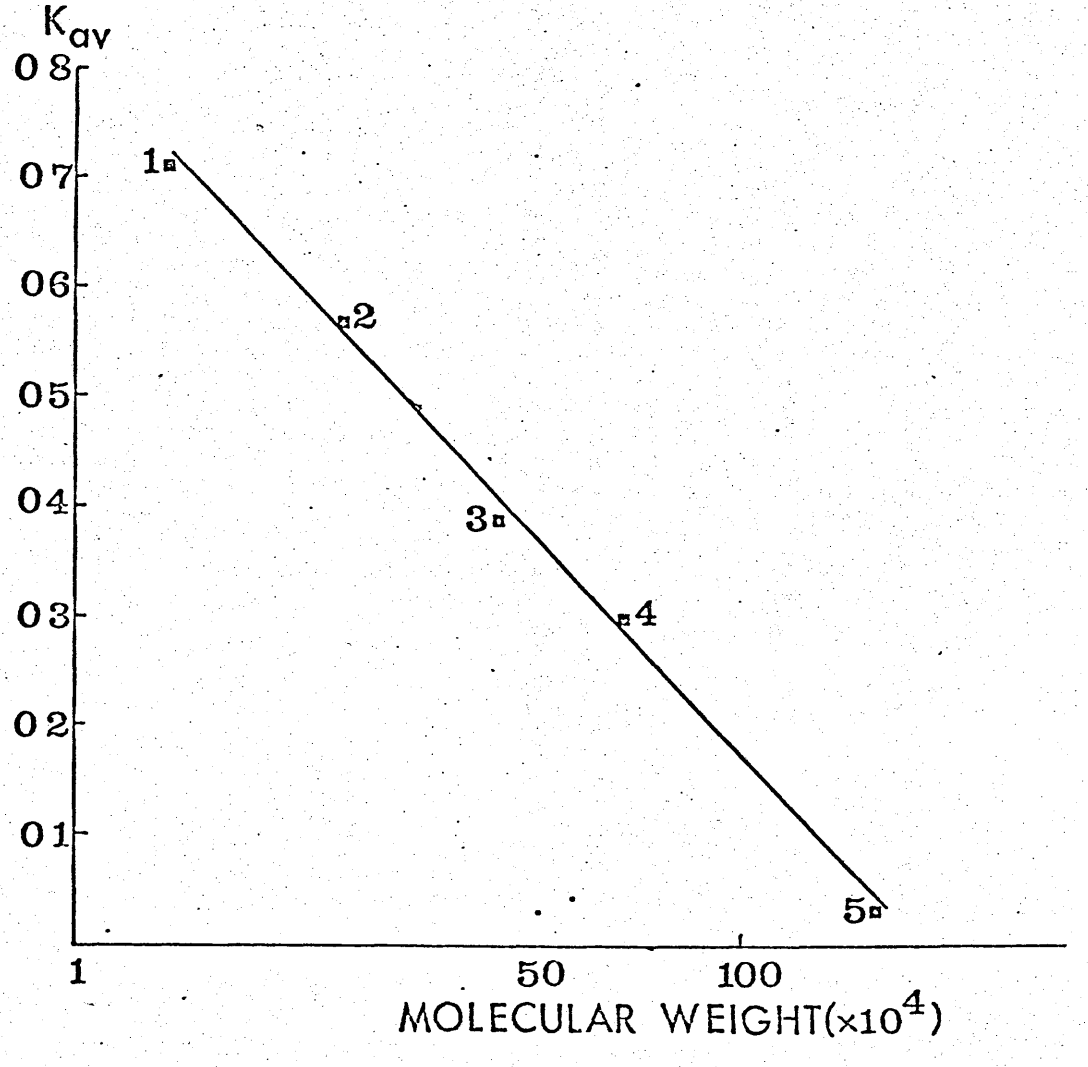

Fig 4 xxiii Calibration line for Sephacryl S200 Chromatography column. The calibrating proteins used were:-

1)	Ribonuclease A	MW 13 700
2)	Chymotrypsinogen A	" 25 000
3)	Ovalbumin	" 43 000
4)	Albumin	" 67 000
5)	Aldolase	" 158 000

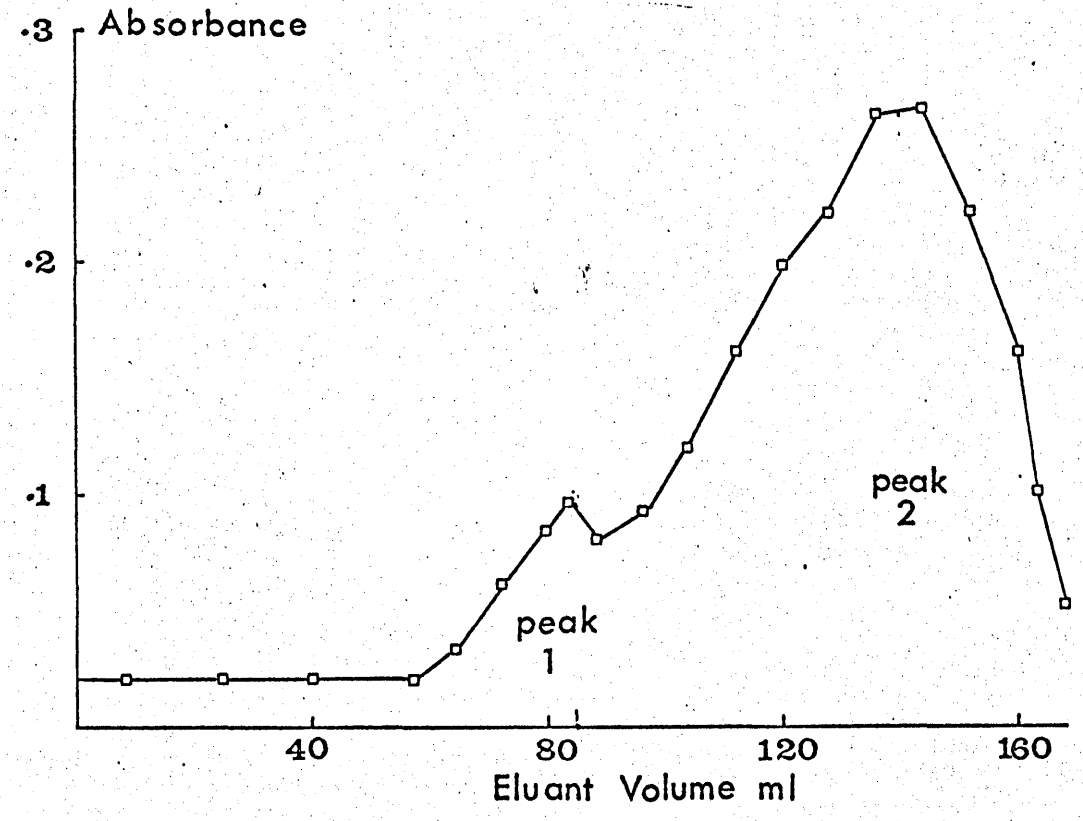

Fig 4 xxiv The elution profile of SP_1 from pooled pregnancy serum applied to Ultragel ACA 34 column.

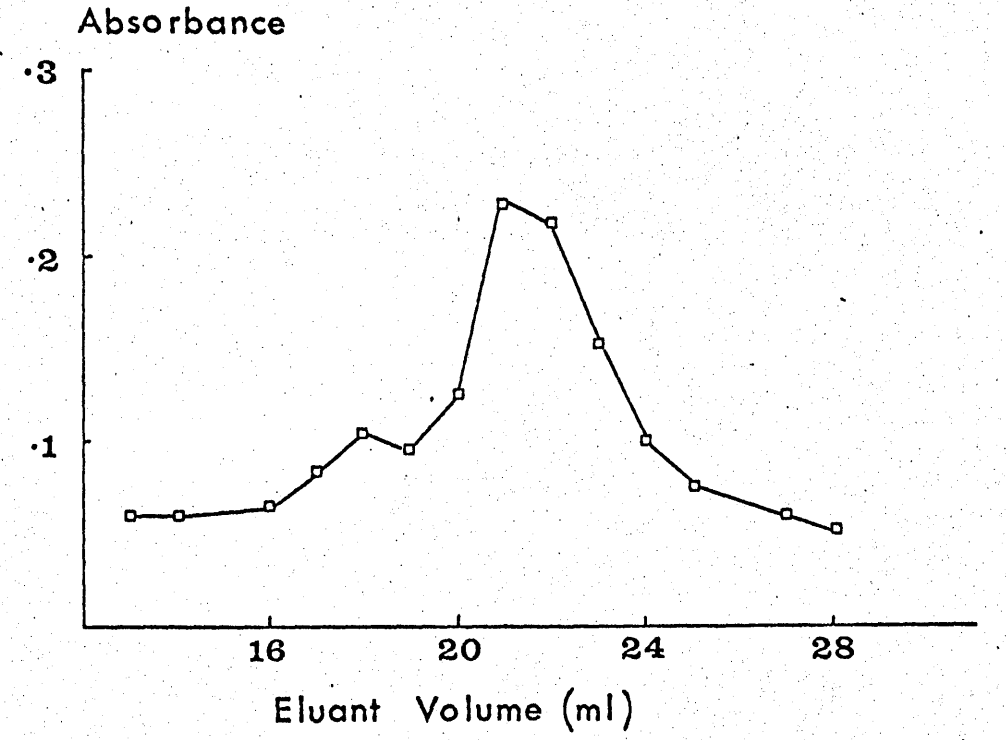

Fig 4 xxv The elution profile of SP_1 from peak 1 of the Ultragel column applied to Sephacryl S200 column.

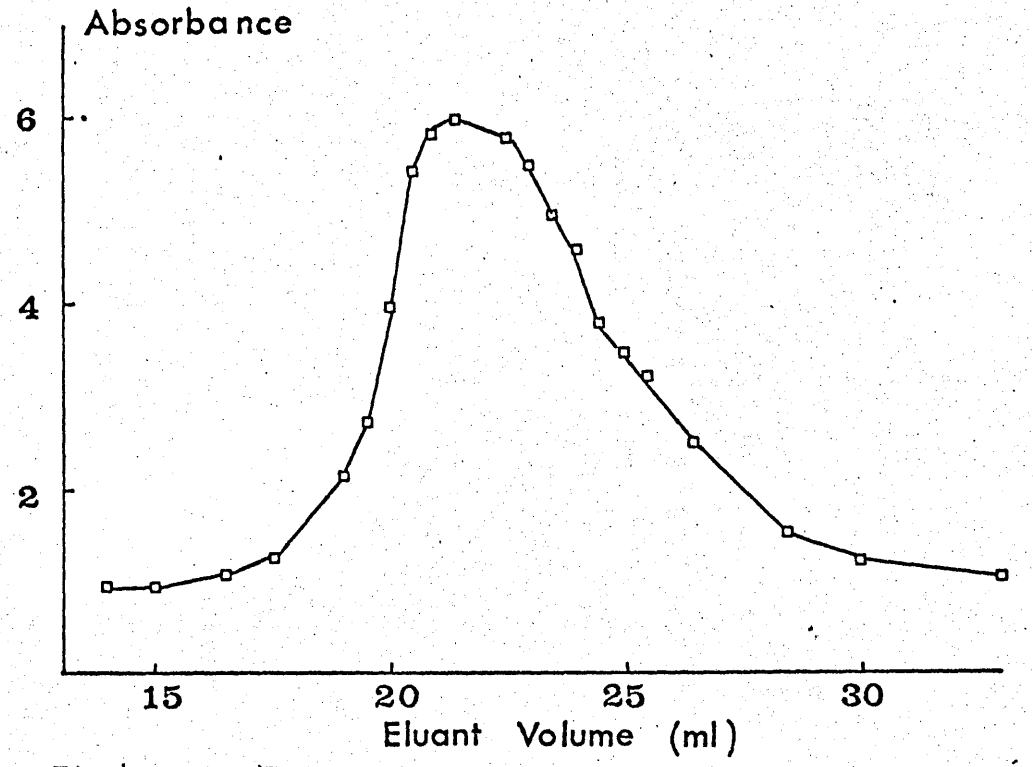

Fig 4 xxvi The elution profile of SP_1 from peak 2 of the Ultragel column applied to Sephacryl S200 column.

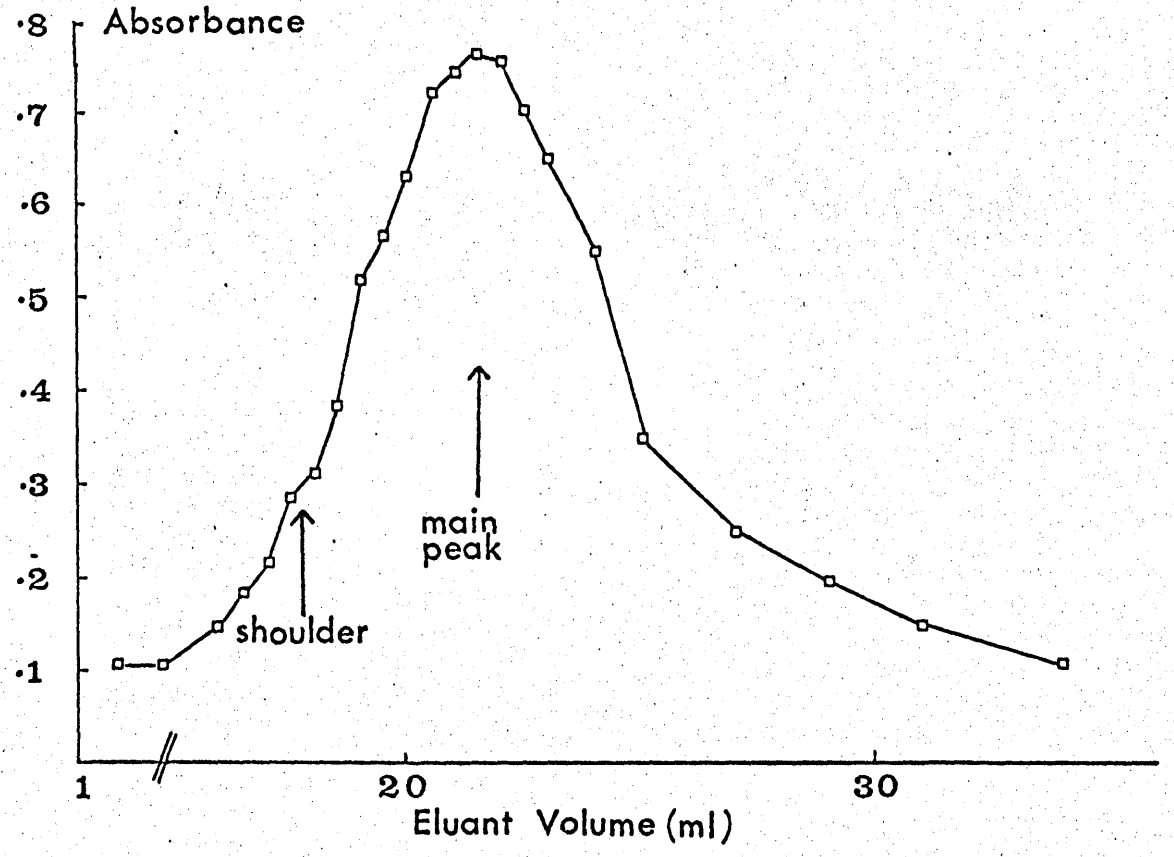

Fig 4 xxvii The elution profile of SP_1 from pooled pregnancy serum applied to Sephacryl S200 column.

4.10.3 Separation of $SP_1\alpha$ and $SP_1\beta$ by chromatography on hydroxyl apatite

Materials and Methods - A column measuring 150 mm x 9 mm was packed with hydroxyl-apatite and equilibriated with 0.005 mol/l potassium dihydrogen phosphate pH 6.8. 200 µl of pooled pregnancy serum was applied to the column, and eluted in the same buffer. The protein content of the eluant was monitored at 280 nm and when the trace returned to the baseline, the eluting buffer was changed to 0.2 M potassium dihydrogen phosphate pH 6.8 and further protein eluted from the column. 1 ml fractions were collected throughout and the SP_1 content of these fractions assessed by ELISA.

4.10.4 Estimation of molecular weights of SP_1 moieties

Calibration of the gel filtration columns was carried out with the use of a protein calibration kit purchased from Pharmacia Chemicals London. After assessing the column void volume by the use of Dextran Blue 6000, the elution volume of each protein was measured and Kav calculated as follows:-

$$Kav = \frac{\text{Elution Volume - Void Volume}}{\text{Column Total Volume - Void Volume}}$$

A calibration line of Kav against $\log_{10}$ molecular weight was made from which the molecular weight of the SP_1 moieties could be estimated (Fig 4 xxii, 4 xxiii).

4.10.5 Results

Fig 4 xxiv shows the SP_1 elution profile from pregnancy serum applied to the Ultragel column. Peaks 1 and 2 were then run separately on the Sephacryl S200 column for improved resolution (Fig 4 xxv and 4 xxvi). The elution profile obtained when pooled pregnancy serum was applied to the Sephacryl column is shown in Fig 4 xxvii.

Table 4 XIII

Kav Values and Molecular Weights of $SP_1 \alpha / \beta$ Separated on Ultragel ACA 34 and Sephacryl S200 as shown in Figs 4 xxiv to 4 xxvii.

Column	Material Loaded	Peak 1		Peak 2	
		Kav	MW	Kav	MW
Ultragel ACA 34	2ml Pooled Pregnancy Serum	0.24	370,000	0.73	84,000
Sephacryl S200	0.3 ml Pooled Pregnancy Serum	0		0.19	95,000
"	Peak 1 from Ultragel column	0.06	180,000	0.18	98,000
"	Peak 2 from Ultragel column	-	-	0.11	125,000

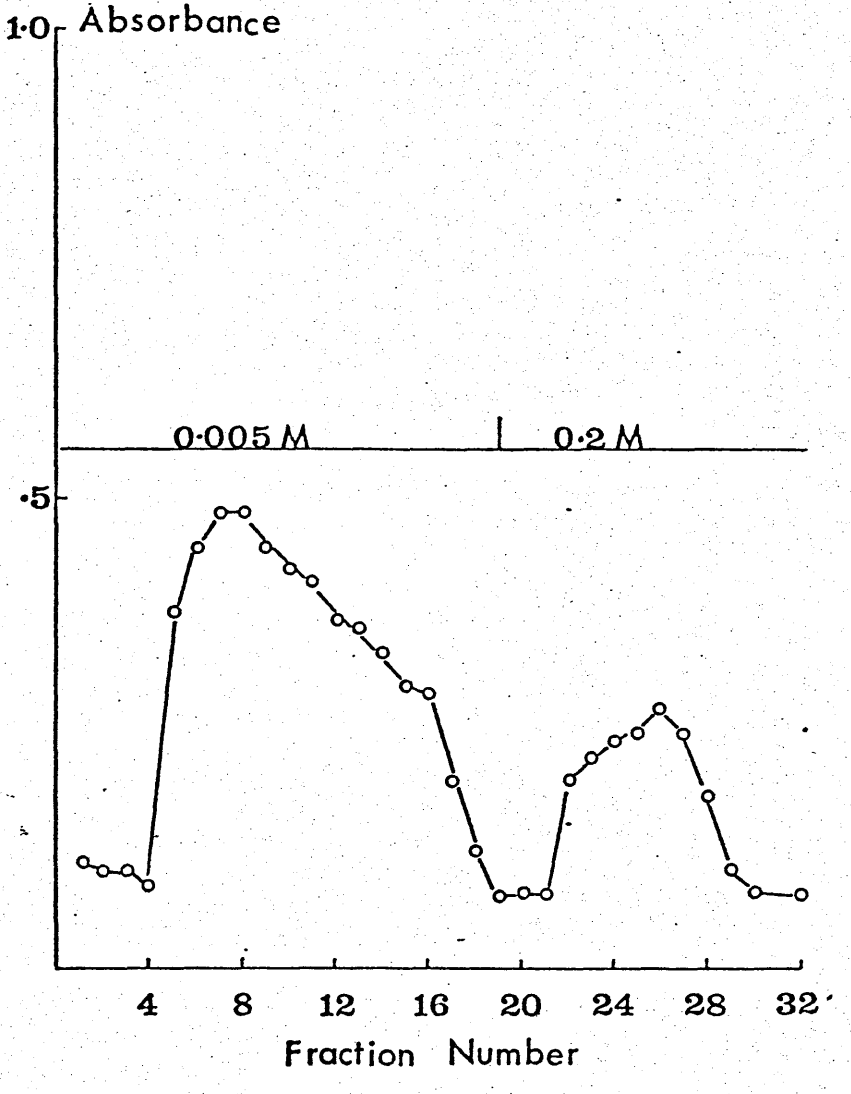

Fig 4 xxviii The elution profile of SP_1 from pooled pregnancy serum applied to hydroxyl-apatite column and eluted with increasing molarity buffer as shown.

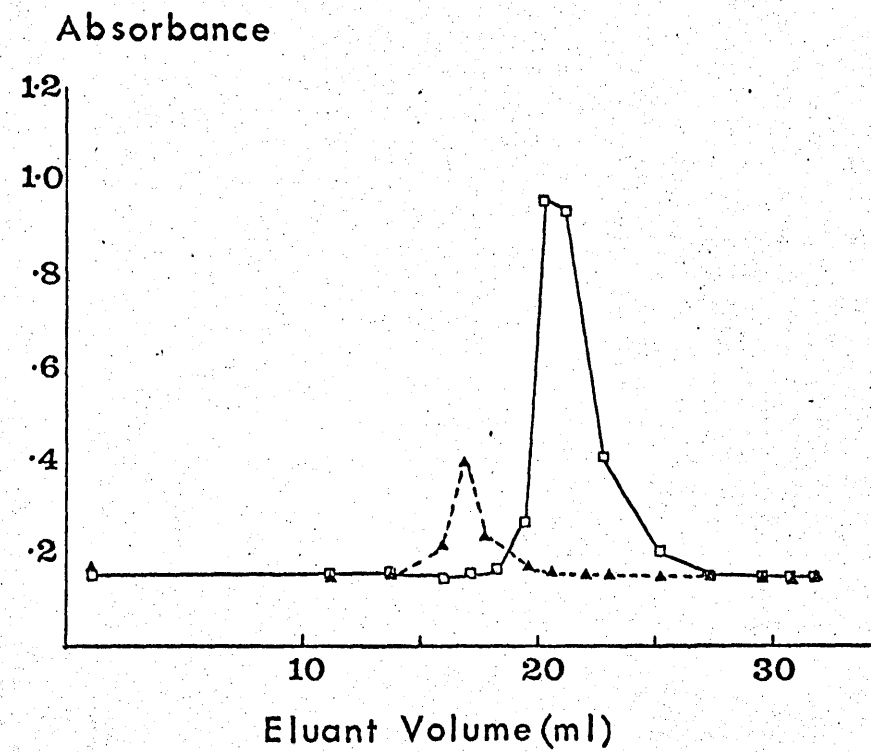

Fig 4 xxix The elution profile of SP_1 peaks obtained from hydroxyl apatite column applied to Sephacryl S200 column.
(▲——▲) represents $SP_1\alpha$ and (□——□) $SP_1\beta$.

The molecular weight estimates of the respective peaks are given in Table 4 XIII.

Figure 4 xxviii shows the elution profile of SP_1 from the hydroxy apatite column. Several column runs were performed and pools of each peak prepared,

4.10.6 Confirmation of Separation of $SP_1\alpha$ and $SP_1\beta$ - Method

Pooled fractions from the two peaks obtained by hydroxylapatite chromatography. After concentration against PEG 6000, 200 µl of the peak materials were applied in separate chromatographic runs to the calibrated Sephacryl S200 column and eluted with PBS. The SP_1 content of the eluant fractions was measured by ELISA.

4.10.7 Results

The results of the SP_1 concentrations of the eluant fractions obtained from the Sephacryl S200 column for each pooled material are shown in Fig 4 xxix. The Kav for each peak on the Sephacryl column was calculated as follows:-

Column Total Volume = 38.2 ml

Void Volume (assessed by Dextran Blue) = 16.5 ml

Peak 1 from hydroxyl-apatite column

$$Kav = \frac{20.5 - 16.5}{38.2 - 16.5} = 0.18$$

From the calibration curve for this column, this corresponds to a molecular weight of 98,000.

Peak 2 from hydroxyl-apatite column - eluted in the void volume and it is thus not possible to assign a molecular weight to this component, other than to state that it is in excess of 160,000.

Neither peak however showed evidence of a secondary SP_1 protein on the Sephacryl separation. It appears, therefore, that the hydroxyl-apatite column chromatography has achieved a clean separation of $SP_1\alpha$ from $SP_1\beta$.

4.10.8 Discussion

Various methods for the separation of $SP_1\alpha$ from $SP_1\beta$ have been proposed. Thus gel filtration using a variety of gels has been described (Ahmed and Klopper 1980, Ahmed, Toop and Klopper 1981, Hindersson et al 1981). The results obtained here however suggest that gel filtration is not the ideal method of separation. Although the β component can be obtained apparently free from $SP_1\alpha$, the reverse is not true and the $SP_1\alpha$ preparation was contaminated by $SP_1\beta$ as evidenced by the double peak obtained on further chromatography of the ACA 34 fraction.

The use of Sephacryl S200 in the separation of $SP_1\alpha$ and β from serum had been reported (Griffiths and Godard 1981, Ahmed and Klopper 1980). Ion exchange chromatography has also been utilized (Ahmed et al 1981, Ahmed and Klopper 1980). However a later statement by this group suggests that in fact they did not achieve a clean separation by any of these techniques - in accordance with the present findings.

The use of hydroxyl apatite reverts to the original work on this protein (Bohn 1972) and was also used by Ahmed and Klopper (1982). Using Laurell rockets as their method of monitoring, they suggested this material allowed clean separation of the moieties. This method of assessing resolution has been criticised (Teisner and Grudzinskas 1982). The use of ELISA as a monitor however gives similar findings in a more objective assay. The molecular weight estimates of 96,000 and $>$ 160,000 are in agreement with the known facts about these moieties. Controversy however

exists as to the exact nature of the components. Klopper's group suggest a single high molecular weight component (MW 430,000) (Ahmed and Klopper 1980). However Hindersson and Teisner (1981) have adduced evidence that an intermediate moiety exists with MW 200,000. The exact situation is not of direct importance to the present study. The question that remains is the way in which the various moieties are recognised by the antiserum used in ELISA.

4.10.9 Measurement of $SP_1 \alpha$ and $SP_1 \beta$ by ELISA - Materials and Methods

$SP_1 \alpha$ and β were prepared from pooled pregnancy serum on hydroxyl apatite columns as previously described. Four series of specimens were investigated:-

a) $SP_1 \alpha$ alone in varying concentrations. The following volumes of SP_1 (100 µl, 200 µl, 300 µl, 400 µl and 600 µl) were made up to a volume of 1000 µl with the requisite volume of PBS.

b) $SP_1 \beta$ alone in varying concentrations - prepared as in a).

c) $SP_1 \beta$ constant at 20% of final volume and $SP_1 \alpha$ varying between 0 and 60% of the final volume.

d) $SP_1 \alpha$ constant at 20% of final volume and SP_1 varying between 0 and 60% of the final volume.

The method of assay used was that of Macdonald et al (1979) as described in Chapter 3.

4.10.10 Results

Fig 4 xxx shows the results for $SP_1 \alpha$ alone at varying concentrations, and Fig 4 xxxi shows the results for $SP_1 \beta$ alone. Measurement of $SP_1 \beta$ with and without $SP_1 \alpha$ is shown in

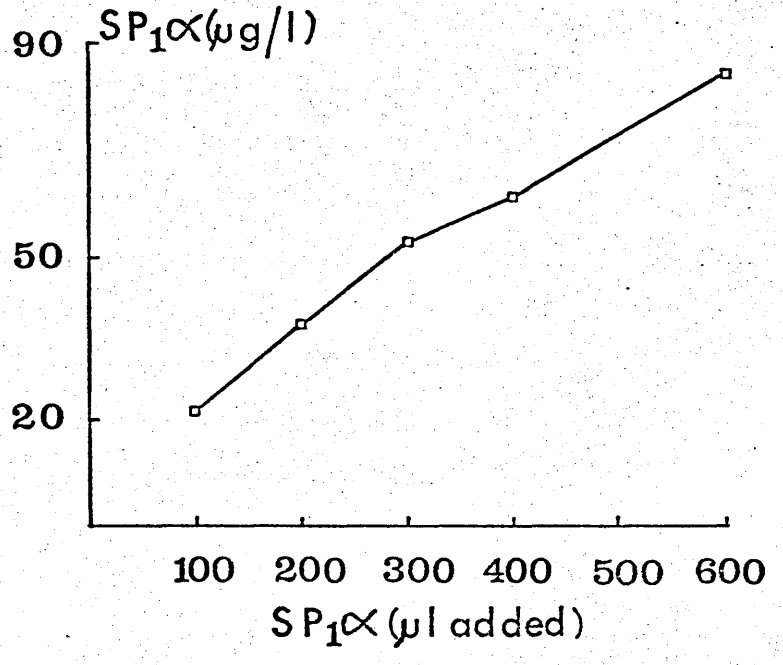

Fig 4 xxx The assay of $SP_1\alpha$ by ELISA.

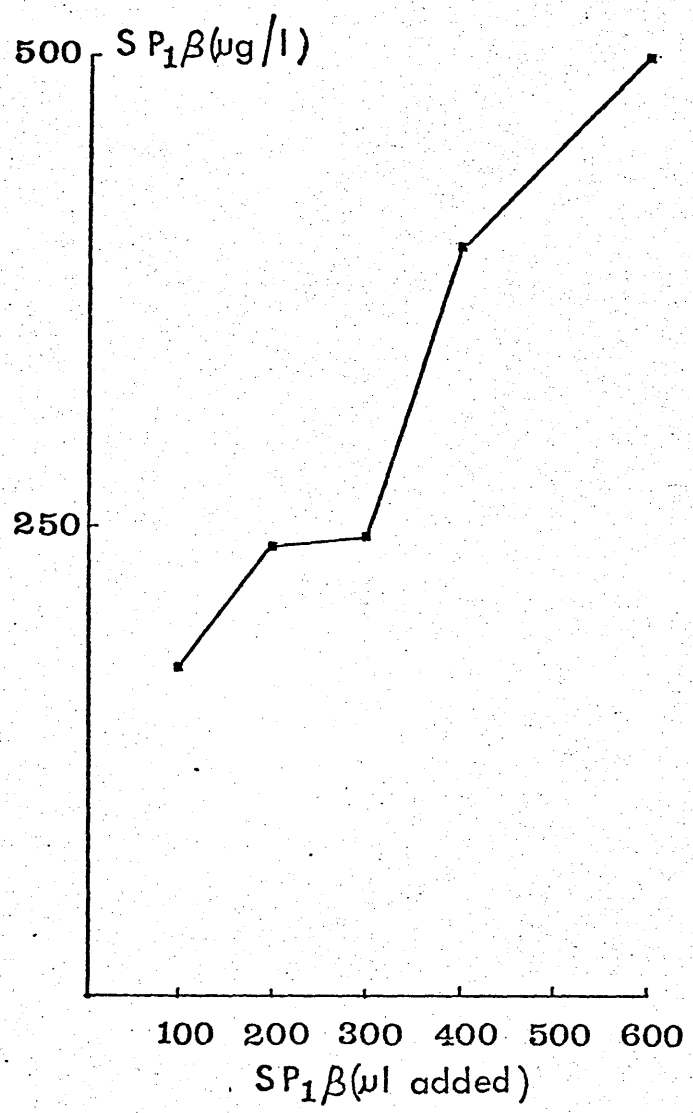

Fig 4 xxxi The assay of SP_1 β by ELISA,

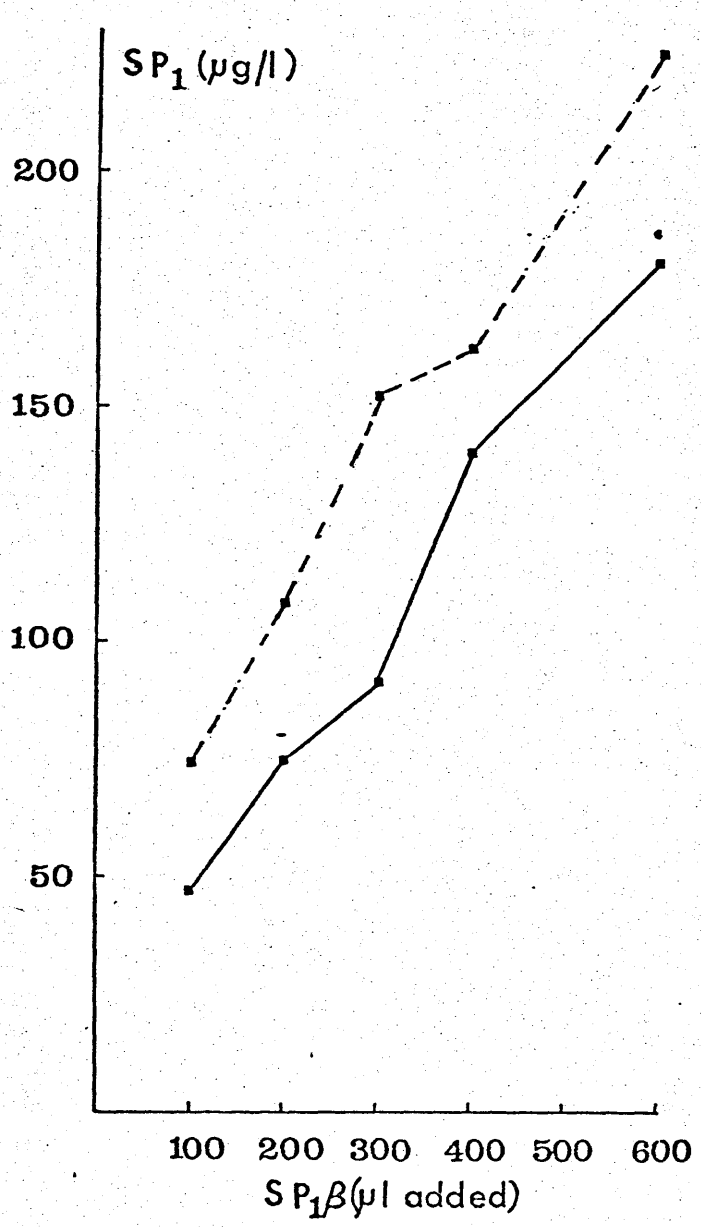

Fig 4 xxxii The measurement of $SP_1\beta$ in the presence of $SP_1\alpha$ (■----■) and without addition of $SP_1\alpha$ (■——■).

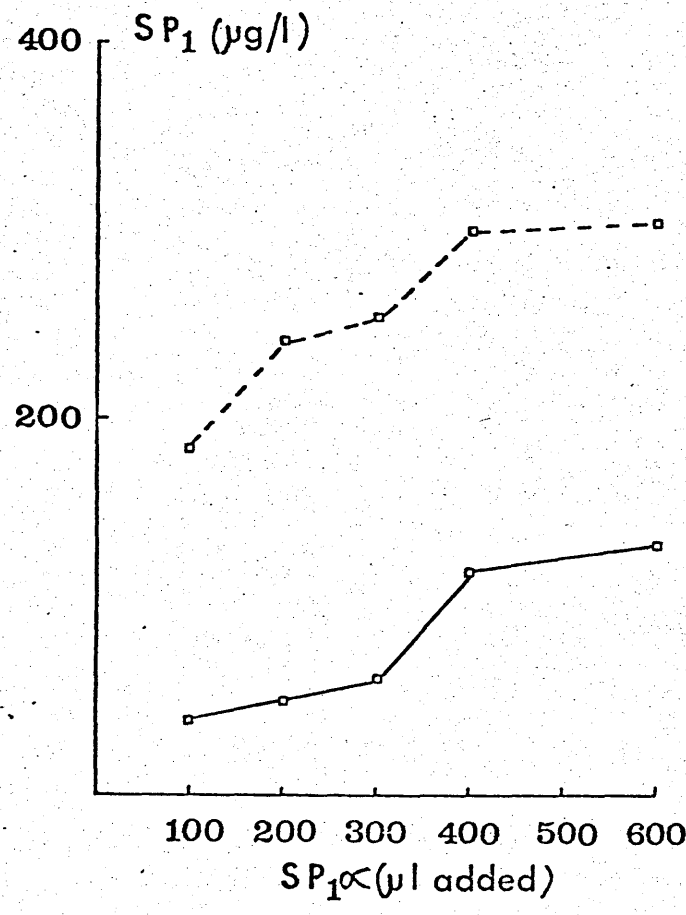

Fig 4 xxxiii The measurement of $SP_1 \alpha$ in the presence of $SP_1 \beta$ (□----□) and without addition of $SP_1 \beta$ (□——□).

Fig 4 xxxii. Addition of $SP_1 \alpha$ causes an increase in the measured SP_1. There is a suggestion that at higher levels of $SP_1 \beta$, the addition of $SP_1 \alpha$ causes a significantly greater increase in the $SP_1 \beta$ reading ie an apparently higher overall SP_1 total. The effect of measuring $SP_1 \alpha$ with and without $SP_1 \beta$ is shown in Fig 4 xxxiii. Again this does not seem to be a purely additive effect ie the addition of 100 µl of SP_1 caused an increase in the total SP_1 of 95 µg/l. However the addition of 600 µl of $SP_1 \alpha$ caused an increment of 140 µg/l in the apparent SP_1 total.

4.10.11 Discussion

With the discovery of the heterogenous nature of SP_1 (Teisner et al 1978) it became apparent that the reaction of these components with antisera varied. Thus Towler et al reported problems encountered in measuring urinary SP_1 by RID, and RIA (1978). Thereafter a number of publications followed along similar lines (Teisner et al 1979, Schultz-Larsen et al 1979, Ho and Jones 1980, Dati et al 1982, Ahmed et al 1982 b). All authors are in agreement that the heterogeneous nature of SP_1 is reflected in its assay methods. Thus to visualize $SP_1 \alpha$ peaks on Laurell rocket electrophoresis it is necessary to incorporate PEG 6000 into the gel (Hindersson 1981). The problem however is complicated by the fact that SP_1 variants give differing dose-response curves in RIA and seem to cause artificially low SP_1 levels when measured by this technique (Schultz-Larsen et al 1979). It is shown elsewhere in this study that whilst a significant correlation exists between SP_1 concentration determined by ELISA and RID, the slope of the regression line was only 0.32. This difference

is perhaps explainable in the light of results obtained in these experiments. Ahmed et al postulate that their findings for the differing slopes in RIA and EIA of SP_1 are explained by two extreme forms within a population of SP_1 antibodies.(Ahmed et al 1982). Their explanation depends on both forms binding SP_1 α and β but differing in their affinities for SP_1 β. Thus in a competitive binding such as RIA or the EIA used in Ahmed's work the findings can be explained.

However, the ELISA used here is not of the competitive type. The fact that the results obtained are similar to those of Ahmed suggests that the hypothesis as given is incorrect. It is difficult to see how the results can be explained in terms of displacement from low affinity antibodies as suggested for competitive systems.

However it is unlikely that the antibody used for coating the plates is sufficient to bind all SP_1 molecules present. Thus a degree of competition may exist at this stage of the assay. It remains to be seen if measurement of the individual components will be useful in clinical practice.

CHAPTER 5

AUTOMATED CURVE FITTING IN ELISA SYSTEMS

5.1 Introduction

Although ELISA is a versatile technique applicable to measurement of a wide variety of antigens (O'Beirne and Cooper 1979, Macdonald et al 1979, Pledger and Belfield 1983), to obtain maximum advantage from rapid analytical throughput, results should be calculated on-line. To achieve this, it is necessary to establish the mathematical relationship between colour produced and antigen concentration.

Whilst hand drawn curves may be suitable for small amounts of data, if the full potential of ELISA is to be exploited, automation of curve fitting will be necessary.

There are two stages of an ELISA which involve antigen-antibody reactions. Firstly antigen is incubated in the presence of immobilised antibody, secondly antibody enzyme conjugate is allowed to react with bound antigen. Neither reaction is allowed to proceed to equilibrium.

These two reactions were investigated in an ELISA for SP_1 (Macdonald et al 1979). Initially an investigation of the reaction between carcinoplacental alkaline phosphatase and its antibody was carried out. This is a simple system to investigate because the bound alkaline phosphatase retains its catalytic activity and no second indicator is needed.

Based on the experience of these experiments, computer programmes were designed to investigate the various methods of curve fitting.

5.2 Investigation of mathematical relationships for the antigen-antibody reaction

5.2.1 Materials

Antisera - Antiserum to pregnancy specific β_1 glycoprotein

(SP_1) and its conjugate with horseradish peroxidase, as detailed in Appendix I.

Anti-carcinoplacental alkaline phosphatase (anti-CPAP) was raised in rabbits as described previously (3.8.2).

Antigen - A standard preparation of SP_1 (OTFL 03), obtained from Hoechst Pharmaceuticals, was used in the experiments described below. Pooled pregnancy serum was used as a source of carcinoplacental alkaline phosphatase. This serum was heated to 56°C for one hour and then assayed in accordance with the recommendations of the Scandinavian Society for Clinical Chemistry (1974) using reagents obtained from Boehringer Corporation Ltd.

5.2.2 Time Course of antigen-antibody reactions - Methods

a) CPAP - anti-CPAP - A range of dilutions of pooled pregnancy sera were pipetted into the wells of microtitre plates (Nunc Grade I) previously coated with antibody. Each dilution was incubated for the the same time at room temperature and the enzyme activity measured using the alkaline phosphatase reagents. The colour produced was measured in a Titertek Multiskan.

b) SP_1 and anti-SP_1 - A range of solutions containing varying amounts of SP_1 were pipetted into the wells of microtitre plates previously coated with anti-SP_1. These solutions were incubated at room temperature for times ranging from 10-80 minutes. After each incubation period the SP_1 standards were replaced by the anti-SP_1 peroxidase conjugate and allowed to

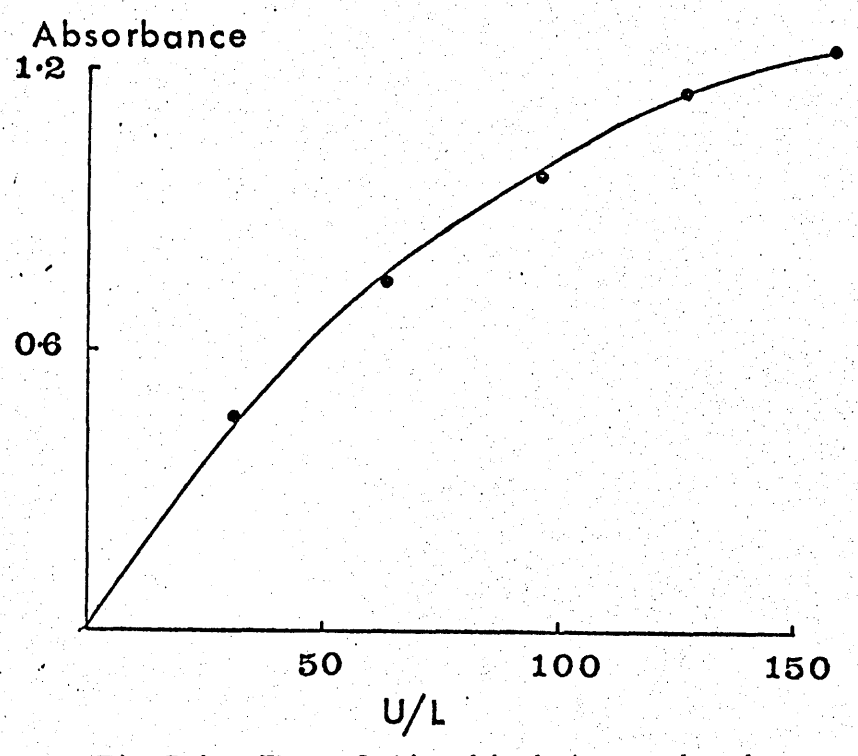

Fig 5 i The relationship between absorbance of chromophore and enzyme activity of CPAP bound to immobilized antibody.

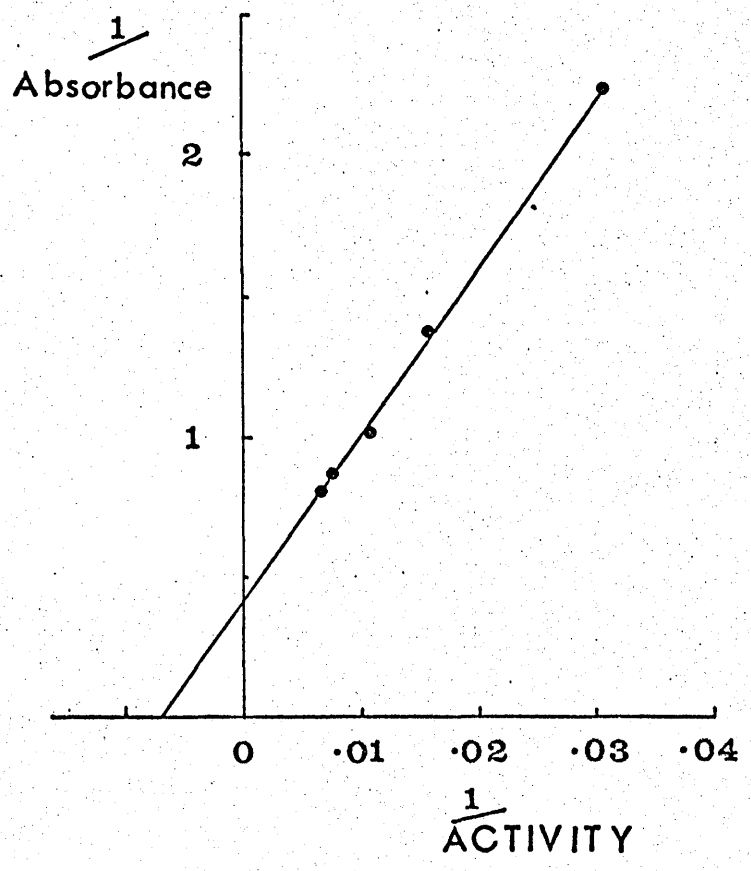

Fig 5 ii Reciprocal plot of the data shown in Fig 5 i.

incubate for 30 minutes. After incubation with the antibody-peroxidase conjugate, chromogen was added and the colour produced measured in a Titertek Multiskan.

c) Immobilised SP_1-antibody enzyme - A range of SP_1 standards were pipetted into the antibody coated wells of microtitre plates and incubated for a constant time, 30 minutes. After incubation the SP_1 solutions were replaced by anti-SP_1 peroxidase conjugate. The time of incubation with anti-SP_1 peroxidase conjugate was varied from 10-80 minutes after which the colour was developed and measured in the manner described above.

5.2.3 Results

Reaction between alkaline phosphatase and its antibody - An apparently hyperbolic relationship was observed between increasing amounts of immobilised alkaline phosphatase activity and colour produced (Fig 5 i). When the reciprocal absorbance was plotted against reciprocal alkaline phosphatase activity a straight line was produced (Fig 5 ii).

5.2.4 Time course of SP_1-anti-SP_1 reaction

At all concentrations of SP_1 examined, the amount of colour development increased with duration of incubation. A maximum colour development was not achieved although the rate of increase diminished with increasing time.

5.2.5 Reaction between antibody bound SP_1 and antibody enzyme

The amount of colour produced increased with duration of incubation but again no maximum colour development was attained

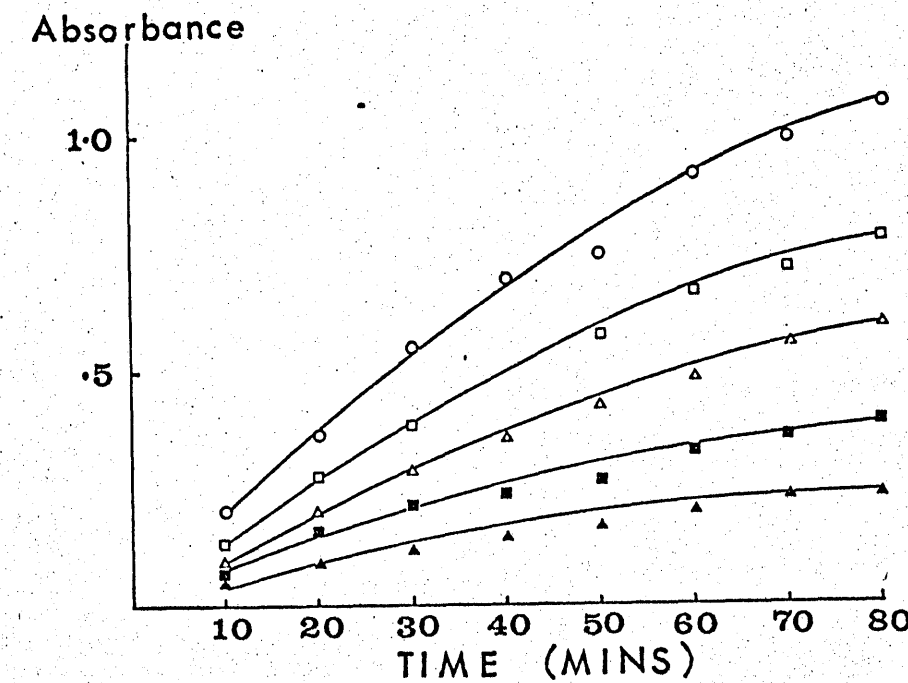

Fig 5 iii Time course of reaction between antibody-enzyme conjugate and SP_1 bound to immobilized anti SP_1 at differing antigen concentrations (O——O) 1000 µg/l, (□——□) 250 µg/l, (△——△) 125 µg/l, (■——■) 62.5 µg/l, (▲——▲) 31.25 µg/l.

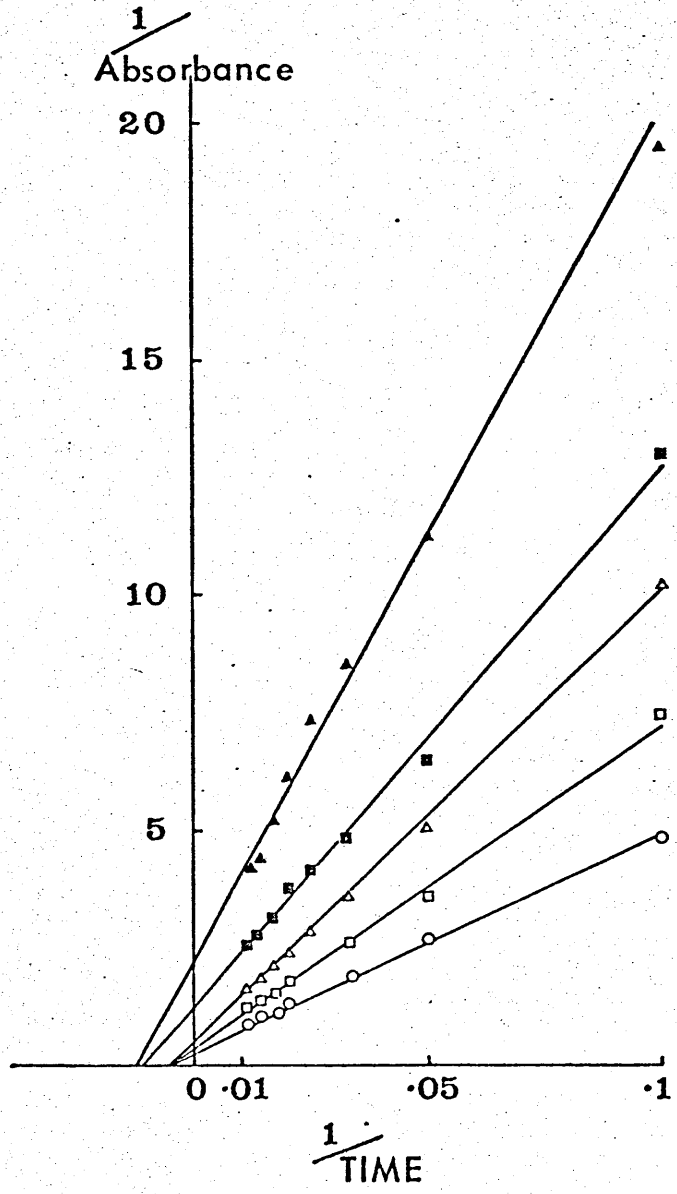

Fig 5 iv Reciprocal plot of the data shown in Fig 5 iii.

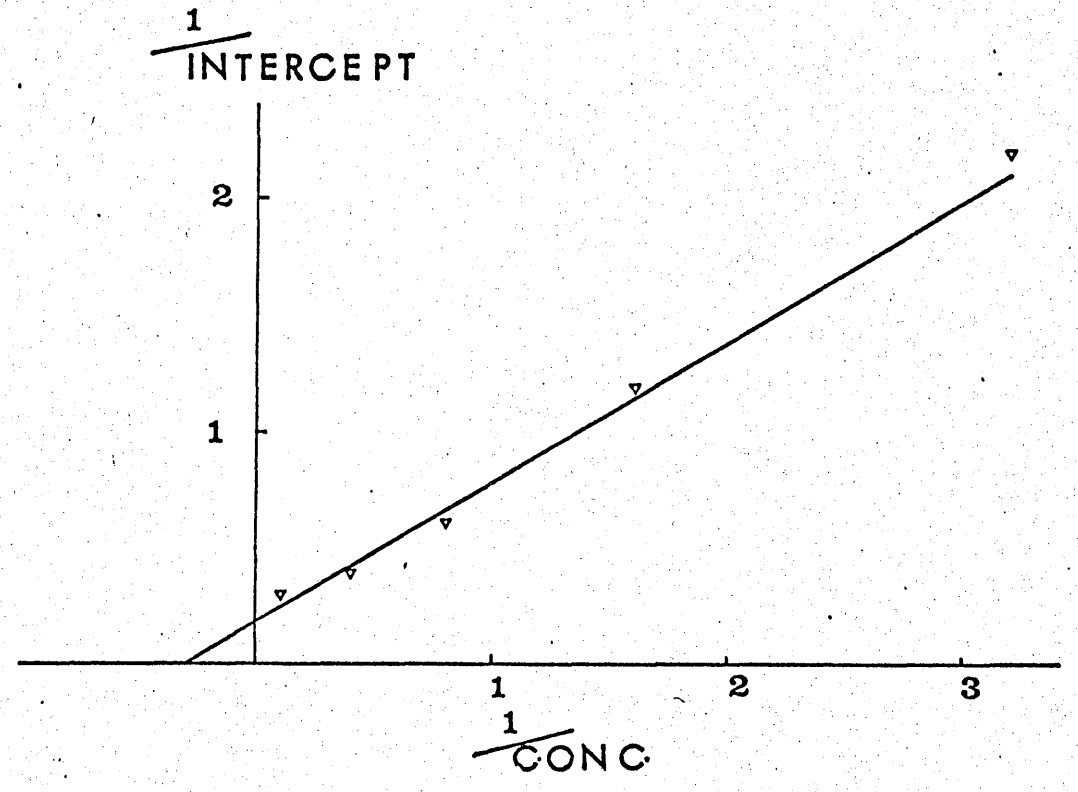

Fig 5 v Reciprocal plot of theoretical maximum absorbance against SP_1 concentration.

(Figure 5 iii). Inspection of the curves suggested an hyperbolic relationship between colour produced and time of incubation. A reciprocal plot of absorbance against time revealed a family of straight lines at each SP_1 concentration (Figure 5 iv) with an intercept corresponding to the reciprocal of the theoretical maximum colour production at that concentration. The intercepts on the vertical axis were replotted against reciprocal concentrations and again a linear relationship was demonstrated (Figure 5 v).

5.2.6 Discussion

Historically, workers employing radioimmunoassay have been concerned with the equilibrium between antigen and antibody, their main concern being to demonstrate that the behaviour of labelled antigen reflects accurately that of the unlabelled antigen which is to be quantified. In ELISA, reactions rarely proceed to equilibrium; careful timing of the reaction and a knowledge of the reaction kinetics are therefore important.

Consideration of the CPAP reaction is uncomplicated by the need for subsequent antibody reactions necessary to label bound CPAP so that it may be detected. Over the range of activities considered the results indicate an hyperbolic relationship between amount of enzyme bound (x) and colour produced (A):-

$$(A) = (A)_{max} \frac{x}{x + const}$$

This equation is analogous to the Hill equation describing the relationship between oxygen pressure and myoglobulin bound oxygen (Wharton and Eisenthal 1981).

The SP_1 assay system is more complex. Antibody-antigen reactions occur in the preliminary capture of antigen and in the sub[illegible] labelling with the antibody-enzyme conjugate.

The reciprocal plots (Figure 5 iii) of absorbance against time enable a theoretical maximum optical density of each antigen concentration to be obtained from the intercept on the vertical axis. This intercept, when replotted against reciprocal concentration (Figure 5 iv), confirms the hyperbolic relationship demonstrated for the CPAP-anti-CPAP reaction and demonstrate that the extent to which the reaction has proceeded towards equilibrium is independant of antigen concentration.

As the objective was to look for a simple numerical relationship between antigen concentration and colour produced, no attempt has been made to provide a theoretical justification for the observations. Over a wide range of antigen concentrations modifications of the affinities of the antibodies for the antigen may occur and the simple hyperbolic relationship may no longer hold. Where the hyperbolic relationship is obeyed, simple derivatives such as the log-logit transformation may form the basis for on-line computation of test results.

5.3 Data Reduction

5.3.1 Introduction

Based on the investigation of kinetics carried out previously a variety of methods were investigated which would allow automated curve fitting.

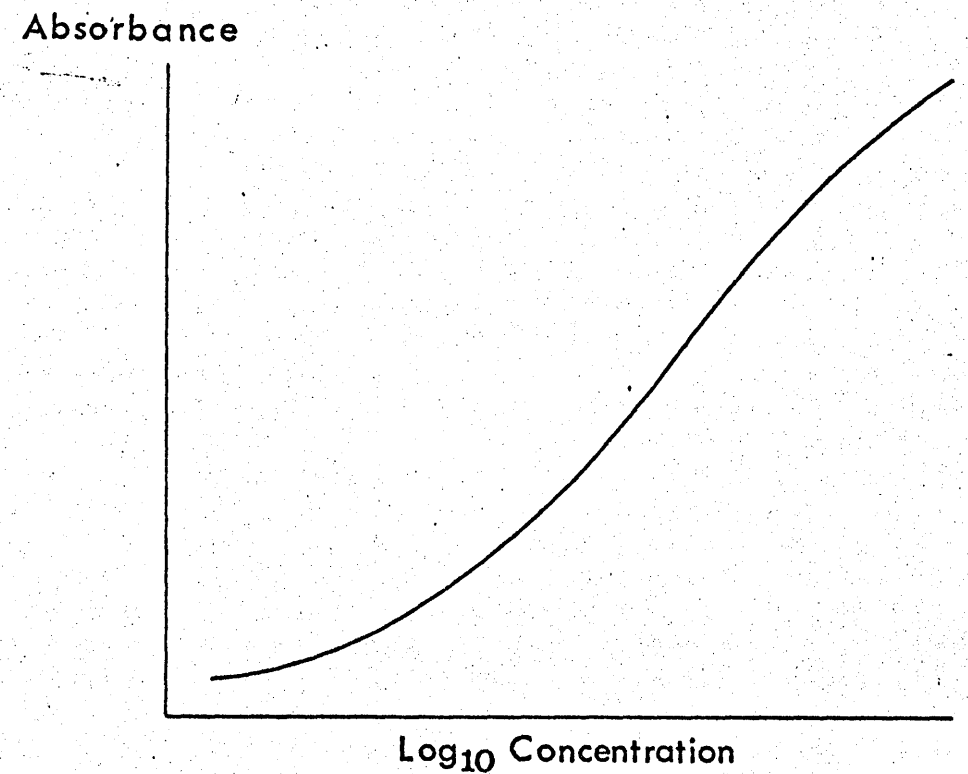

Fig 5 vi ELISA calibration curve type I.

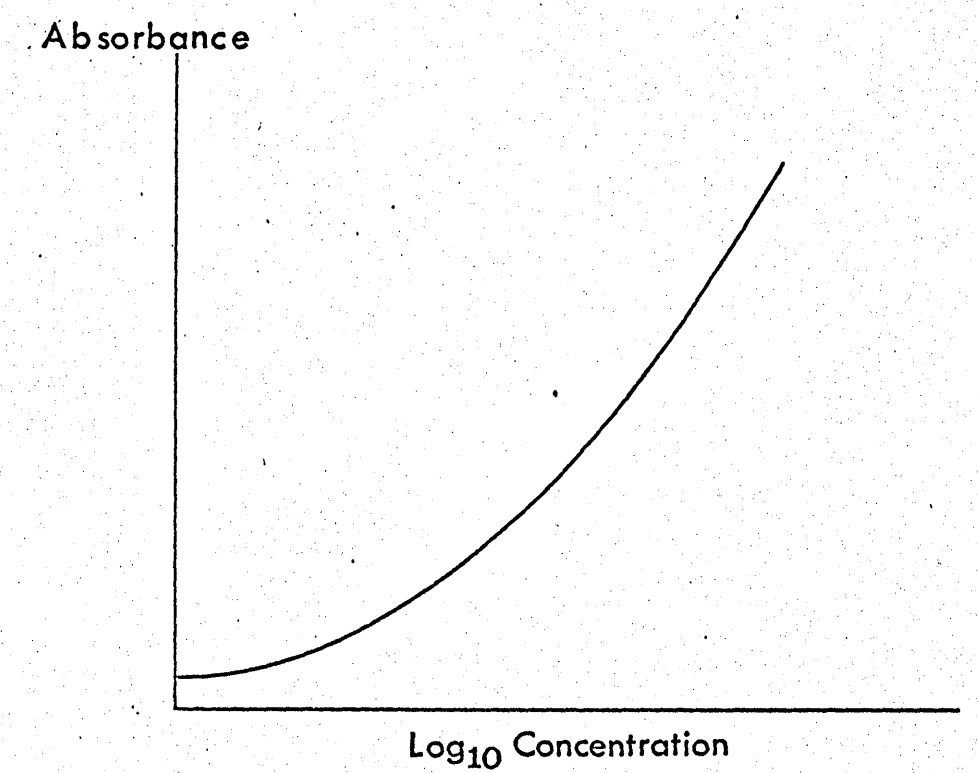

Fig 5 vii ELISA calibration curve type II.

Experience gained during this study suggests that ELISA calibration curves obtained by plotting absorbance against log concentration are of two main types (Figures 5 vi and 5 vii) and may reflect differing optimisation of assays. Each type of curve was considered for a variety of algorithms.

The direct type assay of CPAP when plotted as absorbance against concentration gave a straight line and is amenable to linear regression techniques and will not be discussed further.

5.3.2 Methods

Calibration curves obtained from ELISA for SP_1 and PAPP-A and calibration lines obtained from the immunoassay of CPAP were investigated. The ELISA system consisted of a Multiskan microtitre plate reader (Flow Laboratories, Irvine, Scotland) interfaced with a TNW 2000 interface to a Commodore PET Model 4032 with associated disc drives and printer. Using a data capture system supplied by Flow Laboratories a series of "BASIC" programmes were developed based on this system to allow curve fitting (Appendix 2). The following algorithms were considered. In each case the X axis represents absorbance (A) and the Y axis concentration (or log concentration). No weighting procedures were utilized.

a) For Type I curves (Sigmoid Curves)

i) Hand drawn curve plotted as absorbance against $\log_{10}$ concentration by an experienced operator using French Curves.

ii) Log-logit transform of the data followed by straight line regression of logit A against $\log_{10}$ concentration where logit A =

$$\log_e \frac{A}{1 - A}$$

iii) Healy equation of the form

$$Y = A + B \cdot \frac{\text{Exp}\ (C - D \cdot \log_e X)}{1 + \text{Exp}\ (C - D \cdot \log_e X)}$$

b) For Type II Curves (Parabolic Curves)

i) Hand drawn curve plotted as above.

ii) Log-logit transform as above.

iii) Quadratic equation of the form

$$Y = A\,x^2 + Bx + C$$

iv) Cubic equation of the form

$$Y = Ax^3 + Bx^2 + Cx + D$$

v) Power curve of the form

$Y = A\,x^b$ involving linearization to

$\log_e Y = b.\log_e x + \log_e A$ followed by linear regression.

Curve fitting was performed where applicable by solution of the simultaneous equations by derivation of the triangular matrix using the square root method (Kenney and Keeping 1951), and the Healy equation by Newton's reiterative process for complex equations.

Assessment of "goodness of fit" was made by estimation of the mean sum of residual squares (MRS) where

$$MRS = \frac{(\text{Calculated A} - \text{Actual A})^2}{N}$$

and n is the number of points in the standard curve. Statistical comparison of goodness of fit was made by means of a variance ratio (F) test on the MRS. To investigate the distortion patterns generated during curve fitting, absorbance values covering the entire range of a typical calibration curve were entered manually into the various curve fitting programmes.

Table 5 I

The Mean Residual Squares obtained from 10 assays for SP_1 (Sigmoid type curve)

Assay No	Hand Drawn Curve	Log logit transform	Healy Equation
1	1.8×10^{-3}	2.1×10^{-3}	2.9×10^{-4}
2	8.4×10^{-4}	6.0×10^{-4}	2.6×10^{-4}
3	3.5×10^{-3}	1.1×10^{-3}	2.7×10^{-4}
4	4.7×10^{-4}	1.0×10^{-3}	2.1×10^{-4}
5	3.1×10^{-4}	1.2×10^{-3}	8.6×10^{-5}
6	6.1×10^{-4}	1.3×10^{-3}	1.5×10^{-4}
7	7.2×10^{-5}	5.3×10^{-4}	6.9×10^{-5}
8	1.6×10^{-4}	9.3×10^{-4}	1.4×10^{-4}
9	1.1×10^{-4}	8.3×10^{-4}	7.1×10^{-5}
10	6.3×10^{-5}	9.2×10^{-4}	5.0×10^{-5}

Table 5 II

The Mean Residual Squares obtained from 10 assays for PAPP-A

(parabolic type curve)

Assay No	Hand Drawn Curve	Log-logit transform	Quadratic Equation	Cubic Equation
1	1.5×10^{-4}	1.8×10^{-3}	3.0×10^{-5}	9.1×10^{-5}
2	1.4×10^{-4}	1.1×10^{-3}	1.3×10^{-4}	5.1×10^{-4}
3	1.1×10^{-3}	1.2×10^{-3}	4.3×10^{-4}	1.4×10^{-4}
4	2.2×10^{-4}	1.1×10^{-3}	1.5×10^{-4}	3.5×10^{-5}
5	1.5×10^{-3}	1.2×10^{-3}	2.0×10^{-4}	1.3×10^{-4}
6	1.4×10^{-4}	9.3×10^{-4}	6.5×10^{-5}	5.4×10^{-5}
7	4.0×10^{-5}	1.1×10^{-3}	4.7×10^{-5}	2.2×10^{-5}
8	3.0×10^{-5}	1.7×10^{-3}	1.7×10^{-5}	1.7×10^{-5}
9	1.1×10^{-4}	1.1×10^{-3}	4.7×10^{-5}	3.4×10^{-5}
10	1.8×10^{-4}	7.0×10^{-4}	3.6×10^{-5}	3.4×10^{-5}

Table 5 III

Comparison of Goodness of Fit by F test for Type I Curve

Assay No	F= Log Logit/ Hand Drawn	Healy/Hand Drawn	Healy/Log-logit
1	1.2	6.2	7.2
2	1.4	3.2	2.5
3	3.2	13.0 $p < 0.01$	4.1
4	2.1	1.9	4.8
5	3.9	3.6	14.0 $p < 0.01$
6	2.1	4.1	8.7
7	7.4	1.0	7.7
8	5.8	1.1	6.6
9	7.6	1.6	11.7
10	14.6 $p < 0.01$	1.3	18.4 $p < 0.01$

Table 5 IV

Comparison of Goodness of Fit by F test for Type II Curve

Assay No	F= Quadratic/ Hand Drawn	Cubic/Hand Drawn	Log-logit/ Hand Drawn
1	20.0 $p < 0.01$	1.7	12.0 $p < 0.01$
2	1.1	3.6	7.9
3	3.02	7.9	1.1
4	1.5	6.3	5.0
5	7.5	11.5 $p < 0.01$	1.3
6	2.2	2.6	6.6
7	1.2	1.8	27.5 $p < 0.01$
8	1.8	1.8	56.7 $p < 0.01$
9	2.3	3.2	10.0 $p < 0.01$
10	5.0	5.3	3.9

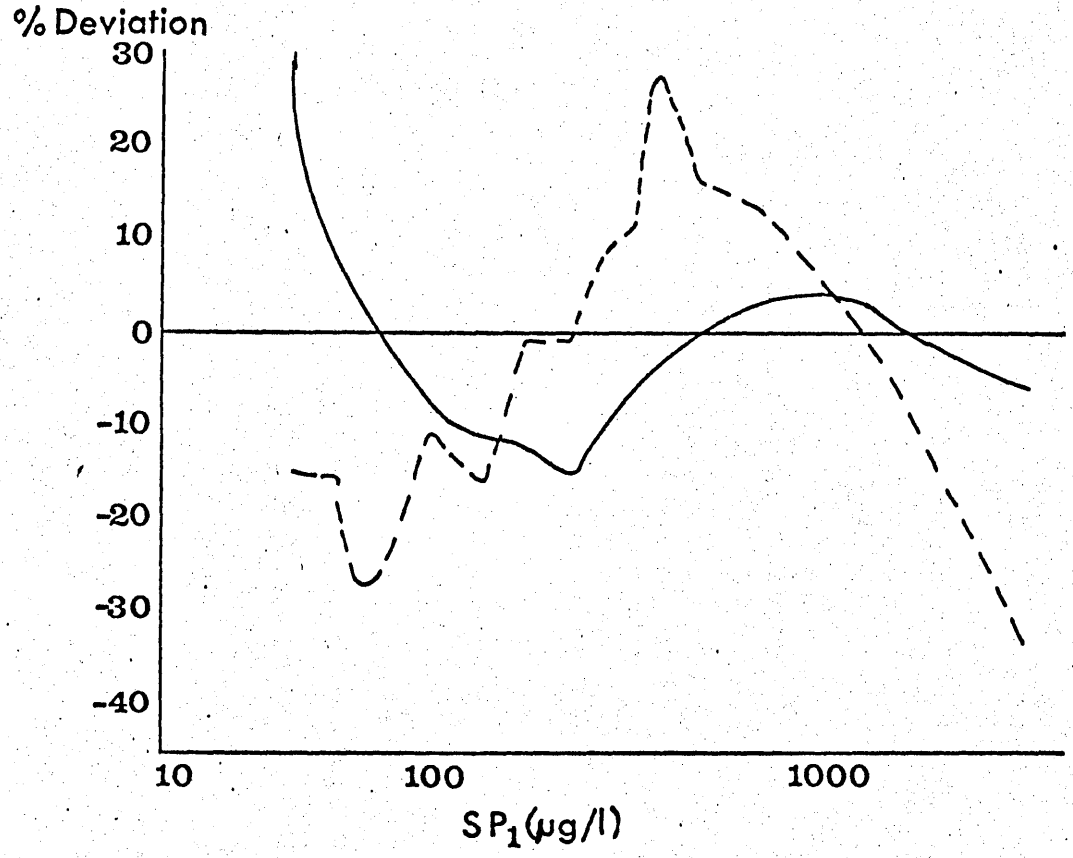

Fig 5 viii The distortion pattern generated by fitting a Type I curve by two different algorithms. 0% deviation represents the hand drawn curve, (———) the Healy fit and (– – –) the log-logit followed by linear regression.

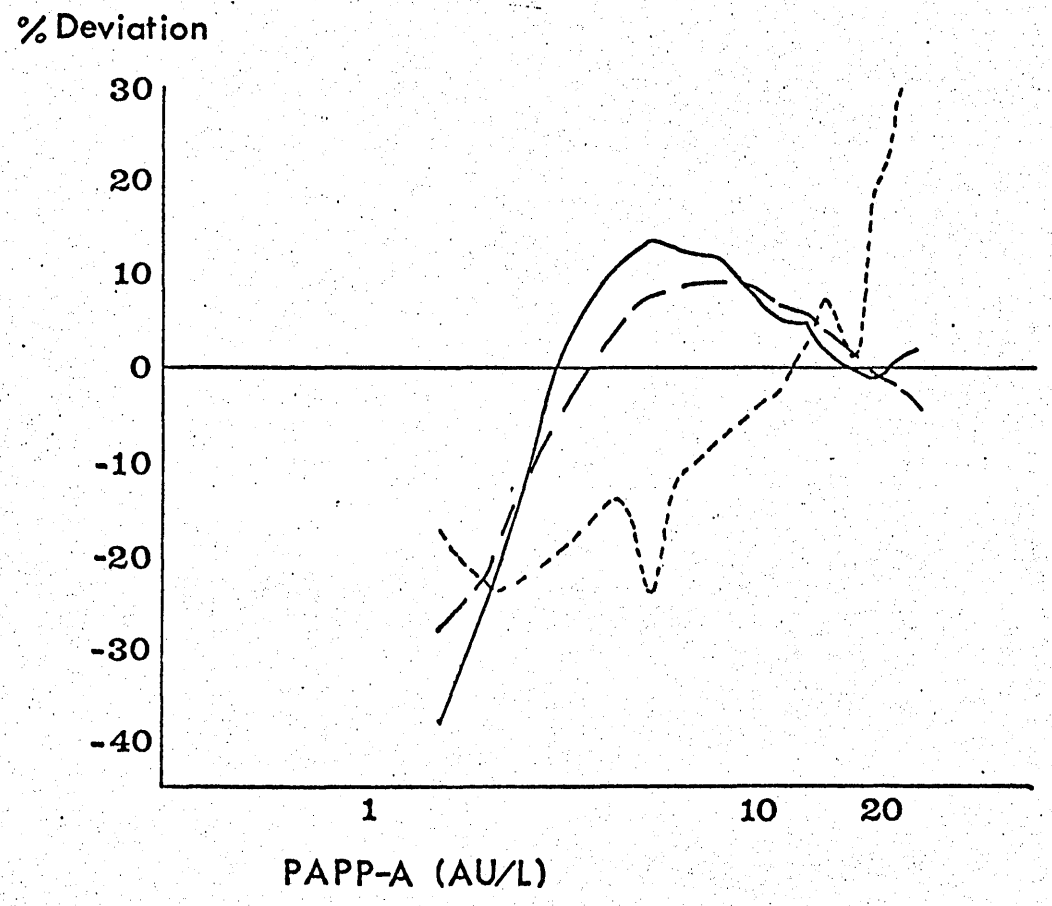

Fig 5 ix The distortion patterns generated by fitting a Type II curve by three different algorithms. 0% deviation represents the hand drawn curve, (———) cubic fit, (— — —) quadratic fit, (------) log-logit followed by linear regression.

Table 5 V

Mean and Standard Deviation of Percentage Distortion for Sigmoid Curves from the Distortion Patterns shown in Figure 5 viii.

	Mean % Distortion	Standard Deviation	Range of % Deviation
Healy/Hand Drawn	-2.5	7.14	-14.6 to 9
Log-Logit/Hand Drawn	+1.3	11.31	-15.8 to 16

(For the range of standards normally employed in the calibration curve)

Table 5 VI

Mean and Standard Deviation of Percentage Distortion for Parabolic Curves from The Distortion Patterns shown in Figure 5 ix.

	Mean % Distortion	Standard Deviation	Range of % Distortion
Quadratic/Hand Drawn	+1.1	10.5	-28 to +9.1
Cubic/Hand Drawn	+2.0	13.3	-38 to +13.7
Log-Logit/Hand Drawn	-6.5	11.0	-17.3 to +19

(For the range of standards normally employed in the calibration curve)

The computed concentrations obtained were expressed as positive or negative % deviation from the results obtained from the hand drawn curves. The mean and SD of the percentage deviations from the hand drawn results were calculated for the various algorithms over the range of standard concentrations normally present in the calibration curve.

5.3.3 Results and Discussion

Tables 5 I and 5 II show the results of the MRS obtained over 10 typical assays for each antigen and tables 5 III and IV show the results of the F tests on the MRS between various methods of fitting. The MRS for log-logit versus hand drawn curves was significantly worse on one occasion (Assay 10) and for Healy versus hand drawn was significantly better on one occasion (Assay 3). For type II curves both quadratic (Assay 1) and cubic (Assay 5) gave significantly lower MRS on these two assays. The power fit for type II curves provided totally unacceptable MRS values.

Figures 5 viii and ix show the distortion patterns generated by application of the algorithms compared with the results obtained from the hand drawn curves and tables 5 V and VI show the mean, standard deviation and range of % distortion from these figures. For type I curves, the Healy equation showed a smaller range of deviation and for type II curves, the smallest range of deviation was observed for the quadratic equation.

In order to automate ELISA systems, it is necessary to utilize suitable curve fitting techniques. For small amounts of data, it is common to use a manual plot of absorbance against log concentration and this type of plot is widely used in immunoassay as it provides a convenient way of handling the wide range of concentrations encountered.

It appears from table 5 I/III that in terms of goodness of fit, there is little difference in the fit achieved using the Healy or log-logit approach compared with a hand drawn curve. Taking the hand drawn curve as a baseline, either approach appears satisfactory.

However, complex distortion patterns were induced by both methods of fitting the curve (Fig 5 viii and ix). Major distortion in the Healy equation was observed only at values below those normally encountered in pregnancy, and over the standard range provided a reasonable fit. Maximal distortion of the log-logit results was observed at both extremes of the standard curve, but also a large positive distortion at the mid point of the curve.

From tables 5 I and 5 IV it appears that either quadratic or cubic equations may be used to describe the type II curve, whilst the log-logit approach differed from the hand drawn curve 40% of the time.

Again considerable distortion was engendered (Fig 5 iv). Whilst the pattern for quadratic and cubic fits was similar, that for the log-logit is more complex. For the polynomial fits, maximal distortion was observed only at lower standard values whilst the log-logit results showed distortion at both extremes.

The method of fitting chosen thus depends on assay conditions. Ideally the distortion imposed over a manually plotted curve should be minimal over the concentration range of interest and particualrly at action limits, where bias in calculating results could have serious implications. The distortion pattern may be reduced by suitable weighting factors (Frazier and Rodbard 1973; Rodbard and Lewald 1970). However, Challand points out

that although weighting will reduce distortion around the mid-point of the standard curve, it will not change the overall pattern (Challand et al 1976). Weighting was not applied in the present study. The variation in distortion patterns imposes one important constraint on the user. It is important that once an algorithm to describe the curve it decided upon, that this method is adhered to. Changing methods merely on the grounds of a reduced MRS will lead ultimately to poor precision and confusion in the results produced.

Providing these constraints are borne in mind, simple algorithms such as those decribed, may be used for data reduction in ELISA systems of this type.

CHAPTER 6

SERUM HPL, SP_1 AND PAPP-A CONCENTRATIONS DURING THE THIRD TRIMESTER OF NORMAL PREGNANCY

One of the milestones of the history of medicine has been the reduction of mortality and morbidity connected with pregnancy. With the virtual elimination of maternal mortality, attention has turned to ensuring optimum development of the foetus. The overall improvement in maternal health has greatly facilitated this. Problem pregnancies however still exist eg diabetic, hypertensive etc. The objectives of biochemical placental function testing are to alert the obstetrician to the possible development of problems affecting the foetus or conversely to issue reassurance viz. that foetal conditions are satisfactory. A variety of foetal or placental products have been used in this context for a number of years (Wilde and Oakey 1975). It is possible that the measurement of maternal concentrations of the "new" placental proteins may offer certain advantages over hPL measurements. This study investigates the use of SP_1 and PAPP-A in certain pregnancy associated problems.

6.2 Clinical Material

The normal group comprised patients attending routine antenatal clinics at the Royal Maternity Hospital. Patients attending these clinics prior to 25 weeks gestation were admitted to this survey after giving their informed consent. Thereafter patients were followed throughout the duration of their pregnancy. Venous blood specimens were drawn at each clinic attendance.

Following delivery, the baby was weighed and the undrained placenta also weighed. Retrospective classification into the normal group was made with the assistance of the consultant obstetrician whose clinic the patient attended.

The normal group was defined as patients having no evidence of pregnancy induced hypertension (PIH), oedema or proteinuria, no

maternal pathology and a satisfactory weight gain throughout pregnancy. The delivered baby had a birth weight > 10th centile corrected for maternal weight and height according to Thomson's figures (Thomson et al 1968). Ninety-one patients were included in this group.

6.3 Analytical Methods

Blood specimens were centrifuged and the serum separated from the clot as soon after collection as possible. Sera were stored at -20°C until assay. HPL, SP_1 and PAPP-A were assayed according to the methods described in chapters 2 and 3.

All specimens from an individual patient were assayed in the same batch to minimize assay variation. Specimens were assayed in duplicate and the mean concentration taken.

6.4 Numerical Analysis of Data

Standard statistical analyses of placental and birth weights were made to give a mean and standard deviation. Standard regression analysis of this data was also performed. Serum protein concentrations obtained from this group of patients were used to construct normal ranges. Although more than one specimen was obtained from some patients, all data points were considered independently.

Normal ranges were prepared in two ways. Initially ranges were expressed as centiles. However, further examination of the data suggested that serum SP_1 and PAPP-A concentrations were positively skewed. A logarithmic transformation was made before mean and SD were calculated for future use. HPL concentrations were normally distributed and untransformed data was used throughout.

Table 6 I

Characteristics of the normal population

a) Birth weight

	Corrected for anthropomorphic factors	Uncorrected
Mean Weight (kg)	3.35	3.41
Std Deviation	0.36	0.42
Number of Patients	91	91

b) Placental Weight

Mean Weight (g)	664
Std Deviation	121
Number of patients	67

c) Linear Regression

	Placental Weight with Corrected Birth Weight	Placental Weight with Uncorrected Birth Weight
Correlation Coefft.	0.49 ($p < .001$)	0.64 ($p < .001$)
Gradient	162	185
Y Axis Int	116	27.8
SE Grad	36.6	27.2
SE Int	123.6	94.2
Number of Patients	67	67

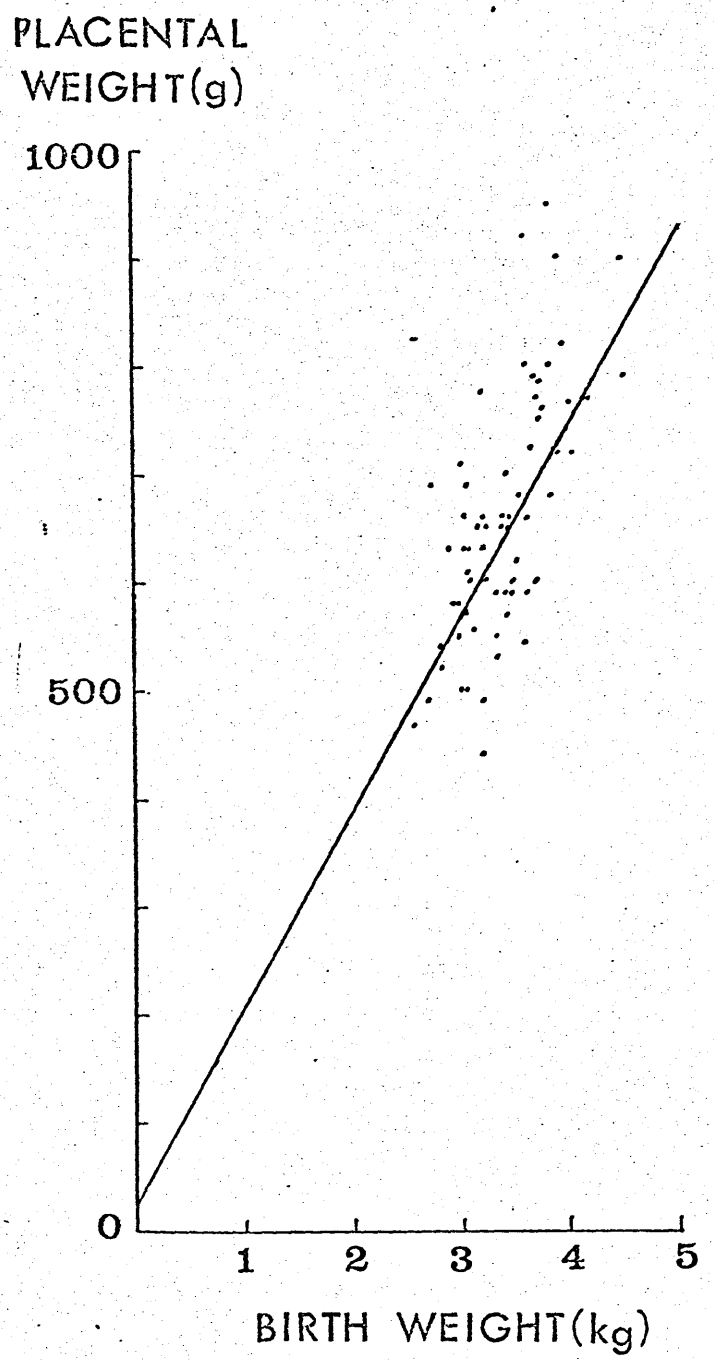

Fig 6 i The correlation between placental weight and birth weight in a normal population.

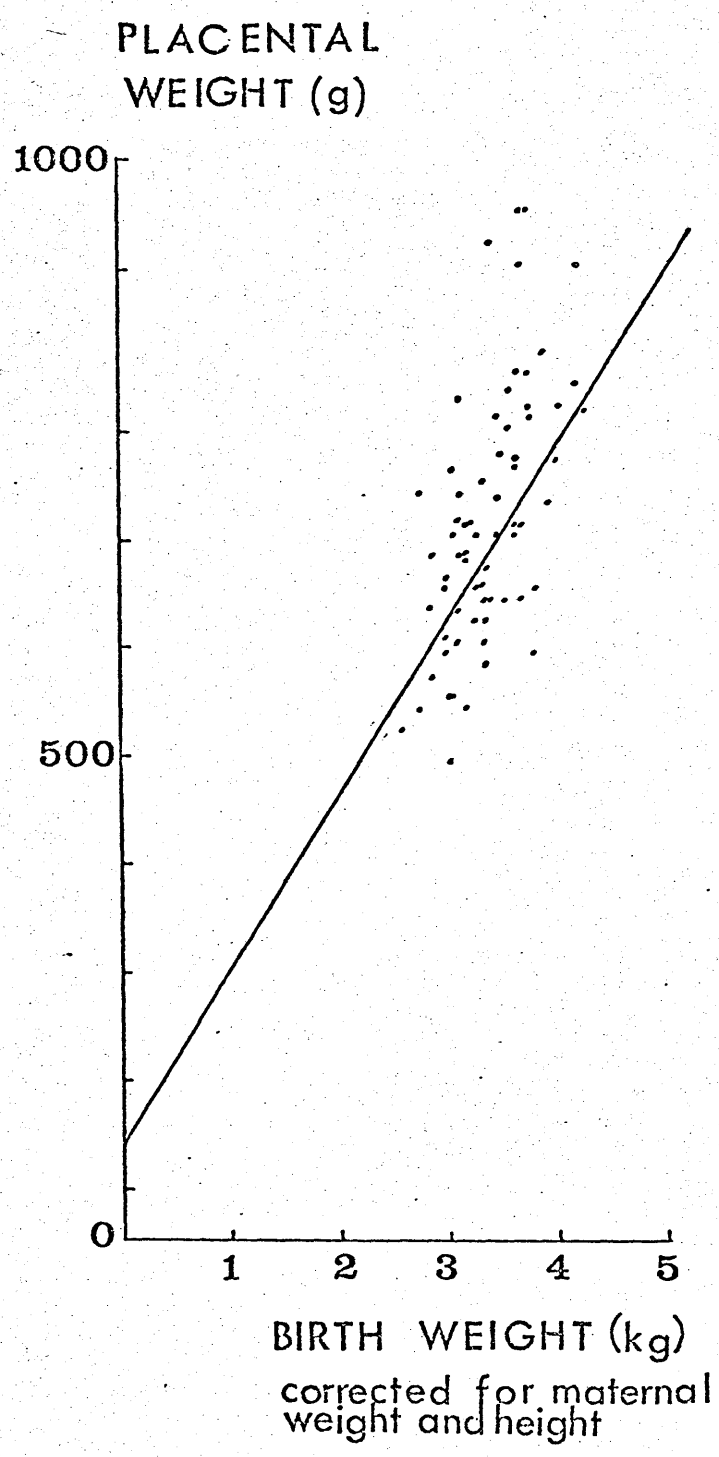

Fig 6 ii The correlation between placental weight and birth weight (corrected for maternal weight and height) in a normal population.

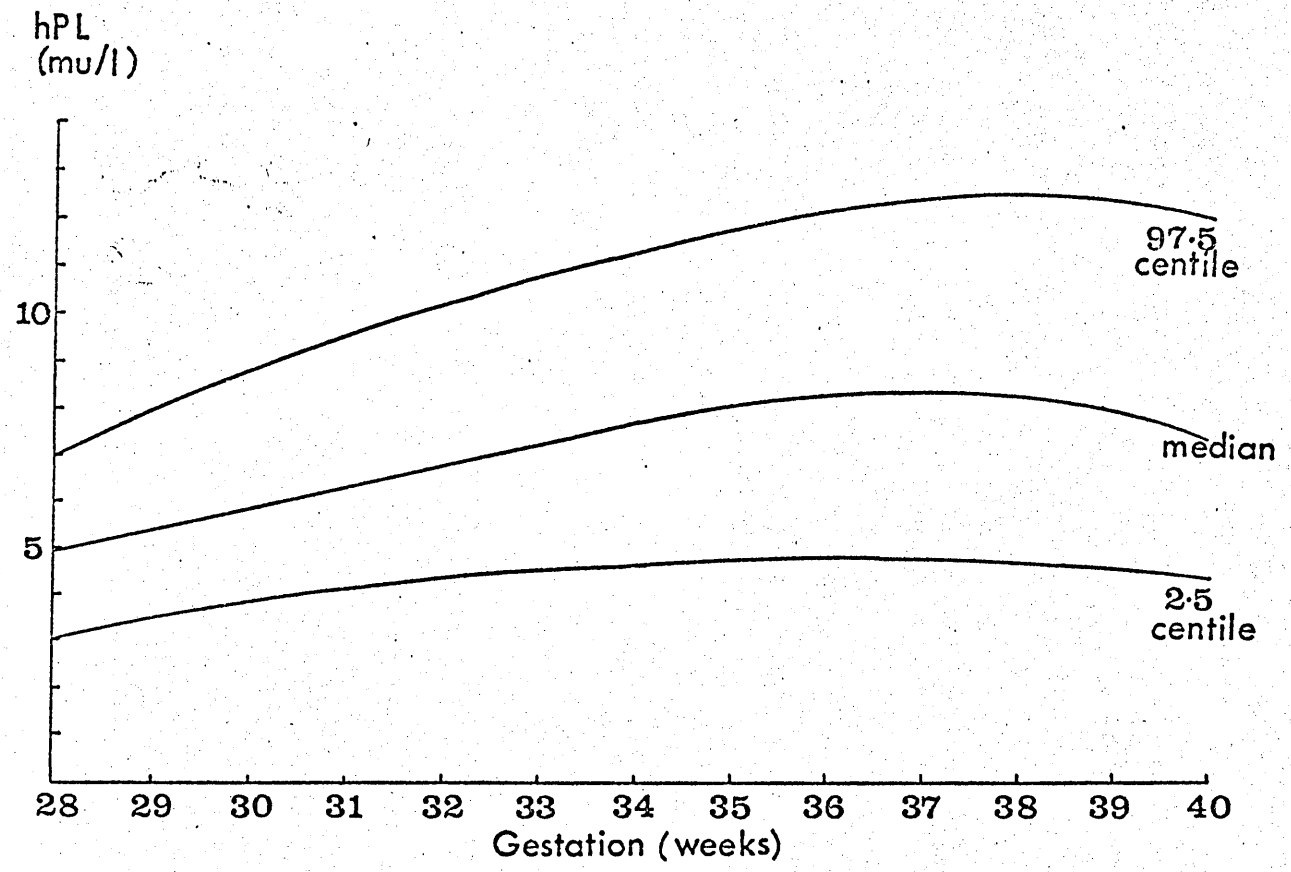

Fig 6 iii Reference range for hPL in the third trimester of pregnancy.

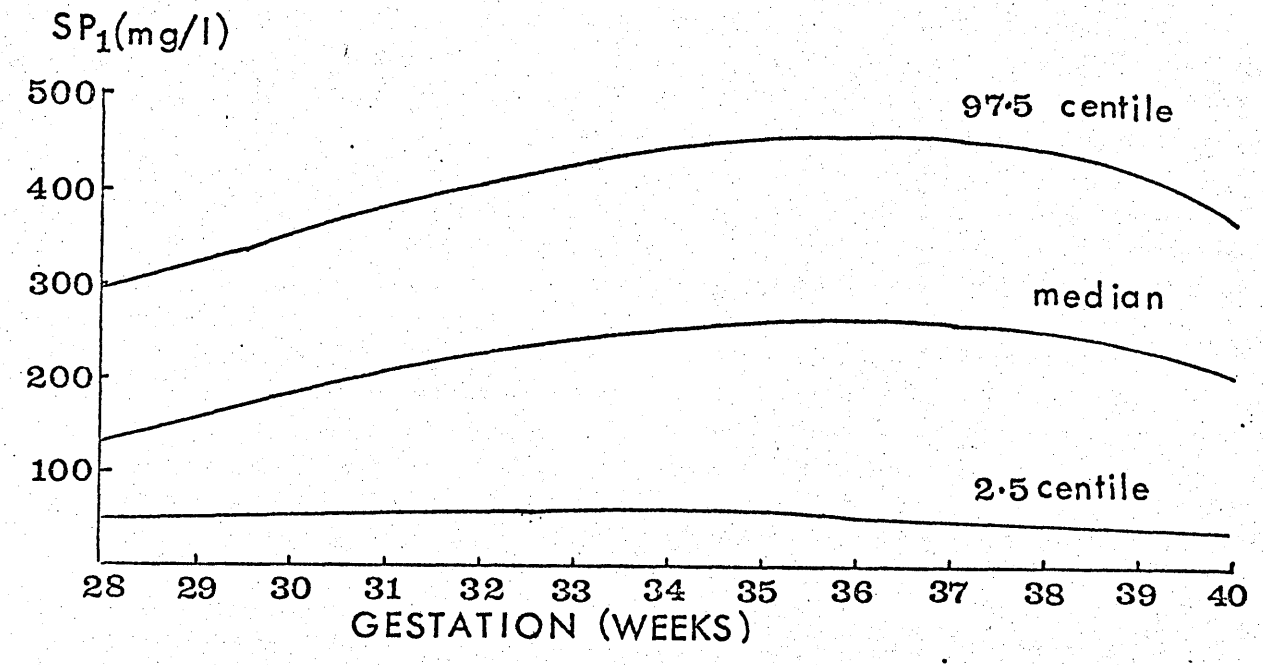

Fig 6 iv Reference range for SP_1 in the third trimester of pregnancy.

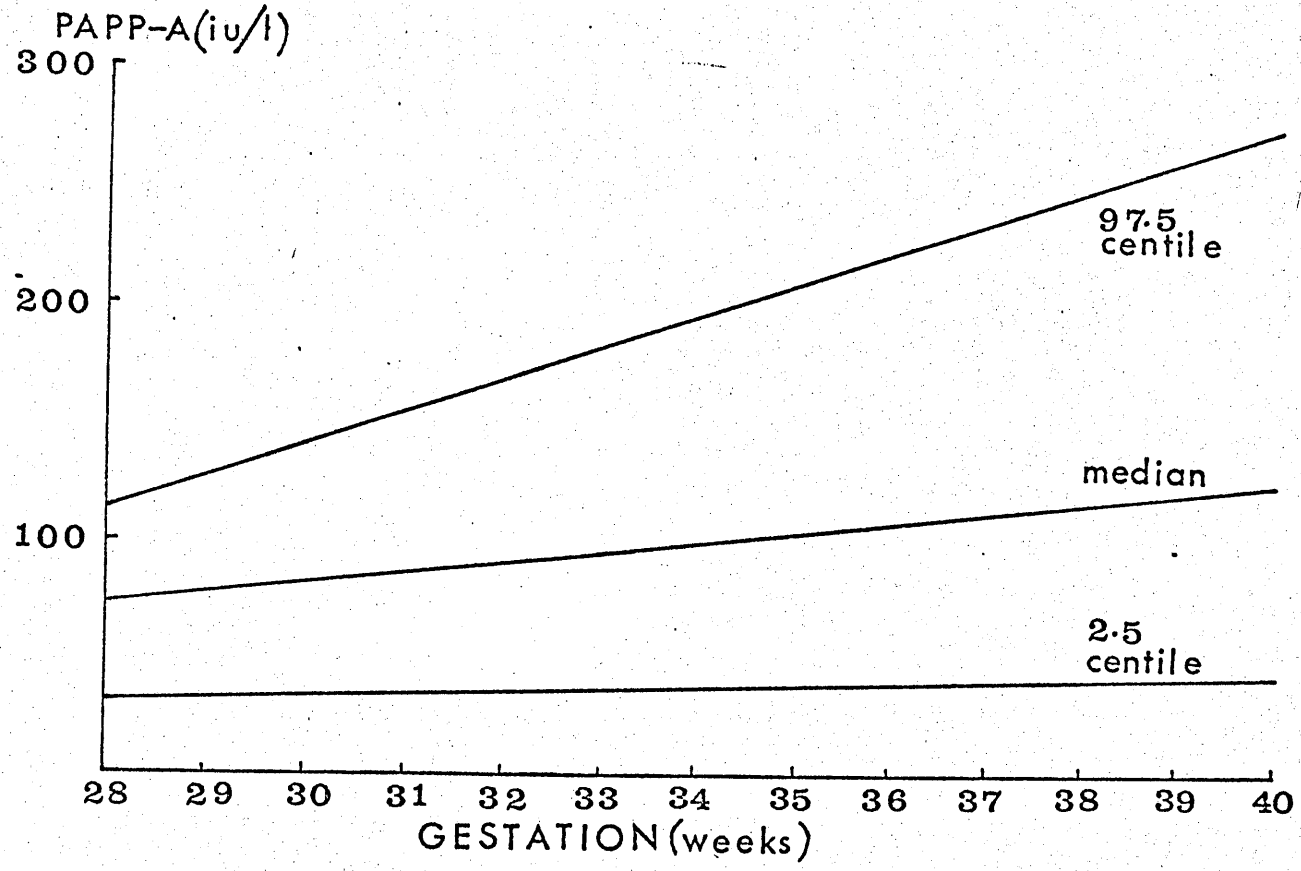

Fig 6 v Reference range for PAPP-A in the third trimester of pregnancy.

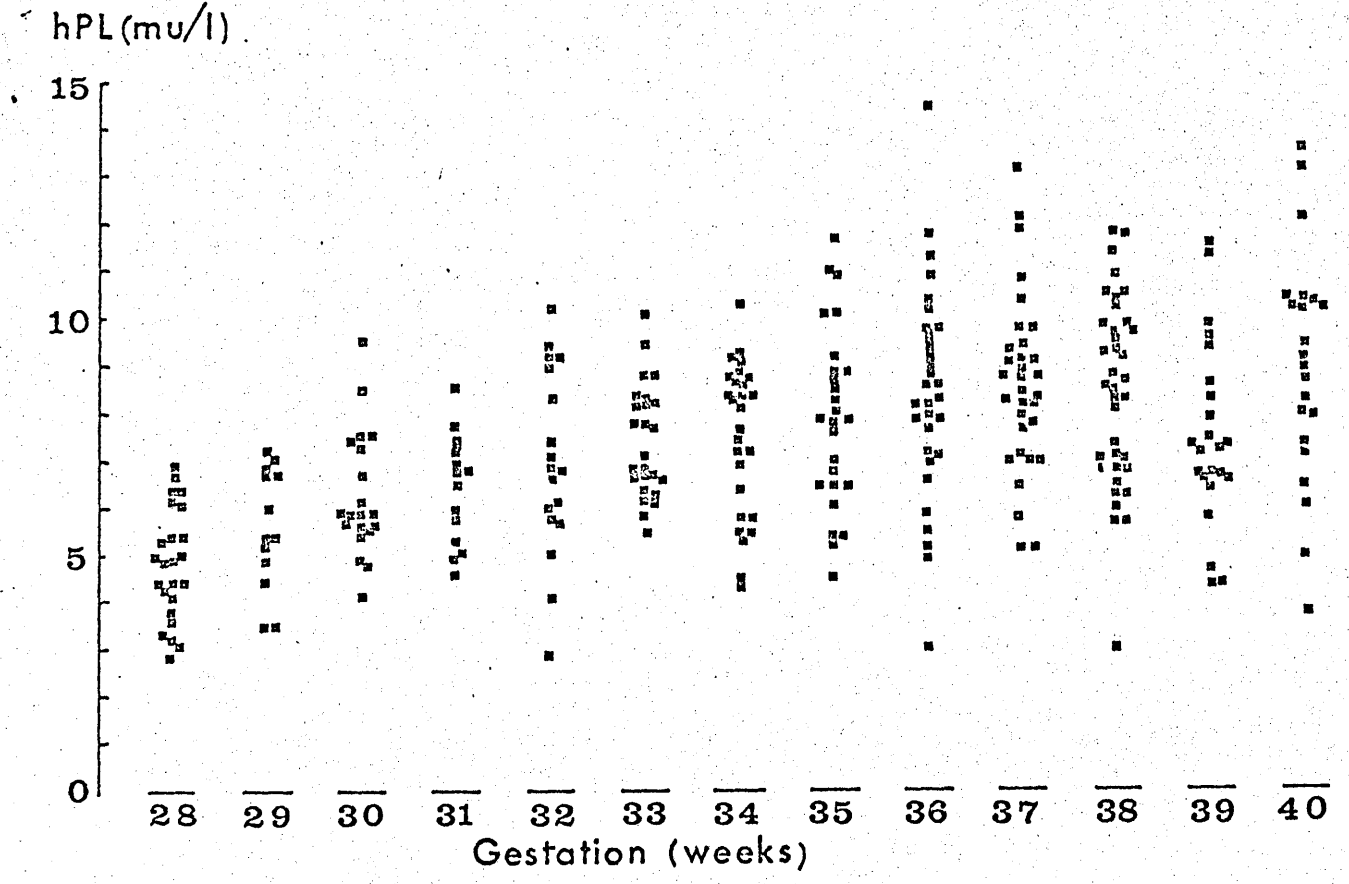

Fig 6 vi Distribution of hPL concentration in the third trimester of pregnancy.

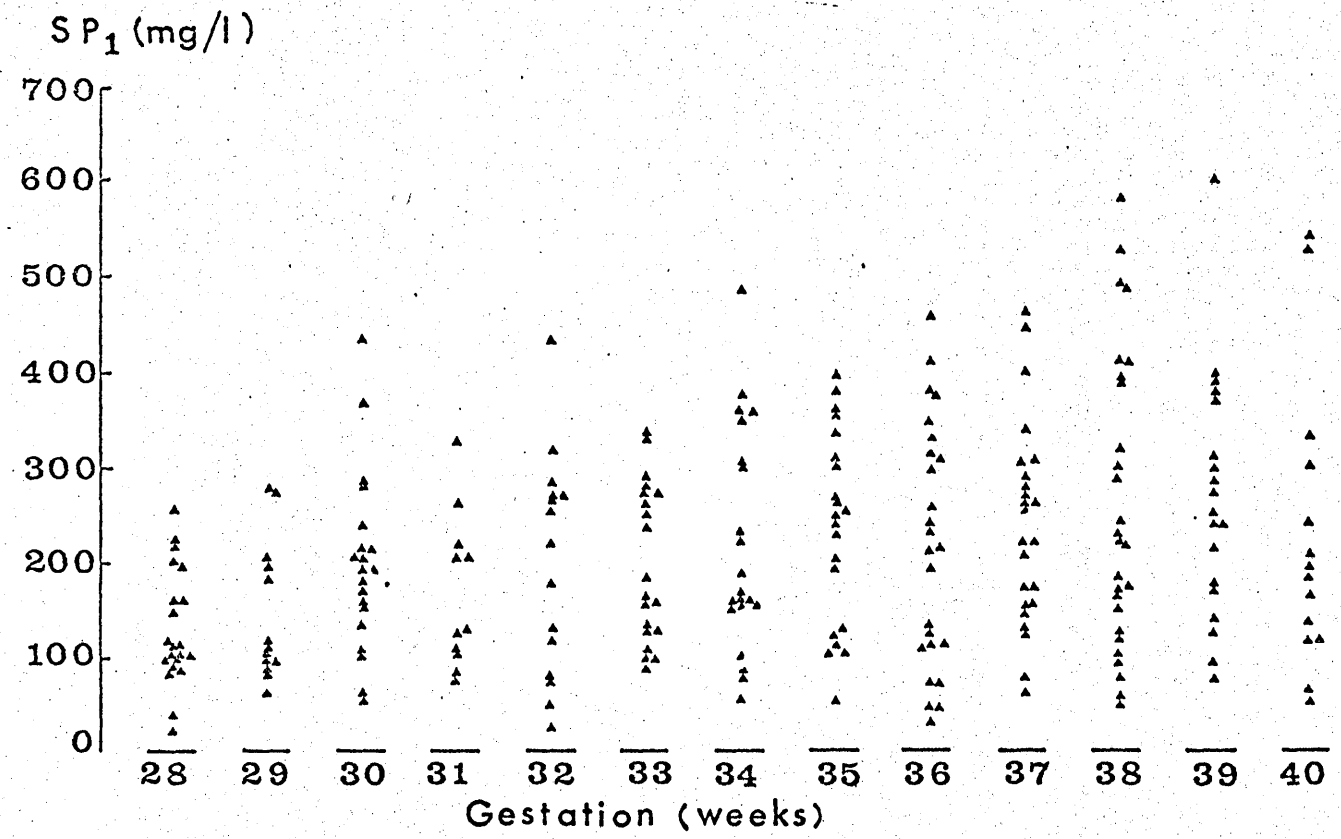

Fig 6 vii Distribution of SP_1 concentrations in the third trimester of pregnancy.

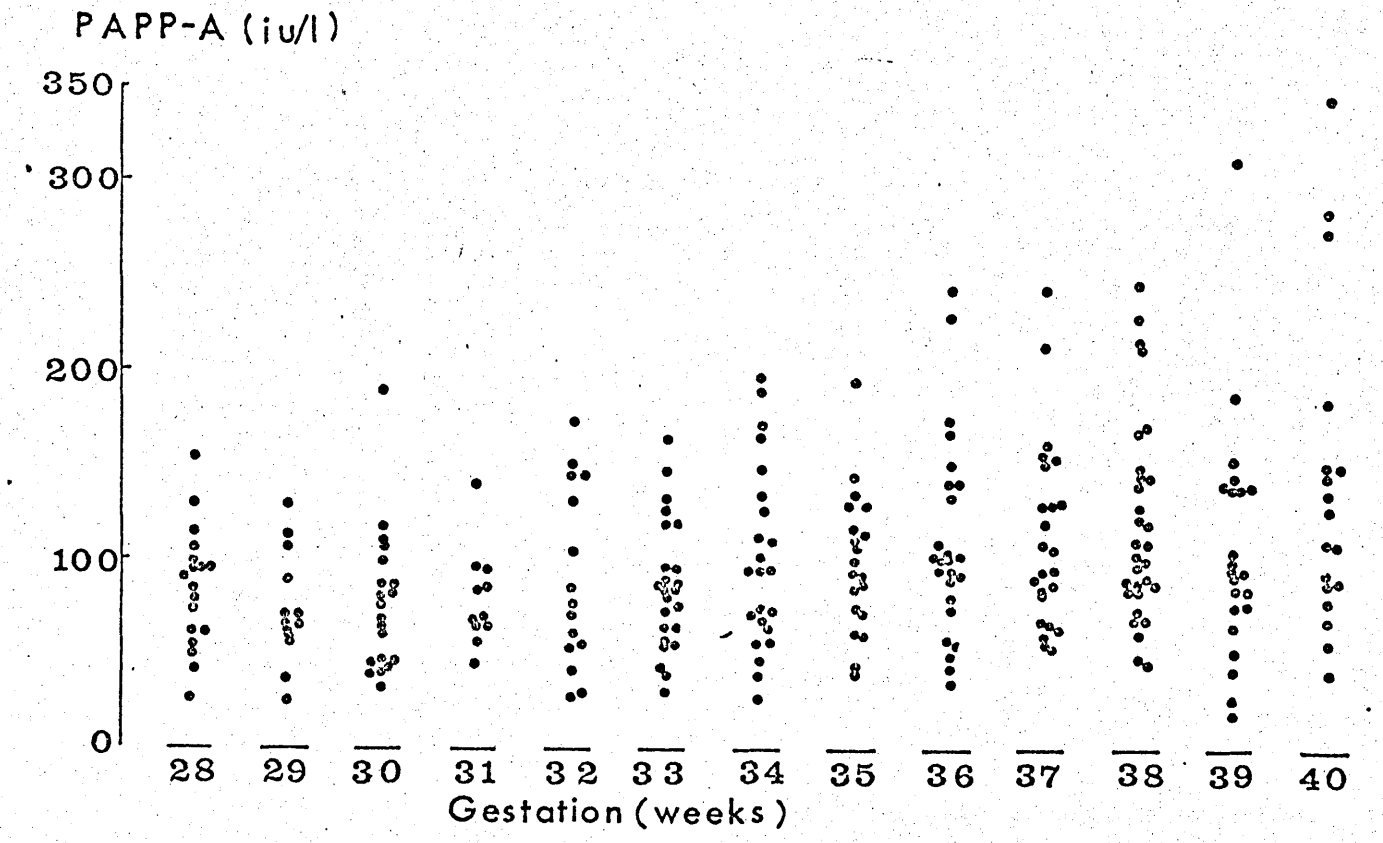

Fig 6 viii Distribution of PAPP-A concentrations in the third trimester of pregnancy.

Table 6 II

The Relationship between peak hPL, birth weight (corrected and uncorrected) and Placental Weight.

	Corrected Birth Weight	Uncorrected Birth Weight	Placental Weight
Mean hPL	8.6 (mu/L)	8.6 (mu/L)	8.6 (mu/L)
Standard Deviation	1.9	2.0	1.8
Standard Error	0.21	0.24	0.24
Mean Weight	3.35 (kg)	3.42	664 (g)
Standard Deviation	0.36	0.41	121
Standard Error	0.04	0.05	15
Correlation Coefft.	0.36 ($p < 0.01$)	0.34	0.52 ($p < 0.01$)
Gradient	2.11	1.66	34.01
Y Axis Intercept	1.55	2.92	373.1
SE Gradient	0.66	0.56	7.0
SE Intercept	2.22	1.93	61
Number of Patients	71	71	67

6.5 Results

Table 6 I shows the statistical analysis of placental weights and birth weights, and Figs 6 i and 6 ii show the regression analysis of this data. There was a significant correlation between both actual birth weight and corrected birth weight with placental weight.

Figures 6 iii - 6 v show the normal ranges for the 3 proteins expressed as centiles. For all 3 proteins, an increase in concentration was noted with increasing gestation, although for hPL and SP_1 a plateau was noted at about 38 weeks followed by a decrease in concentration.

Figures 6 vi - 6 viii show the distributions of data points used to construct the normal ranges and on which the logarithmic transformation was based.

Regression statistics between placental weight, foetal weight and each of the pregnancy proteins were calculated (Table 6 II). Only for hPL was a significant association with either foetal or placental weight found.

No correlation between the peak concentrations (taken between 36 and 38 weeks gestation) of any pair of proteins found.

6.6 Discussion

The supreme test of placental function may be regarded as the birth of a well nourished, adequately oxygenated baby. Since the placenta is the organ responsible for transfer of nutrients, it seems likely that a large placenta has a greater transfer capacity than a small placenta (Fox 1979). However, to what extent placental adequacy can be inferred from placental weight is questionable (Baird and Walker 1959, Aherne 1966, Browne 1973, Dawes 1968). In an extensive survey of births, Thomson found

correlation coefficients of between 0.5 - 0.6 indicating that birth weight is rather poorly predicted by placental weight (Thomson et al 1969). The results obtained in the present study although drawn from a smaller population accord well with Thomson's figures (Birthweight mean 3.41 kg compared with Thomson's value of 3.30 kg and mean placental weight 664 g compared with 649 g). Younozzai and Haworth (1969) found similar birthweights but a lower mean placental weight. This difference may be accounted for by the thoroughness with which blood and membranes are removed before weighing (Garrow and Hawes 1971).

The low correlation coefficient between birth weight and placental weight is perhaps not surprising in view of the fact that the placenta has considerable functional reserve. If foetal growth were rigidly limited by placental mass, it would imply that the placenta is normally working at full capacity - a concept that appears incorrect. It seems more probable that the small placenta is small because the baby is small and placental function is therefore adequate for a foetus the growth of which is retarded by extraplacental factors. Correction of foetal weight to obviate these maternal effects will lower the predictive value of placental weight.

The pattern of pregnancy protein concentrations observed for the 3 proteins under consideration should be considered in the light of previous findings.

The wide range of normal has been recognized by many groups of workers in this field (Sciarra et al 1968). Many groups have found skewed distributions for hPL concentrations, (Letchworth et al 1971, Guibal et al 1980, Pluta et al 1980) although some have not (Janisch and Spona 1980).

The skewed distribution observed for SP_1 is also in accordance with other groups and the normal range is in agreement with that previously obtained by this method (Pluta et al 1979, Chard and Grudzinskas 1980, Gordon et al 1977, Macdonald et al 1979 and Gemmell 1982). The median peak value also agrees with that of Gordon et al (1977) using RIA. Other groups however have found lower peak values (Karg et al 1981, Pluta et al 1979). These differences may result from the use of different mass standards - a problem seen with many of the pregnancy proteins. For SP_1 however, the problem is even greater because of the molecular heterogeneity (Towler et al 1978, Teisner et al 1978). This fact and the differing assay systems used makes strict comparison of results almost impossible. The future use of an International Reference Preparation may obviate this problem. At present the continued use of mass standards for SP_1 may be considered acceptable. This problem is discussed further in Chapter 4. In contrast to the results obtained for hPL and SP_1, serum PAPP-A concentrations increased with advancing pregnancy in a less marked manner, and no peak concentration was observed. A similar pattern has been observed previously (Smith et al 1979, Bischof et al 1982a).

Synthesis of hPL appears related to placental mass as shown by the elevated serum levels found in pregnancies complicated by diabetes mellitus or Rhesus isoimmunisation (Selenkow et al 1971). The correlation of hPL concentrations with placental and foetal weight is not unexpected therefore, although not all groups of workers have found a correlation (Grumbach et al 1968, Sciarra et al 1968, Letchworth et al 1971b, Spellacy et al 1971).

Similar disagreements exist over the correlation of SP_1 with placental and foetal weight, some groups finding a correlation

(Braunstein et al 1980, Vermeulen et al 1982, Gamberini et al 1977) or no correlation (Chapman et al 1981, Mandruzzato et al 1980). The discordance of results between hPL and SP_1 may be explained by differing modes of production.

A clear concentration gradient from the retroplacental blood to peripheral blood has been shown for hPL (Klopper and Hughes 1978). SP_1 however has been shown to exhibit a reverse gradient (Smith et al 1979, Grudzinskas et al 1980b). These findings led Klopper et al to hypothesize that very little of the SP_1 found in the maternal circulation is directly secreted by the villous trophoblast, but rather is the effect of lysis of the maternal trophoblast. If this is so, the lack of correlation of maternal SP_1 concentration with placental weight is hardly surprising. The lack of correlation between hPL and SP_1 concentrations is also explainable.

PAPP-A has also been shown to exhibit a reverse concentration gradient similar to that for SP_1. It is interesting that no correlation between PAPP-A and foetal or placental weight was found. By analogy with SP_1, this lack of correlation can be explained and similarly the lack of correlation between PAPP-A and the other pregnancy proteins is also explained.

The consistency of comparisons between this and other surveys suggests that although relatively small in numbers, this normal group represents a suitable basis for construction of reference ranges.

CHAPTER 7

THE USE OF PREGNANCY ASSOCIATED PROTEINS IN MONITORING PLACENTAL FUNCTION IN LIGHT FOR DATES PREGNANCIES AND IN PATIENTS WITH POOR WEIGHT GAIN

7.1 The Light for Dates Pregnancy

7.1.1 Objectives

The detection of the LFD baby and the monitoring of this type of pregnancy has been the aim of obstetricians and biochemists for many years. The reasons behind this are several. The obstetricians' concern is directed towards causation; placental failure towards the end of gestation carrying intimations of mortality (Brook 1983). Furthermore these babies are at increased risk in the neonatal period and are also disadvantaged into childhood, being less well developed both physically and intellectually (Neligan et al 1976). The clinical evaluation of fetal risk due to IUGR remains difficult however. Only the most severe cases of poor fetal growth are likely to be recog-nized clinically (Aickin et al 1983). Even the advent of ultrasonographic monitoring whilst being an ideal non-invasive technique has problems with the number of patients that can be screened. Furthermore this type of examination facility tends to be confined to major centres.

Biochemical methods therefore still play an important part in the detection and monitoring of the LFD pregnancy.

7.1.2 Clinical Material

Patients who delivered a baby of $< 10^{th}$ centile for weight corrected for gestational age (Thomson et al 1968) were included in this group. Some pregnancies were normotensive whilst others were hypertensive - all were considered as a single LFD group however.

7.1.3 Analytical Methods

The assay methods given previously (Chapters 2, 3) for hPL,

Table 7 I

Characteristics of LFD Pregnancies

	LFD	Normal	
Mean Birth Weight (kg)	2.38	3.40	
SD	0.46	0.42	t = 12.0
SE	0.07	0.05	p < 0.01
n	38	91	
Mean Placental Weight (kg)	464	632	
SD	111	145	t = 6.1
SE	18.4	17.3	p < 0.001
n	35	67	

Table 7 II

Definitions of the terms used in comparing LFD and normal pregnancies.

True Positive (TP) A serum pregnancy protein concentration below the decision threshold with subsequent delivery of a LFD baby.

False Positive (FP) A serum pregnancy protein concentration below the decision threshold with subsequent delivery of a normal birth weight baby.

True Negative (TN) A serum pregnancy protein concentration above the decision threshold with subsequent delivery of a normal birth weight baby.

False Negative (FN) A serum pregnancy protein concentration above the decision threshold with subsequent delivery of a LFD baby.

Specificity The proportion of subjects without the disease who give a negative result =

$$\frac{TN}{TN + FP}$$

Sensitivity The proportion of subjects with the disease who give a positive test result =

$$\frac{TP}{TP + FN}$$

Predictive value of a positive test result = $\frac{TP}{TP + FP}$

Predictive value of a negative test result = $\frac{TN}{TN + FN}$

Prevalence The proportion of the population under investigation who have the disease which we wish to detect.

SP_1 and PAPP-A were used. The weight of the baby and the weight of the placenta were noted at delivery and the characteristics of these pregnancies compared to the normal group are shown in Table 7 I. Birth weights were not corrected for maternal weight and height for the reasons described previously (Chapter 6).

7.1.4 Numerical Analysis of Data

Comparison of the pregnancy protein concentrations in LFD and normal pregnancies were made by comparing the median concentration at each week of gestation. For objective assessment of the usefulness of an individial protein in detecting a LFD baby, the following approach was adopted:-

All samples were considered independently although several samples were obtained from most patients throughout gestation, the objective being to assess the value of an individual measurement rather than a series. The serum concentrations of the 3 proteins considered are all dependent on the duration of pregnancy, so the decision thresholds used to predict IUGR were expressed as centiles of the control distribution. Prediction of IUGR may thus be made at any week of gestation. Centiles were calculated at each weeks gestation using standard statistical methods. Logarithmic transforms of serum SP_1 and PAPP-A concentrations were made before calculation of the mean and SD. For hPL, untransformed data was used.

After calculation of true/false positives and negatives, sensitivity, specificity and predictive values for each test were calculated in accordance with the methods described by Galen and Gambino (1975). These parameters are defined in Table 7 II.

7.1.5 Results

There was a significant difference in birth weights between the

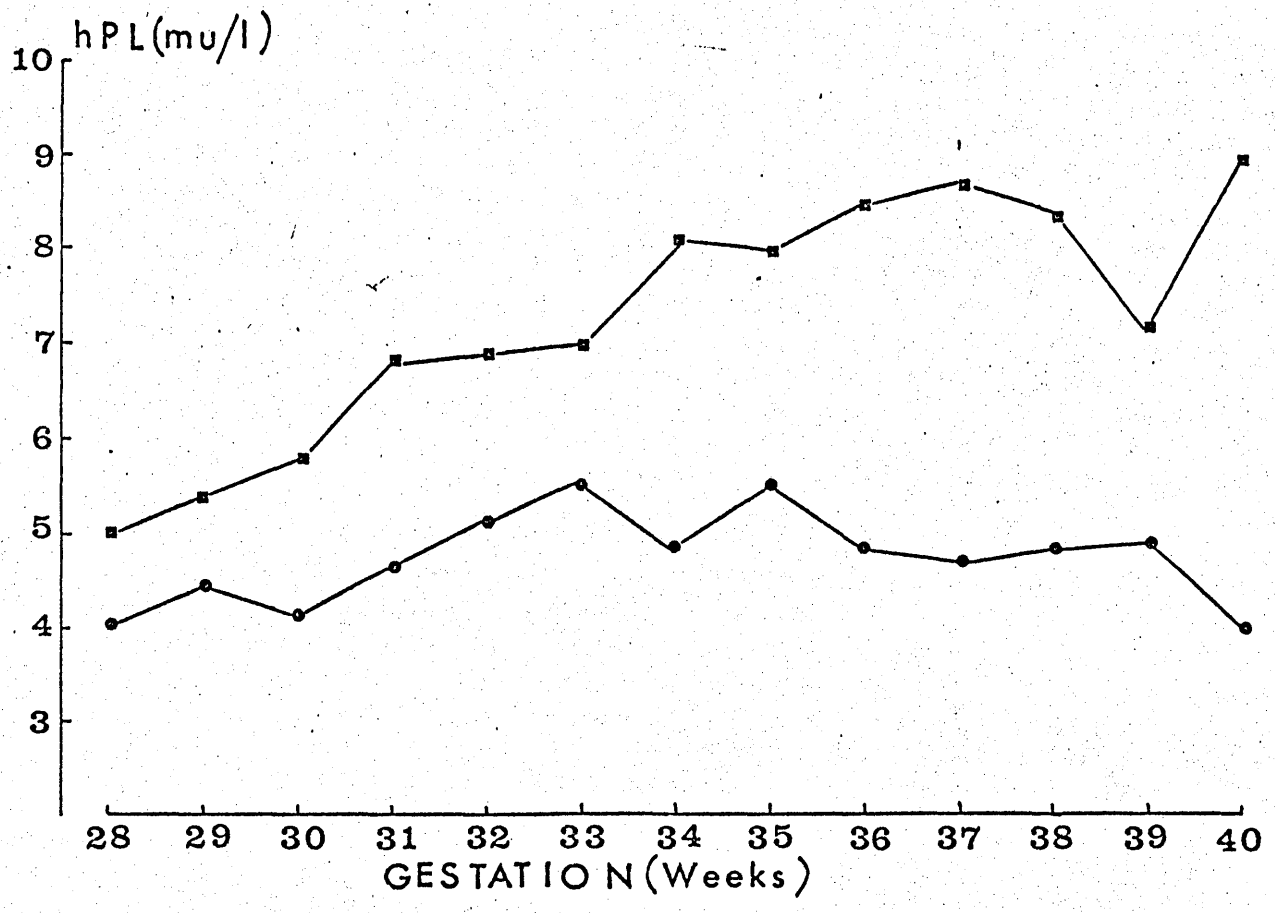

Fig 7 i Median hPL concentration during the third trimester of pregnancy in two groups of patients - (■) normal pregnancy, (●) pregnancy resulting in a LFD baby.

Table 7 III

Intersextile Ranges for Concentrations of hPL, SP_1 and PAPP-A in LFD and Normal Pregnancies

		LFD Group			Normal Group		
	Week of Gestation	Lower Sextile	Median	Upper Sextile	Lower Sextile	Median	Upper Sextile
HPL miu/l	28	3.1	4.0	4.8	3.6	5.0	6.2
	29	3.2	4.4	5.1	4.4	5.4	6.8
	30	3.2	4.1	4.5	4.9	5.8	7.5
	31	4.4	4.6	5.0	5.0	6.8	7.4
	32	3.9	5.1	6.5	5.7	6.9	9.2
	33	3.2	5.5	6.1	6.3	7.0	8.3
	34	3.7	4.9	5.6	5.5	8.1	8.9
	35	4.4	5.5	5.9	6.1	7.9	9.2
	36	4.3	4.8	6.1	6.6	8.4	9.8
	37	3.6	4.7	6.6	7.0	8.7	9.8
	38	3.0	4.8	7.4	6.5	8.3	10.4
	39	3.5	4.9	6.0	6.4	7.2	9.4
	40	2.3	4.0	4.3	6.4	8.9	10.4
SP_1 mg/l	28	75	97	151	85	112	200
	29	96	137	179	87	152	207
	30	112	171	196	107	190	280
	31	109	143	390	83	127	264
	32	122	190	219	73	218	284
	33	113	144	238	109	181	282
	34	111	149	208	102	167	359
	35	97	130	332	113	245	355
	36	105	131	188	74	226	350
	37	104	164	273	145	259	309
	38	106	151	235	95	222	411
	39	117	142	267	141	230	382
	40	95	157	167	64	185	331
PAPP-A iu/l	28	32	54	122	56	86	106
	29	54	89	199	55	67	107
	30	21	70	107	44	74	102
	31	20	55	123	56	68	97
	32	48	72	105	41	77	145
	33	40	124	190	54	84	119
	34	41	87	159	56	74	165
	35	80	128	176	62	96	128
	36	45	70	107	57	101	167
	37	60	127	194	68	96	154
	38	57	87	110	73	109	167
	39	48	87	133	51	93	141
	40	14	115	191	79	123	184

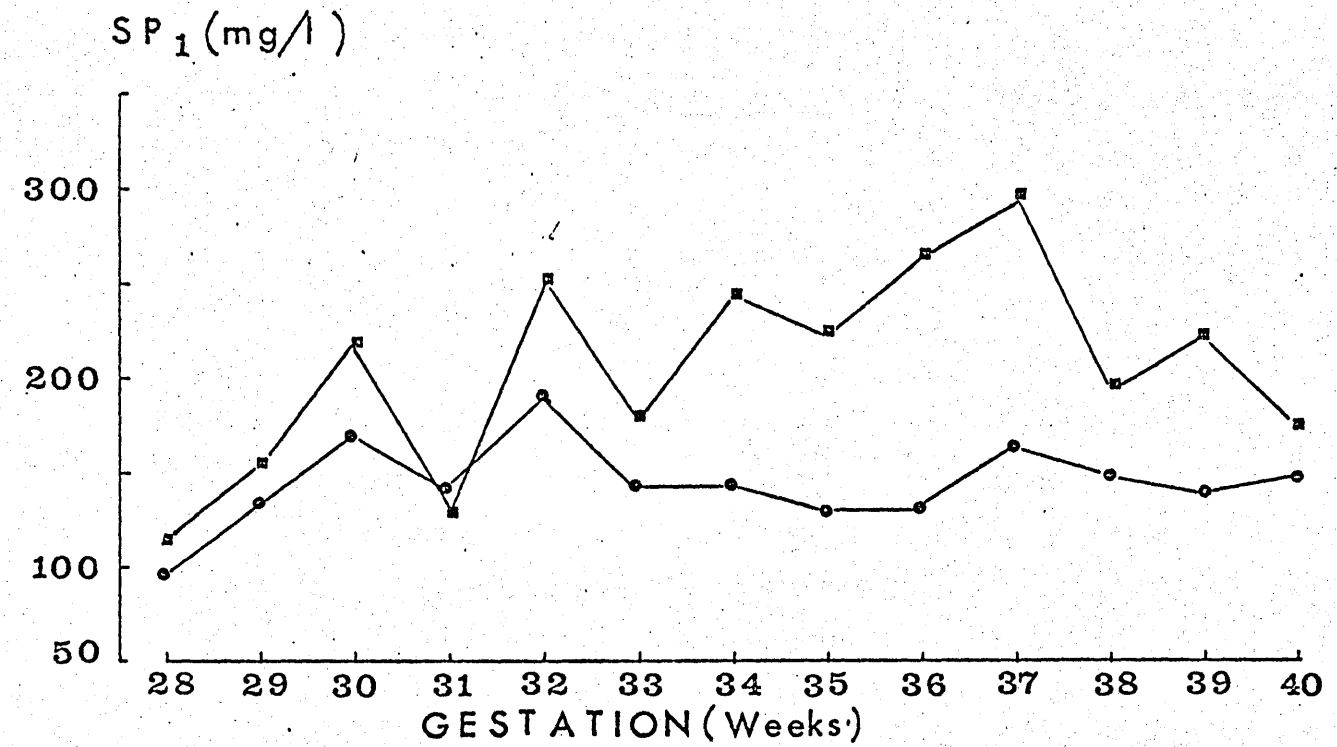

Fig 7 ii Median SP_1 concentration during the third trimester of pregnancy in two groups of patients - (■) normal pregnancy, (●) pregnancy resulting in a LFD baby.

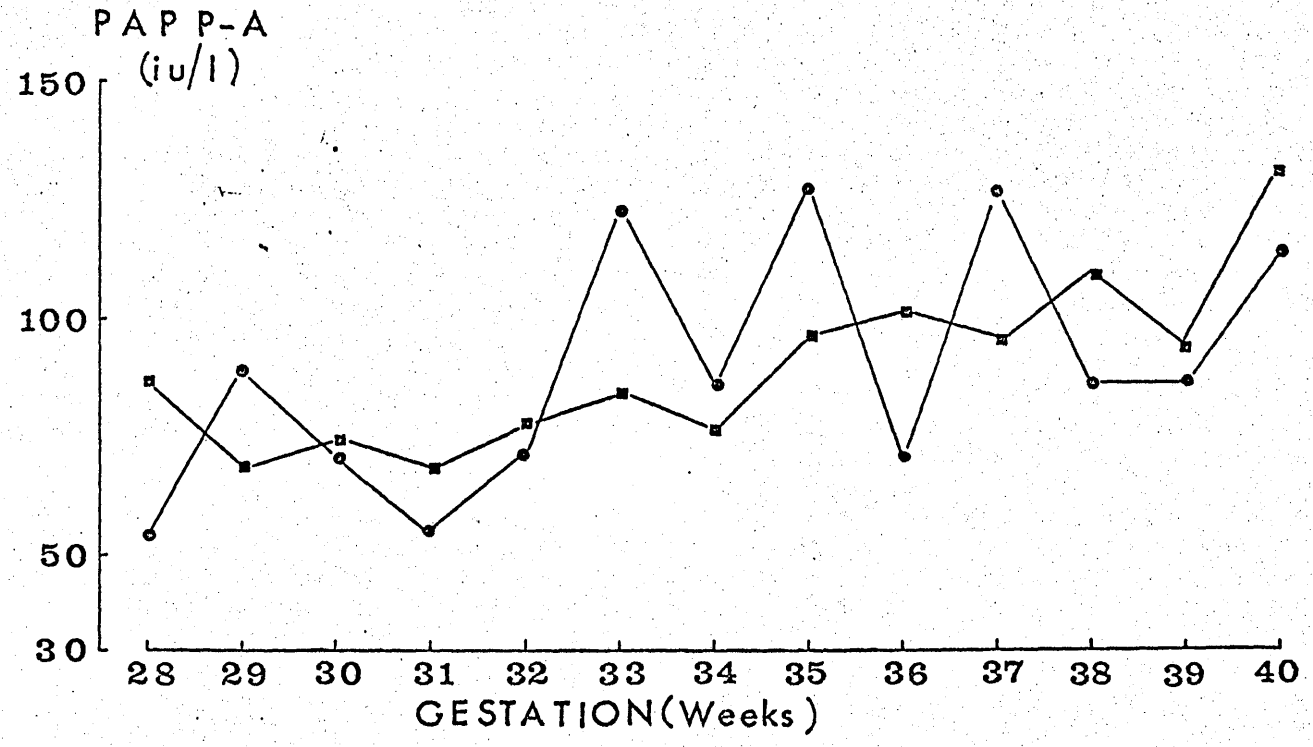

Fig 7 iii Median PAPP-A concentration during the third trimester of pregnancy in two groups of patients - (■) normal pregnancy, (●) pregnancy resulting in a LFD baby.

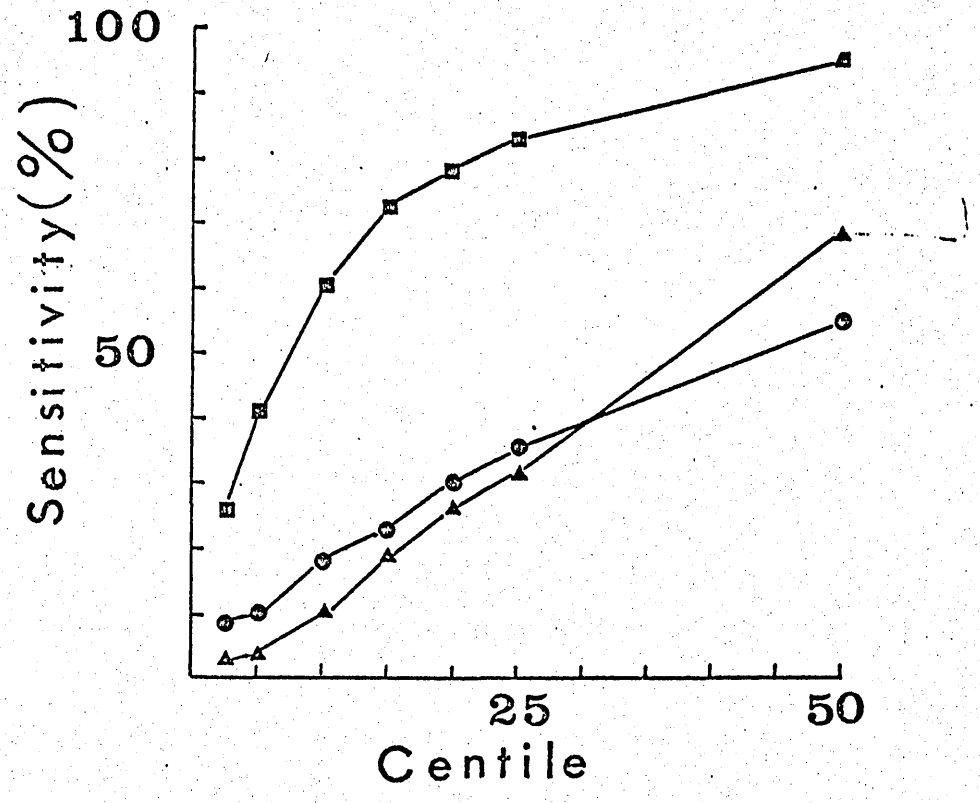

Fig 7 iv The variation of test sensitivity with decision threshold expressed as centiles for three placental proteins (■) hPL, (▲) SP_1, (●) PAPP-A.

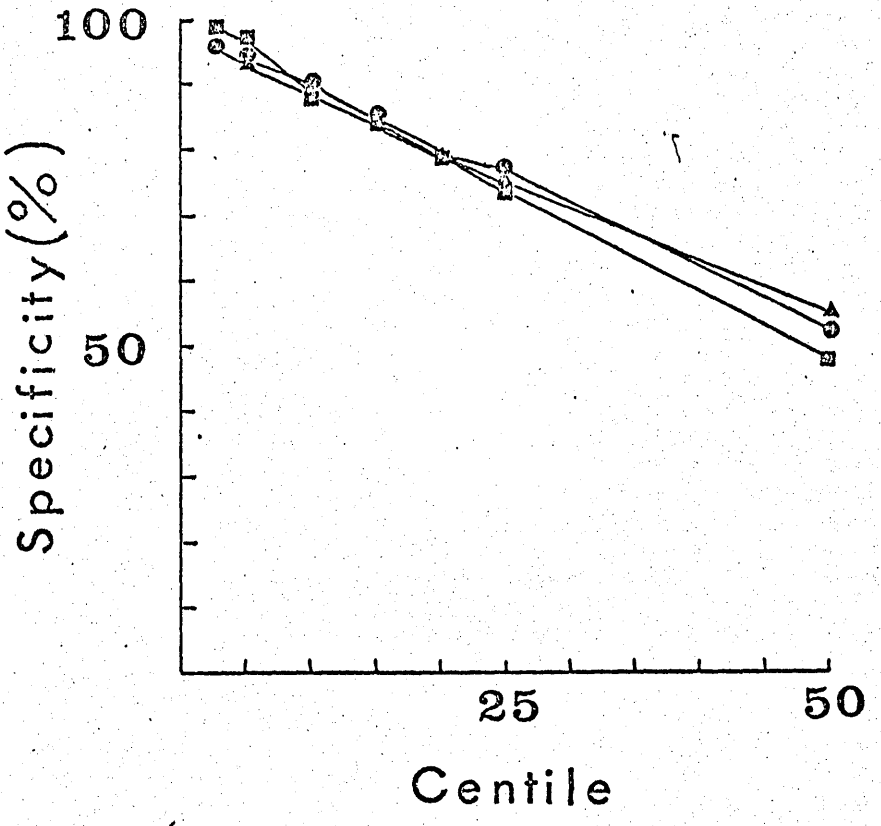

Fig 7 v The variation of test specificity with decision threshold expressed as centiles for three placental proteins (■) hPL, (▲) SP_1, (●) PAPP-A.

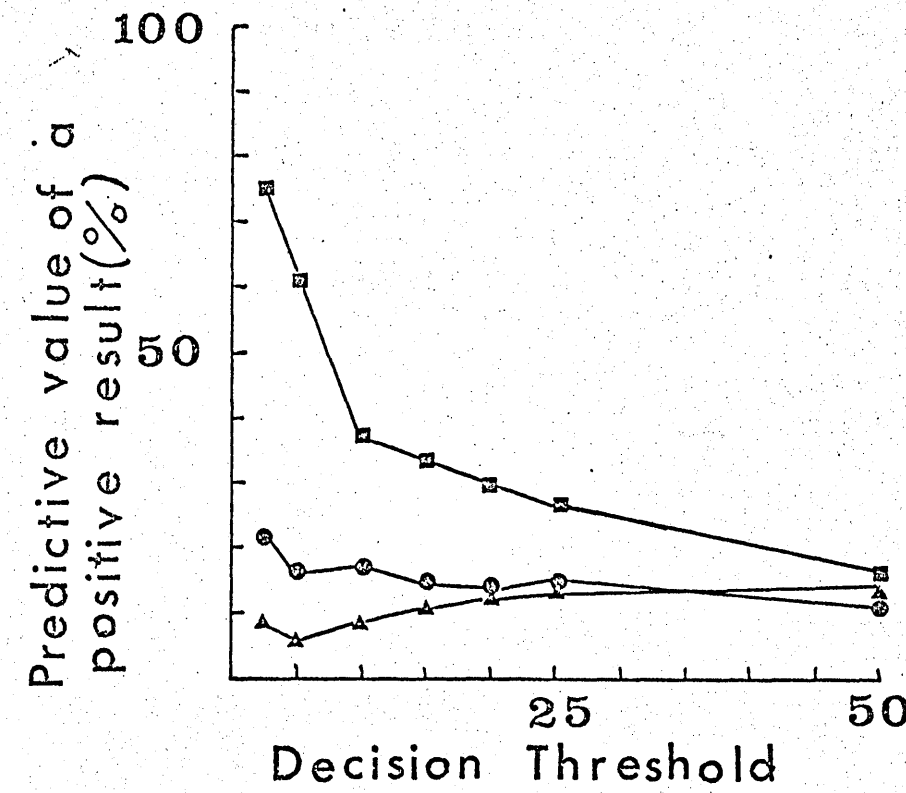

Fig 7 vi The variation of predictive value of a positive result with decision threshold for three placental proteins (■) hPL, (▲) SP_1, (●) PAPP-A.

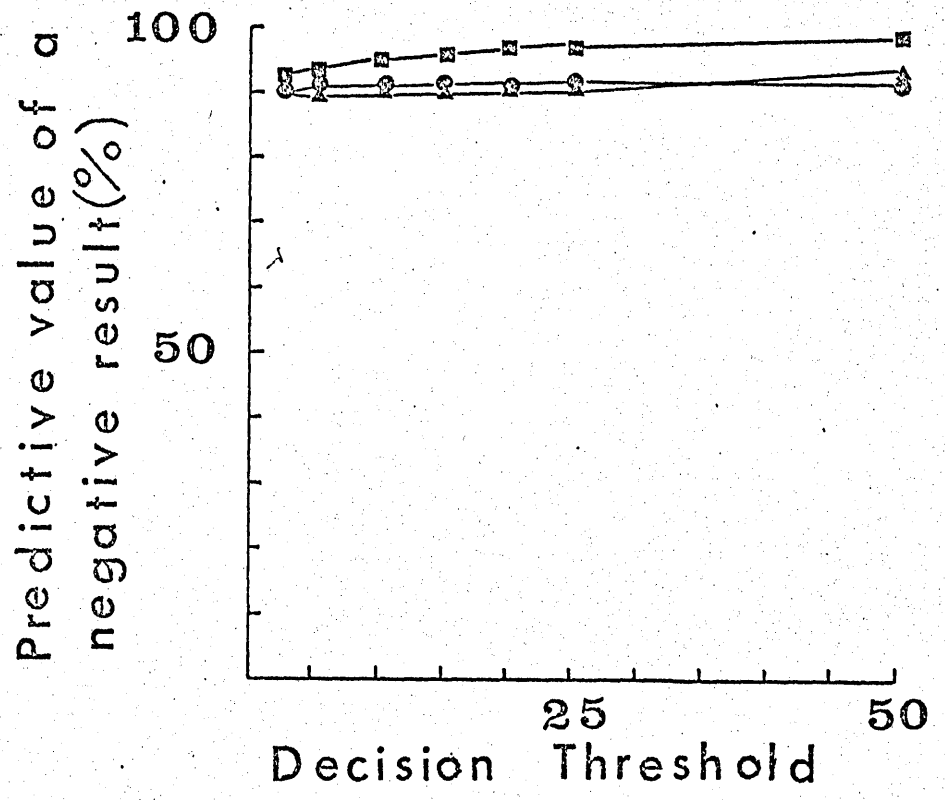

Fig 7 vii The variation of predictive value of a negative result with decision threshold for three placental proteins (■) hPL, (▲) SP_1, (●) PAPP-A.

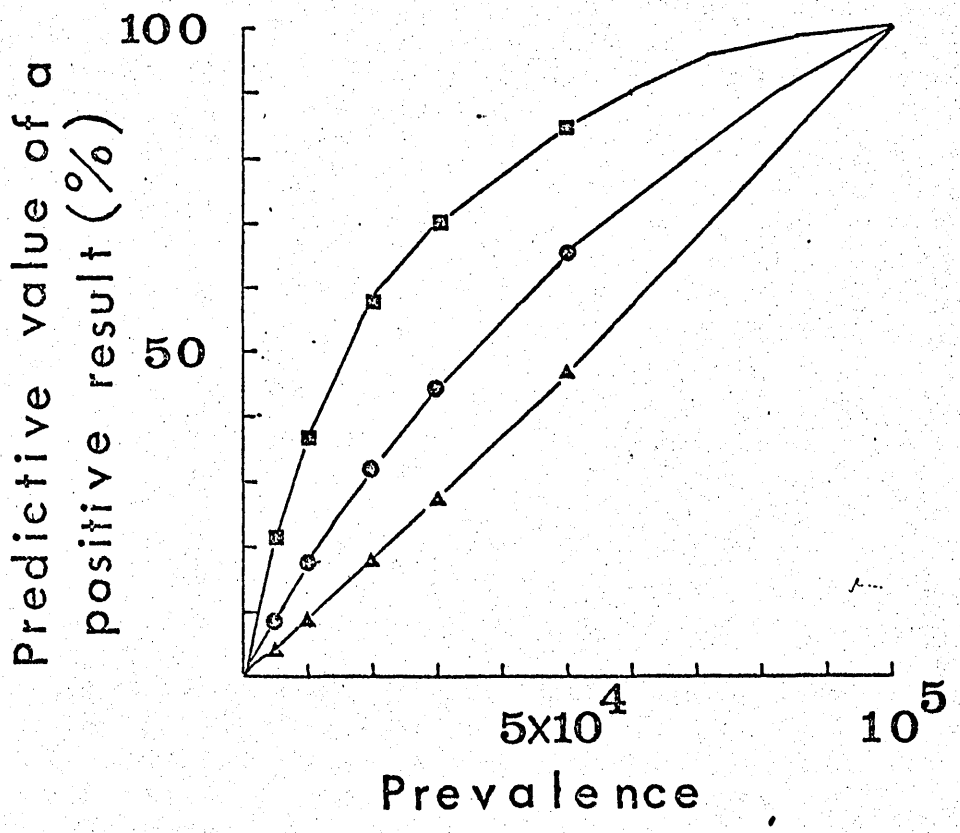

Fig 7 viii The variation of predictive value of a positive result with prevalence for three placental proteins (■) hPL, (▲) SP_1, (●) PAPP-A.

normal and LFD group and also in placental weights (Table 7 I) ($p < 0.01$).

HPL in the normal group was significantly higher at each week of gestation than in the LFD group ($p < 0.01$) (Figure 7 i, Table 7 III). There was no significant difference in SP_1 and PAPP-A concentrations (Figure 7 ii, 7iii and Table 7 III).

Figures 7 iv - 7 vii show the values for sensitivity, specificity and predictive values of positive and negative results at several decision thresholds and Figure 7 viii depicts the dependence of predictive value upon prevalence of the disease in the population examined.

7.1.6 Discussion

The need for objective assessment of predictive placental function tests has been emphasized (Chard and Klopper 1982). All too often a subjective opinion is formed based on the observation that hPL is generally higher in 1 group of patients than another.

Three variables determine the predictive value of a test - specificity, sensitivity and prevalence (Table 7 II). Because the serum concentrations of the three proteins under investigation are dependent on the duration of pregnancy, decision thresholds have been described as centiles of the distribution of the control results. Therefore specificity has been defined by selection of the decision threshold.

hPL clearly provides the most sensitive test when using this approach. As the decision threshold approaches the median, more growth retarded babies are included in the group as a whole but at the cost of including a number of normal subjects. The value of a test in assessing the individual patient is best described in terms of the prevalence of the disease. Assuming

a prevalence of 10,000 per 100,000 population (10%), SP_1 and PAPP-A are of less value than hPL in predicting IUGR.

For the individual patient, hPL remains the most useful of the pregnancy proteins under study in the detection of the growth retarded foetus. Whilst hPL is clearly of use in detection of the LFD foetus, it remains to be seen whether it will be superceded by physical techniques. Whilst these methods may have certain attractions their use at present remains limited to specialized centres. Conversely hPL assays are easy and cheap to perform and are likely to remain an important part of the biochemical approach to the LFD pregnancy.

7.2 Poor weight profile pregnancies

7.2.1 Objectives

The patient with either low weight gain throughout the duration of pregnancy, or with a static weight for several weeks poses a problem for the obstetrician. Does this syndrome represent a growth retarded foetus, a foetus that has initially grown at a satisfactory rate and then ceased growth for some reason, or is the foetus healthy and of good size with only a lack of maternal weight gain to concern the physician? It is in this light that monitoring should be seen. Primarily monitoring in this situation will be carried out to reassure the physician that placental function is proceeding apace.

7.2.2 Clinical Material

A poor weight gain was defined as a static weight over a 2-3 week period or an overall weight gain of < 8 kg throughout pregnancy regardless of the weight of the baby.

7.2.3 Analytical Methods

HPL, SP_1 and PAPP-A were measured by the methods described

Table 7 IV

Characteristics of Patients with Poor Weight Profile During Pregnancy

	Poor Weight Profile	Normal	
Mean Birth Weight (kg)	3.30	3.41	
SD	0.5	0.42	$p > 0.1$
n	18	80	
Mean Placental Weight (g)	654	664	
SD	150	121	$p > 0.1$
n	18	67	

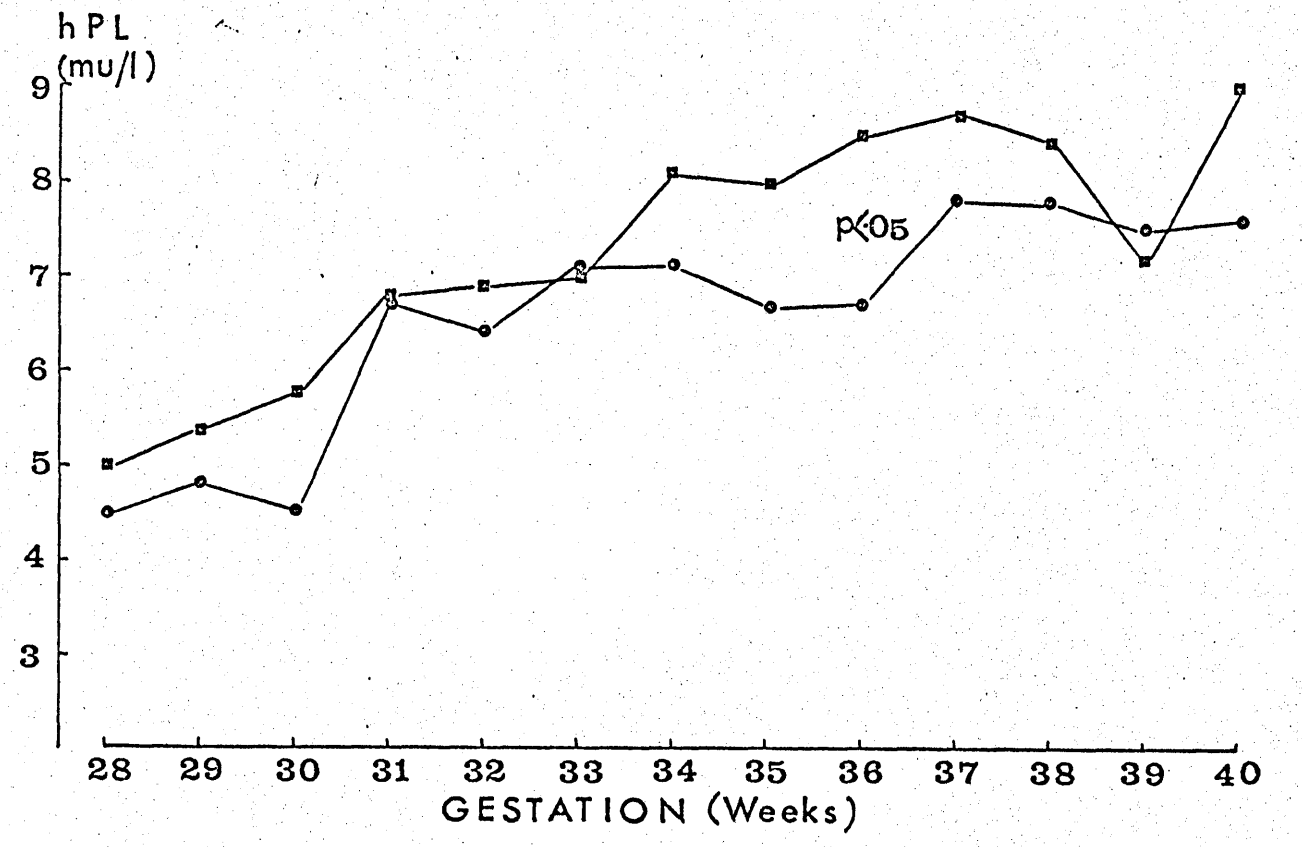

Fig 7 ix Median hPL concentration during the third trimester of pregnancy in two groups of patients - (■) normal pregnancy, (●) poor weight profile pregnancy.

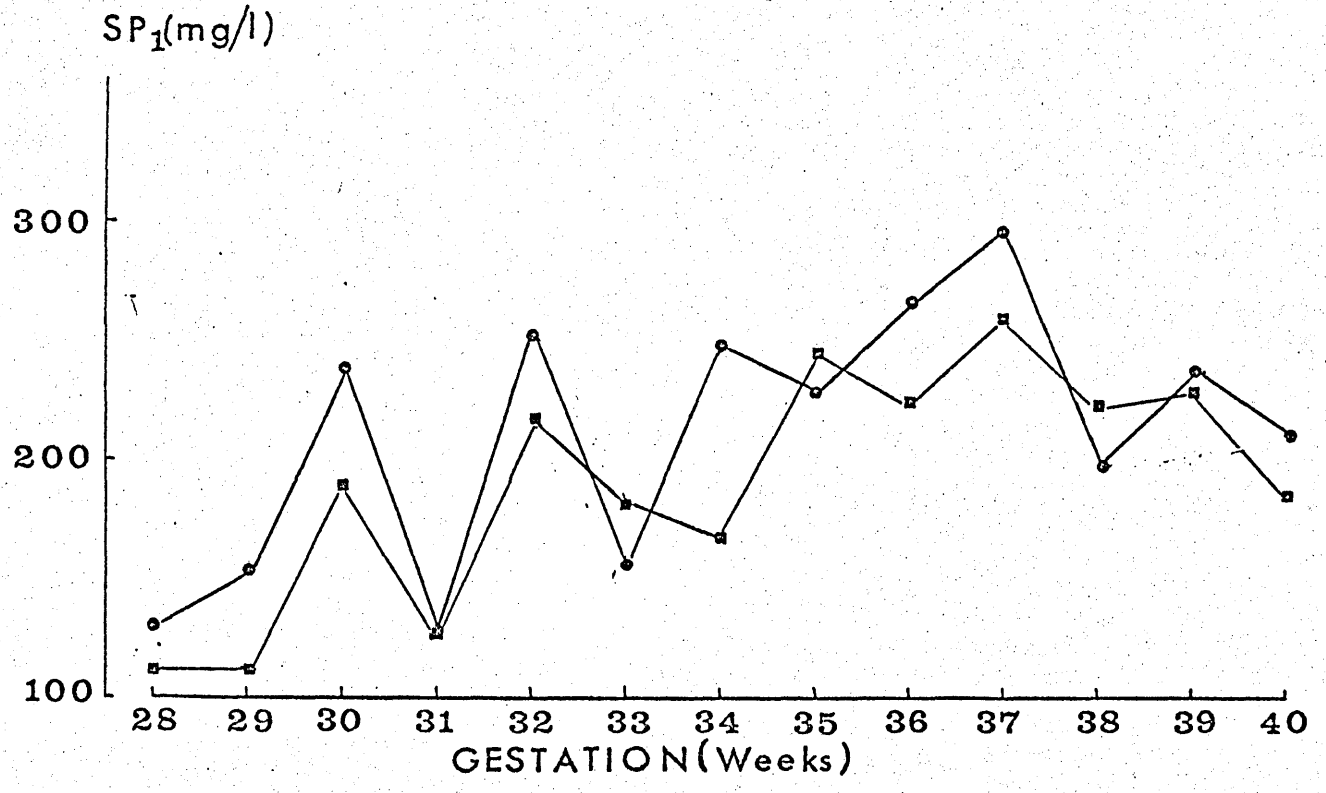

Fig 7 x Median SP_1 concentration during the third trimester of pregnancy in two groups of patients - (■) normal pregnancy, (●) poor weight profile pregnancy.

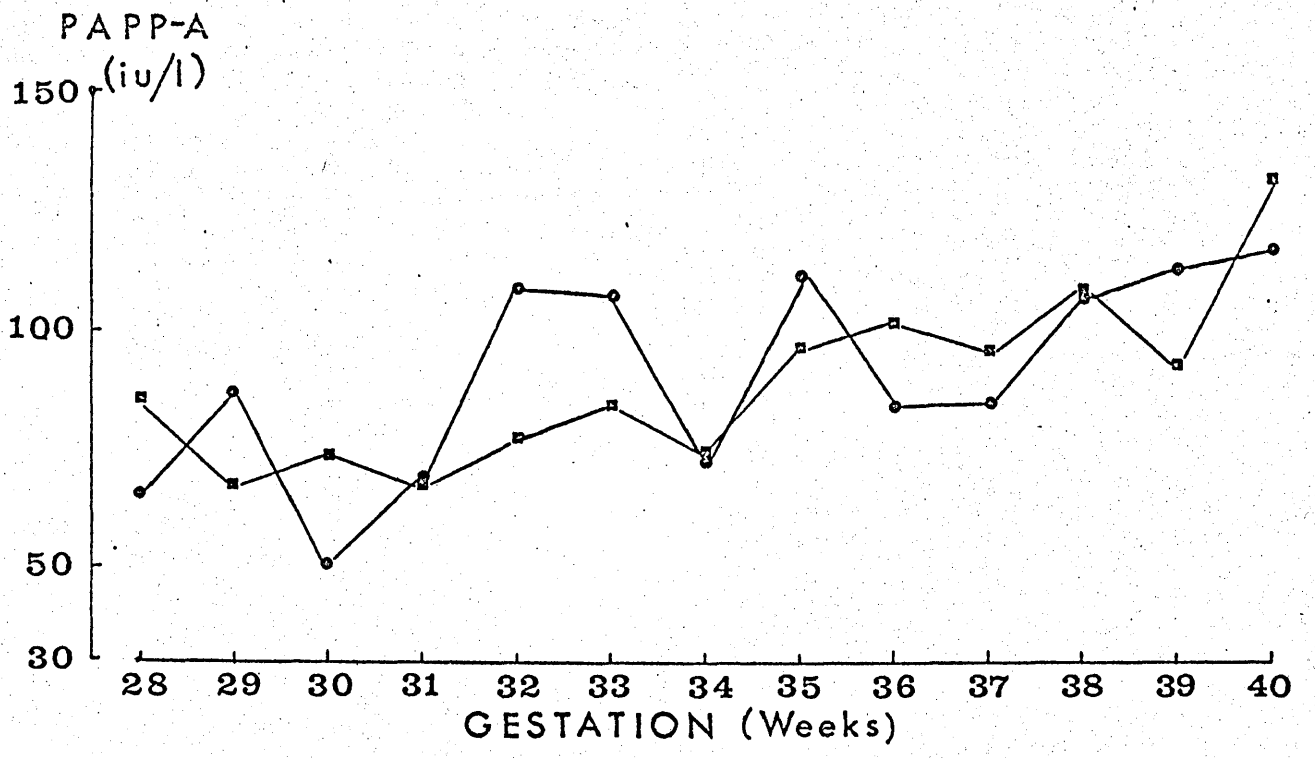

Fig 7 xi Median PAPP-A concentration in the third trimester of pregnancy in two groups of patients - (■) normal pregnancy, (●) poor weight profile pregnancy.

Table 7 V

Intersextile Ranges for concentrations of hPL, SP_1 and PAPP-A in Poor weight Profile and Normal Pregnancies

	Poor Weight Profile Group			Normal Group		
Week of Gestation	Lower Sextile	Median	Upper Sextile	Lower Sextile	Median	Upper Sextile
HPL miu/l						
28	3.7	4.5	4.8	3.6	5.0	6.2
29	4.3	4.8	5.8	4.4	5.4	6.8
30	3.8	4.5	6.3	4.9	5.8	7.5
31	5.1	6.7	7.6	5.0	6.8	7.4
32	5.2	6.4	7.6	5.7	6.9	9.2
33	4.9	7.1	8.8	6.3	7.0	8.3
34	5.8	7.1	8.7	5.5	8.1	8.9
35	4.9	6.7	8.7	6.1	7.9	9.2
36	5.4	6.7	7.8	6.6	8.4	9.8
37	5.9	7.8	9.4	7.0	8.7	9.8
38	6.3	7.8	8.2	6.5	8.3	10.4
39	6.3	7.5	9.4	6.4	7.2	9.4
40	6.6	7.6	10.8	6.4	8.9	10.4
SP_1 mg/l						
28	85	131	202	85	112	200
29	97	156	188	87	152	207
30	171	240	277	107	190	280
31	99	128	242	83	127	264
32	218	255	396	73	218	284
33	133	157	313	109	181	282
34	161	250	296	102	167	359
35	163	227	368	113	245	355
36	128	268	351	74	226	350
37	139	298	350	145	259	309
38	130	199	307	95	222	411
39	153	238	469	141	230	382
40	151	213	230	64	185	331
PAPP-A iu/l						
28	53	65	89	56	86	106
29	27	87	91	55	67	107
30	43	50	94	44	74	102
31	69	69	142	56	68	97
32	70	108	158	41	77	145
33	67	106	140	54	84	119
34	48	71	96	56	74	165
35	65	110	155	62	96	128
36	51	83	181	57	101	167
37	61	84	146	68	96	154
38	89	107	160	73	109	167
39	98	112	142	51	93	141
40	77	116	158	79	123	184

previously, all specimens from an individual patient being assayed in a single batch.

7.2.4 Numerical analysis of Data

Comparison of the pregnancy characteristics between the poor weight profile and normal groups was made by use of gaussian statistics. Uncorrected birth weights were used throughout. Comparison of the pregnancy protein concentrations was made by use of the median concentration at varying weeks of gestation, and the Mann Witney U test.

7.2.5 Results

The characteristics of the poor weight profile group compared with the normal group are shown in Table 7 IV. No significant difference in either placental weight or birth weight was observed between the two groups ($p > 0.1$). Figures 7 ix - xi show the median pregnancy protein concentrations throughout the 3^{rd} trimester of pregnancy in the two groups and Table 7 V, the intersextile ranges. For SP_1 and PAPP-A no significant difference in concentrations was observed, whilst for hPL a significant difference was observed only at 1 week (36 weeks gestation $p < .05$).

7.2.6 Discussion

It has been observed that a negative finding in placental function testing may be as useful as a positive result (Chard 1982b). This situation is ideally seen in the present context - reassurance can be issued to the clinician based on a normal hPL concentration in a woman with poor weight gain or static weight. The poor weight group had babies of an equivalent weight to the normal group, suggesting that their lack of maternal weight gain was not central to foetal growth. The

corollary of course is that a low maternal hPL concentration in a woman with poor weight gain is suggestive of placental malfunction and reduced foetal growth. It is shown elsewhere that SP_1 and PAPP-A concentrations do not correlate with placental or birth weight. Therefore these proteins would be or less use in delineating the poor weight profile pregnancy truly at risk as an IUGR pregnancy.

CHAPTER 8

THE USE OF PREGNANCY ASSOCIATED PROTEINS IN THE MONITORING OF PLACENTAL FUNCTION IN THE THIRD TRIMESTER OF PREGNANCIES COMPLICATED BY PREGNANCY INDUCED HYPERTENSION OR DIABETES MELLITUS

8.1 Hypertensive Patients

8.1.1 Objectives

Hypertension in pregnancy has been recognized for many years as jeopardizing the foetus. In hypertensive pregnancies, placental function may become impaired due to pathological narrowing of the spiral arterioles which supply the intervillous space (Robertson et al 1967) and may result in reduced placental perfusion by maternal blood. This in turn may lead to the development of a dysmature baby or more acutely, fetal hypoxia and distress. Monitoring of placental function in this disorder should therefore be able to predict the development of these problems and varying claims have been made (for a review see Curzon 1979). The possible use of SP_1 and PAPP-A in monitoring hypertensive pregnancies is less clearly defined although it has been claimed that PAPP-A is elevated before clinical signs of pre eclampsia occur (Lin et al 1977, Hughes et al 1980, Klopper and Hughes 1980).

8.1.2 Clinical Material

Patients comprising this group were drawn from clinics held at the Royal Maternity Hospital and Stobhill Hospital, Glasgow. Patients from Stobhill Hospital were also involved in a trial of the drug Atenolol (a β blocker) in the reduction of hypertension. Thus some Stobhill patients were on drug treatment and others received a placebo. Preliminary investigation had shown that the varying treatments did not affect the parameters under discussion here.

Pregnancy induced hypertension (PIH) was defined either as a sustained diastolic blood pressure of $\geq$ 90 mmHg with or without evidence of oedema or proteinuria, or for Stobhill patients

as a systolic pressure of $>$ 140 mmHg/diastolic pressure of $>$ 90 mmHg on 2 occasions 24 hours apart.

Venous blood specimens were taken at several stages throughout pregnancy.

Hypertensive pregnancies resulting in a light for dates baby (LFD) were excluded from this data as they will be considered elsewhere (7.1).

8.1.3 Analytical Methods

HPL, SP_1 and PAPP-A were measured by the methods given previously, all specimens from an individual patient being assayed in a single batch.

8.1.4 Numerical Analysis of Data

Comparison of the hypertensive pregnancy characteristics with normal pregnancies was made by use of gaussian statistics.

As the serum concentration of SP_1 and PAPP-A was found to be skewed (Chapter 7), comparison of protein concentrations was made by use of the median and Mann-Witney test.

8.1.5 Results and Discussion

The hypertensive population comprised a heterogeneous group in that some were treated with Atenolol, some with placebo and some were not involved in any drug trial. However no significant differences could be shown between these groups for any of the parameters under consideration. It is thus valid to combine these patients into a single group. Further since many of the patients in this group were under "shared care" with their GP, it was not possible to obtain the maternal weight details required to correct the baby's birth weight. Actual birth weights have therefore been used in this section.

Table 8 I

Characteristics of Hypertensive Pregnancies and Normal Pregnancies

	Hypertensive	Normal	
Mean Birth Weight (kg)	3.27	3.41	
SD	0.5	0.42	$p > 0.1$
n	82	80	
Mean Placental Weight (g)	587	664	
SD	154	121	$t = 2.9$
n	76	67	$p < 0.01$

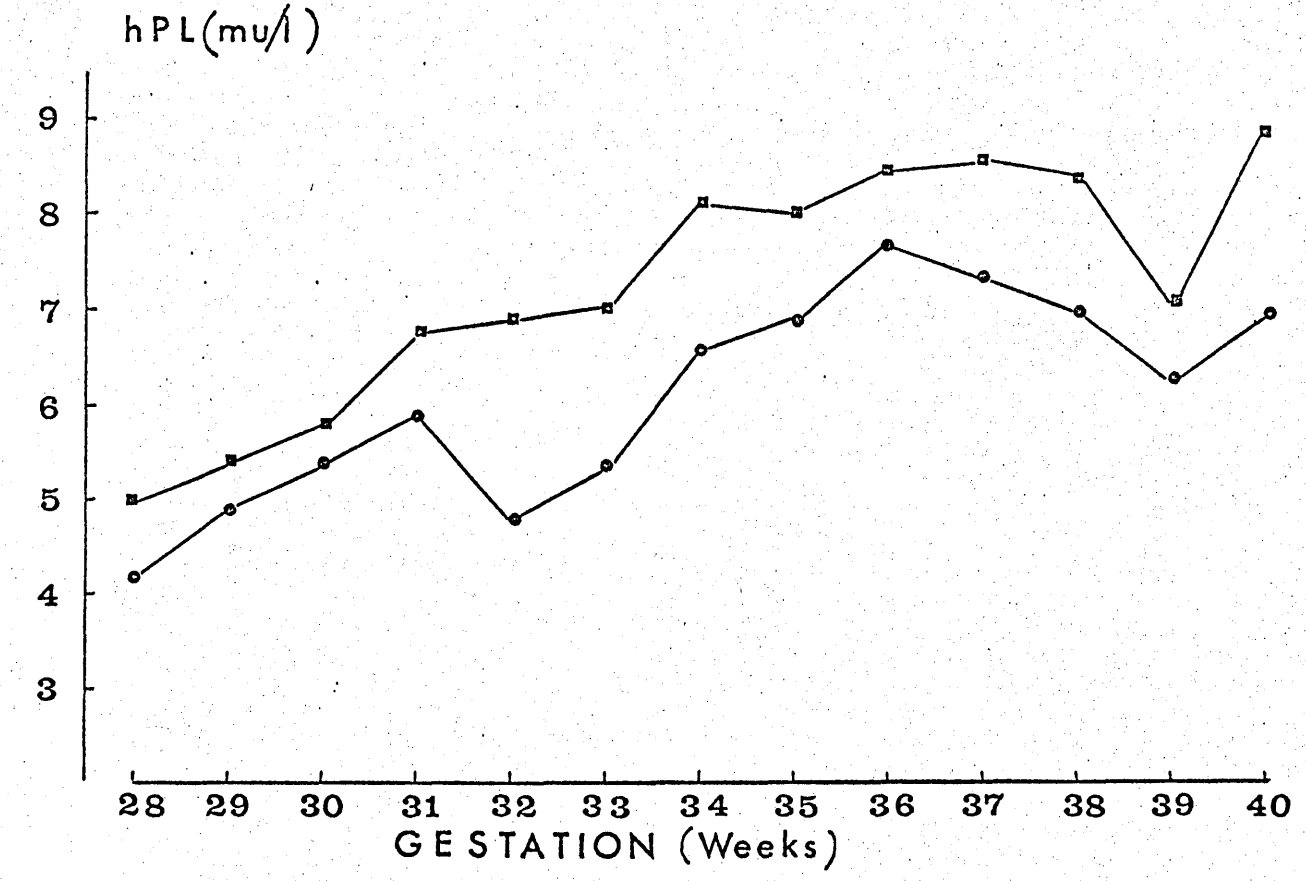

Figure 8 i Median hPL concentration in the third trimester of pregnancy in two groups of patients - (■) normal pregnancy, (●) pregnancy complicated by PIH (after exclusion of LFD pregnancies).

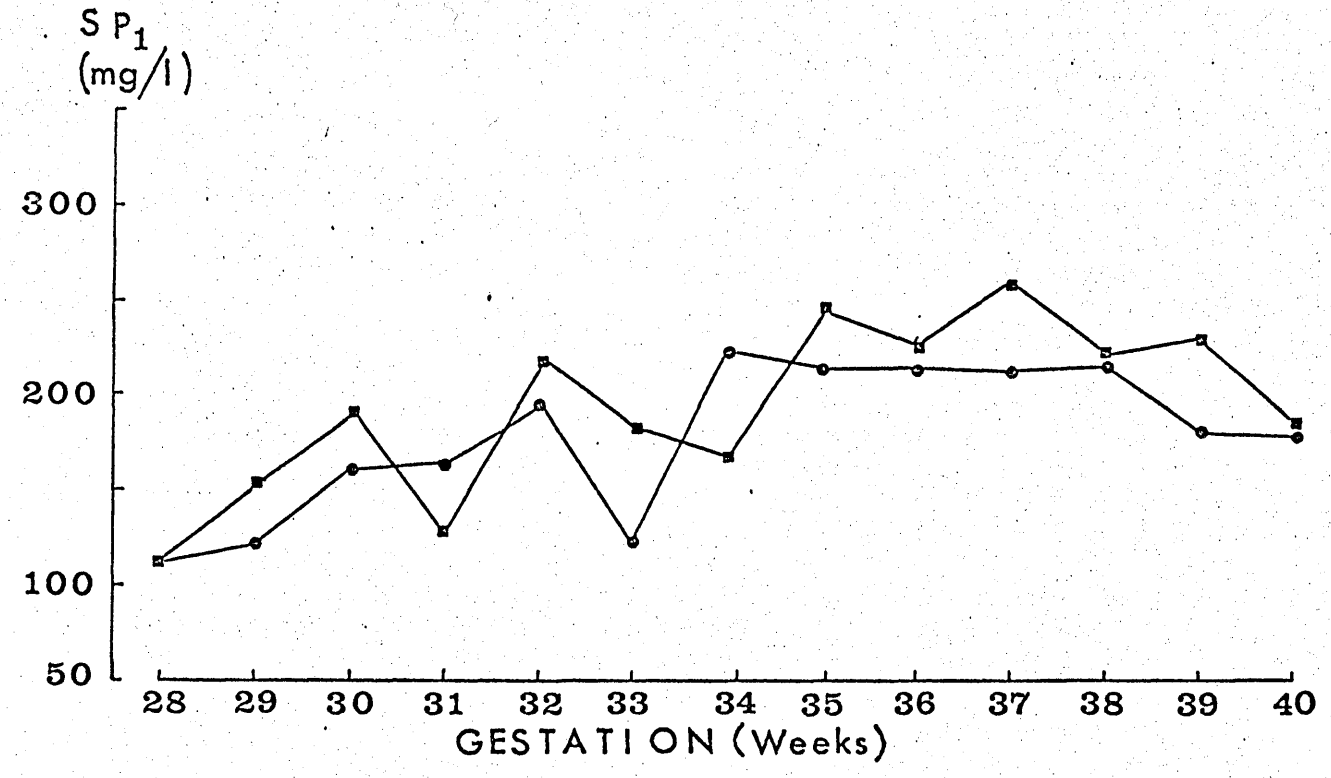

Figure 8 ii Median SP_1 concentration in the third trimester of pregnancy in two groups of patients - (■) normal pregnancy, (●) pregnancy complicated by PIH (after exclusion of LFD pregnancies).

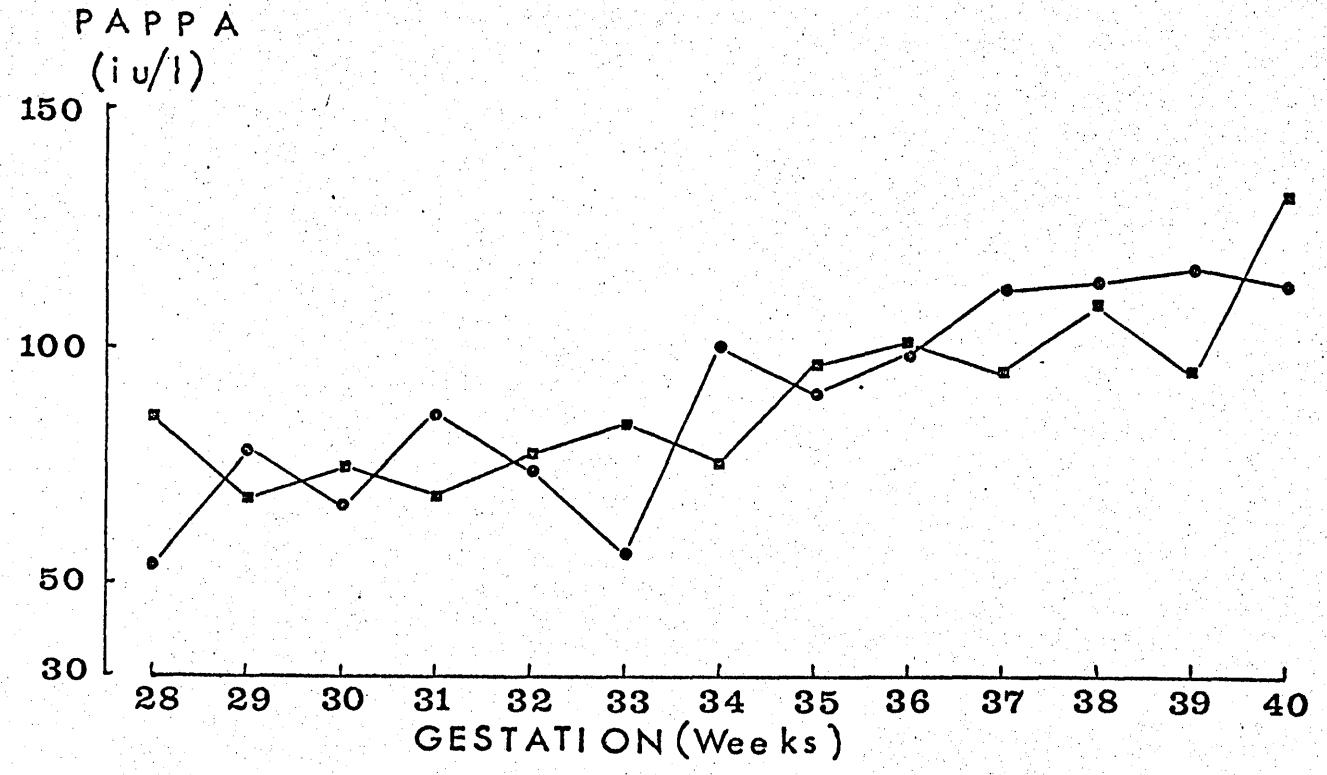

Figure 8 iii Median PAPP-A concentration in the third trimester of pregnancy in two groups of patients - (■) normal pregnancy, (●) pregnancy complicated by PIH (after exclusion of LFD pregnancies).

Table 8 II

Intersextile Ranges for Concentrations of hPL, SP_1 and PAPP-A in PIH and Normal Pregnancies

	Week of Gestation	PIH Group			Normal Group		
		Lower Sextile	Median	Upper Sextile	Lower Sextile	Median	Upper Sextile
HPL miu/l	28	2.9	4.2	5.4	3.6	5.0	6.2
	29	4.2	4.9	7.1	4.4	5.4	6.8
	30	2.5	5.4	6.3	4.9	5.8	7.5
	31	4.8	5.9	6.5	5.0	6.8	7.4
	32	4.2	4.8	6.7	5.7	6.9	9.2
	33	4.7	5.4	7.3	6.3	7.0	8.3
	34	4.9	6.6	8.2	5.5	8.1	8.9
	35	5.0	6.9	9.2	6.1	7.9	9.2
	36	4.5	7.7	10.3	6.6	8.4	9.8
	37	4.7	7.4	9.8	7.0	8.7	9.8
	38	4.2	7.0	8.9	6.5	8.3	10.4
	39	4.0	6.3	7.9	6.4	7.2	9.4
	40	4.1	7.0	8.5	6.4	8.9	10.4
SP_1 mg/l	28	88	112	186	85	112	200
	29	98	122	149	87	152	207
	30	104	161	199	107	190	280
	31	103	165	298	83	127	264
	32	112	195	246	73	218	284
	33	96	120	256	109	181	282
	34	147	225	270	102	167	359
	35	116	212	275	113	245	355
	36	121	214	330	74	226	350
	37	120	213	333	145	259	309
	38	137	221	350	95	222	411
	39	136	179	267	141	230	382
	40	157	178	373	64	185	331
PAPP-A iu/l	28	24	56	98	56	86	106
	29	45	79	108	55	67	107
	30	10	68	131	44	74	102
	31	49	86	116	56	68	97
	32	48	74	131	41	77	145
	33	25	56	110	54	84	119
	34	41	100	119	56	74	165
	35	53	90	128	62	96	128
	36	66	98	135	57	101	167
	37	72	113	158	68	95	154
	38	63	114	151	73	109	167
	39	71	117	170	51	93	141
	40	44	113	134	79	123	184

Table 8 I shows the characteristics of the hypertensive group compared with the normal group. Although placental weight was significantly lower in the hypertensive group, no significant difference in birth weight was found.

A positive correlation between peak (36-38 week) hPL concentration and birth weight was obtained ($r = 0.35$ $p < 0.01$ $n = 76$), but no correlation between birth weight or placental weight and either SP_1 or PAPP-A was found.

Figures 8 i - iii show the trend in placental protein concentration in the hypertensive group compared with the normal population and Table 8 II shows the intersextile ranges. From week 32 onwards, for hPL there was usually a significant difference in concentrations between the two groups, the hypertensive population having lower concentrations. No difference in SP_1 or PAPP-A concentrations was found between the groups. The decreased hPL concentrations in the hypertensive group may be related to the significantly lower placental mass observed for this group. As SP_1 and PAPP-A have been shown not to correlate with placental weight, the lack of difference is not surprising. However, several reports have alluded to elevated serum PAPP-A concentrations in hypertensive patients (Lin et al 1977, Hughes et al 1980, Klopper and Hughes 1980).

This finding is not confirmed here and is in agreement with Westergaard and Teisner (1982). The differences cannot be accounted for by the fact that some patients were treated with antihypertensive agents, as no differences in PAPP-A concentrations were found comparing placebo treated patients with drug treated patients. The answer may lie in the fact that many earlier studies utilized plasma specimens. The observation

that heparin levels affect the PAPP-A concentrations must cast doubt on the original hopeful observations regarding PAPP-A in hypertensive pregnancies.

From the findings presented here it is apparent that SP_1 or PAPP-A concentrations have little to offer in the monitoring of placental function in hypertensive pregnancies. HPL is likely to remain the mainstay therefore.

8.2 Diabetic Patients

8.2.1 Objectives

The advent of insulin therapy for diabetics meant that more diabetic women reached childbearing age. The pregnant diabetic however posed many problems. She was at risk of developing ketoacidosis, the foetus was at risk of congenital malformation or intrauterine death. If delivered successfully, the baby often encountered problems of respiratory distress and hypoglycaemia.

With improved preparations of insulin, improved dietary instruction and obstetric care many of these problems are no longer as serious. However most obstetricians still monitor their pregnant diabetic patients very closely.

There is however no consensus opinion on what levels of hPL represent "normality" for the diabetic. There have also been links suggested between SP_1 and carbohydrate metabolism (Tatra 1976, Singh 1979).

The possible use of pregnancy proteins in monitoring diabetic pregnancies still requires further investigation.

8.2.2 Clinical Material

27 pregnancies complicated by diabetes mellitus were studied,

Table 8 III

Characteristics of Patients Studied

a) Diabetic patients with placental weight > 90th centile

	Placental Weight (g)	Corrected Birth Weight (kg)	Gestation (weeks)	Peak hPL (mu/l)	Peak SP_1 (mg/l)	Peak PAPP-A (IU/L)
1	680	3.39	38	15.6	331	199
2	800	3.27	38	12.0	262	70
3	820	3.27	37	11.5	568	115
4	1000	4.18	38	11.2	687	154
5	700	4.03	38	14.4	267	68
6	730	2.64	36	11.6	392	225
7	750	3.34	36	12.8	634	164
8	630	2.43	37+	12.0	155	55
9	950	3.74	38	6.3	486	114
10	860	3.63	37	10.3	527	117
11	670	4.18	38	7.6	483	163
12	840	3.69	38+	9.2	377	97
13	700	3.26	38	9.0	233	177

b) Non diabetic controls with placental weight > 90th centile

1	680	3.43	39+	8.2	276	87
2	800	3.60	39	9.1	390	141
3	770	3.71	40	9.7	287	90
4	900	4.16	38	9.5	433	244
5	690	3.10	40	10.4	210	113
6	775	3.17	40	7.7	289	96
7	765	4.21	40+	6.5	123	109
8	590	3.50	37+	8.6	400	141
9	920	3.58	39+	9.0	125	101
10	800	4.03	40	9.9	324	128
11	650	3.23	39+	8.0	376	78
12	775	3.07	40	7.8	287	140
13	770	3.71	40	9.7	269	88

Table 8 IV

Characteristics of Patients Studied

a) Diabetic patients with placental weight < 90th centile

	Placental Weight (g)	Corrected Birth Weight (kg)	Gestation (weeks)	Peak hPL (mu/l)	Peak SP_1 (mg/l)	Peak PAPP-A (IU/L)
1	430	3.14	38	6.8	437	84
2	450	3.30	37+	5.9	130	78
3	620	2.77	37+	9.6	362	80
4	600	3.05	37	6.0	470	27
5	610	3.40	38	8.8	485	196
6	450	2.20	37	6.9	326	70
7	540	3.72	38	7.8	364	120
8	580	3.75	38	5.1	279	129
9	630	3.06	38	5.2	555	90
10	520	2.92	38	6.3	202	181
11	620	2.93	40	9.7	495	142
12	640	3.59	37+	6.8	183	89
13	530	3.13	37+	5.3	213	127
14	580	3.66		7.1	475	38

b) Non diabetic controls with placental weight < 90th centile

1	440	3.24	39+	5.4	306	130
2	490	2.72	38	8.9	145	129
3	600	2.94	40	8.1	166	104
4	580	2.80	40	9.3	60	128
5	600	3.21	38+	6.3	175	175
6	470	2.56	37+	7.9	261	128
7	550	3.04	39+	5.7	181	129
8	540	2.96	40	7.7	300	124
9	620	3.31	40	6.1	204	91
10	500	2.98	39+	7.1	216	145
11	600	3.24	39	5.2	243	17
12	650	3.43	40+	8.0	300	107
13	640	3.77	40	7.0	125	113
14	590	3.64	37	5.9	235	80

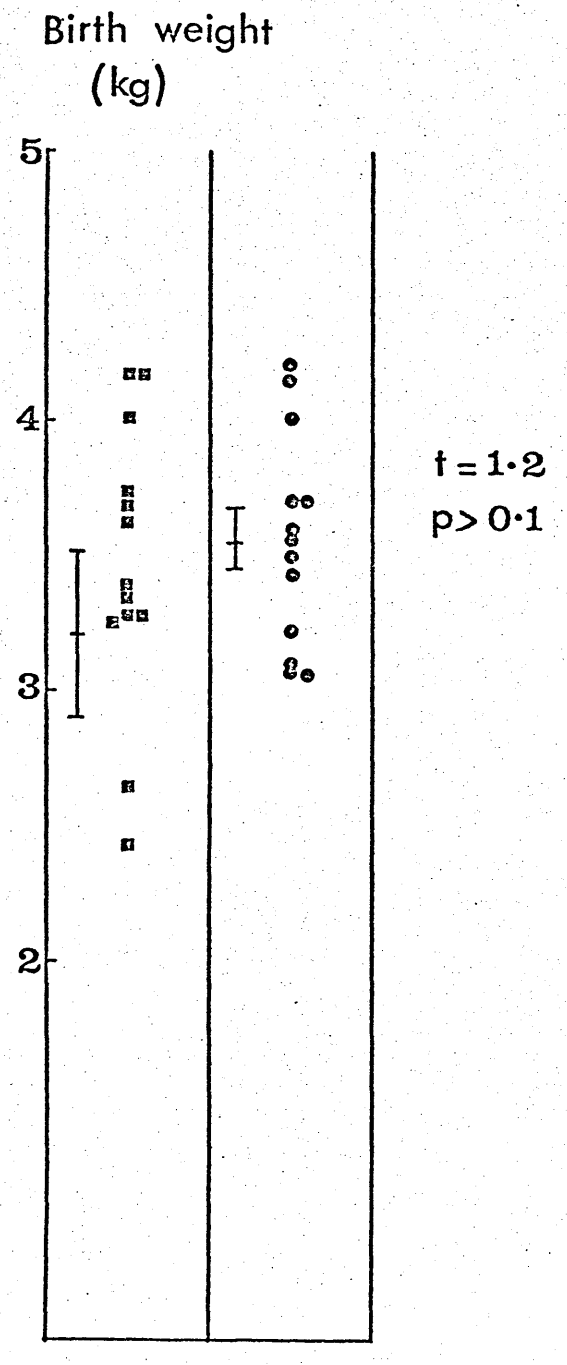

Figure 8 iv Birth weight of babies born to diabetic mothers with placental weight > 90th centile (▪) and their non-diabetic controls (●). The bars represent mean ± SE.

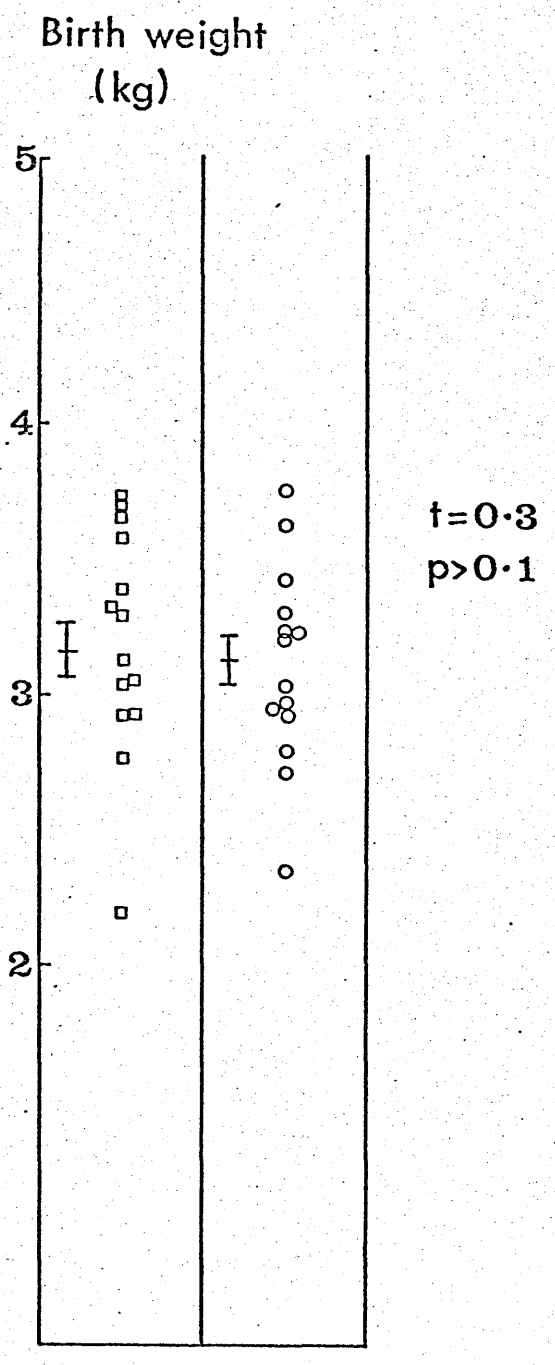

Figure 8 v Birth weights of babies born to diabetic mothers with placental weight < 90th centile (□) and their non-diabetic controls (o). The bars represent mean ± SE.

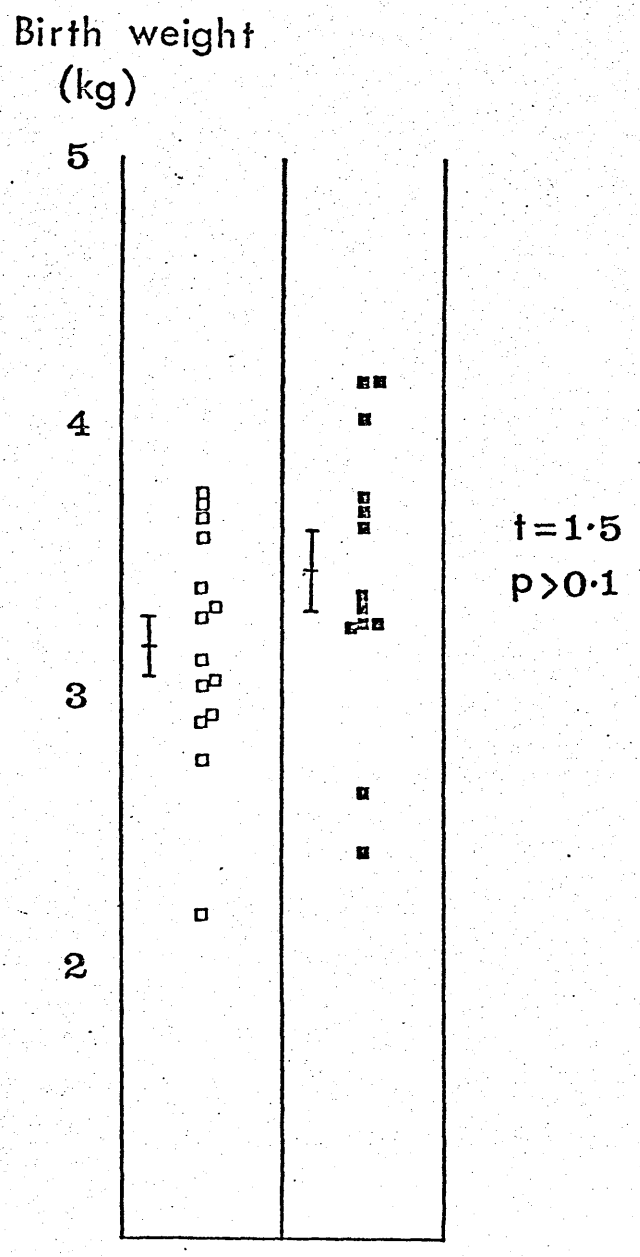

Figure 8 vi Birth weights of babies born to diabetic mothers with placental weight < 90th centile (□) and diabetic mothers with placental weight > 90th centile(■). The bars represent mean ± SE.

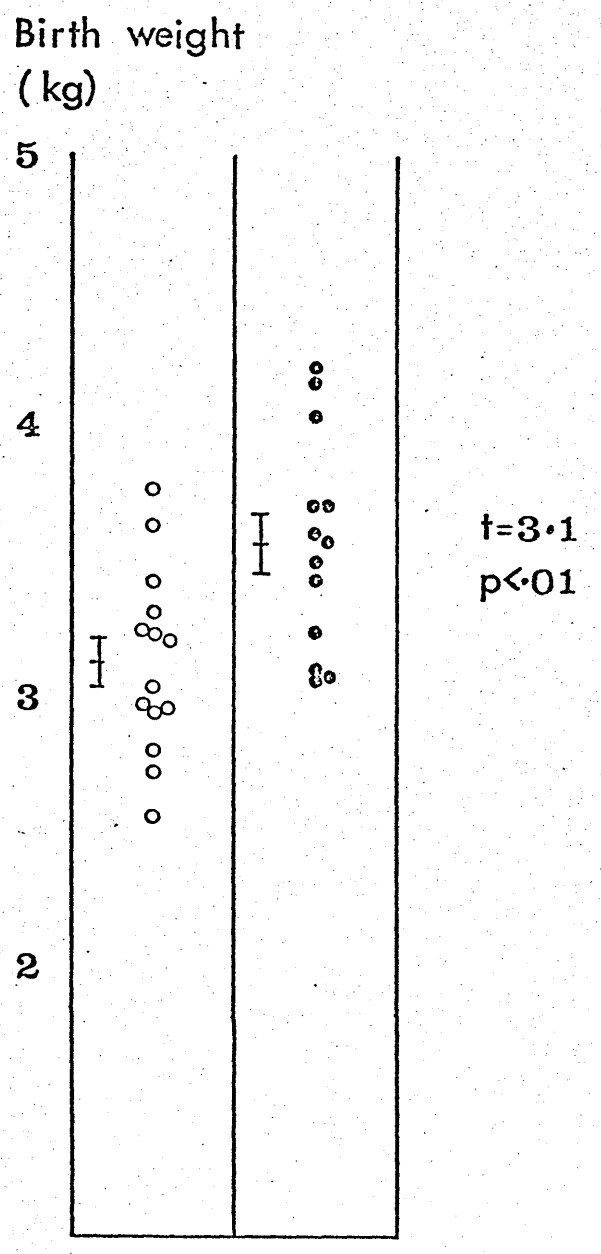

Figure 8 vii Birth weight of babies born to non-diabetic mothers with placental weight < 90th centile (o) and non-diabetic mothers with placental weight > 90th centile (●). The bars represent mean ± SE.

all patients being insulin dependent during pregnancy. All were under the care of a single consultant obstetrician and received normal clinical care associated with this condition. Patients were routinely admitted to hospital for stabilization of their diabetes, and most were delivered prior to term either by syntocinon induction or by Caesarian Section (LUSCS).

8.2.3 Analytical Methods

hPL, SP_1 and PAPP-A concentrations were assayed as described previously. The birth weight and placental weight were recorded at delivery.

8.2.4 Numerical Analysis of Data

After delivery, patients were allocated into one of two groups on the basis of their placental weight being greater than or less than the 90^{th} centile for their gestation. Each individual was matched as closely as possible for placental weight to a non diabetic control subject. For each group of diabetic patients therefore there was a corresponding control group. The characteristics of these groups are shown in Table 8 III and 8 IV. All birth weights were corrected for maternal weight and height (Thomson et al 1968). The corrected birth weights of all babies born to the control group were all $> 10^{th}$ centile for weight.

The presence of significant differences between the groups was tested by students t test, a significant finding being taken as $P \leq 0.05$.

8.2.5 Results

There was no difference in the placental weights of the diabetic patients compared with their respective control groups, so the groups were well matched. Figures 8 iv - 8 vii show the birth

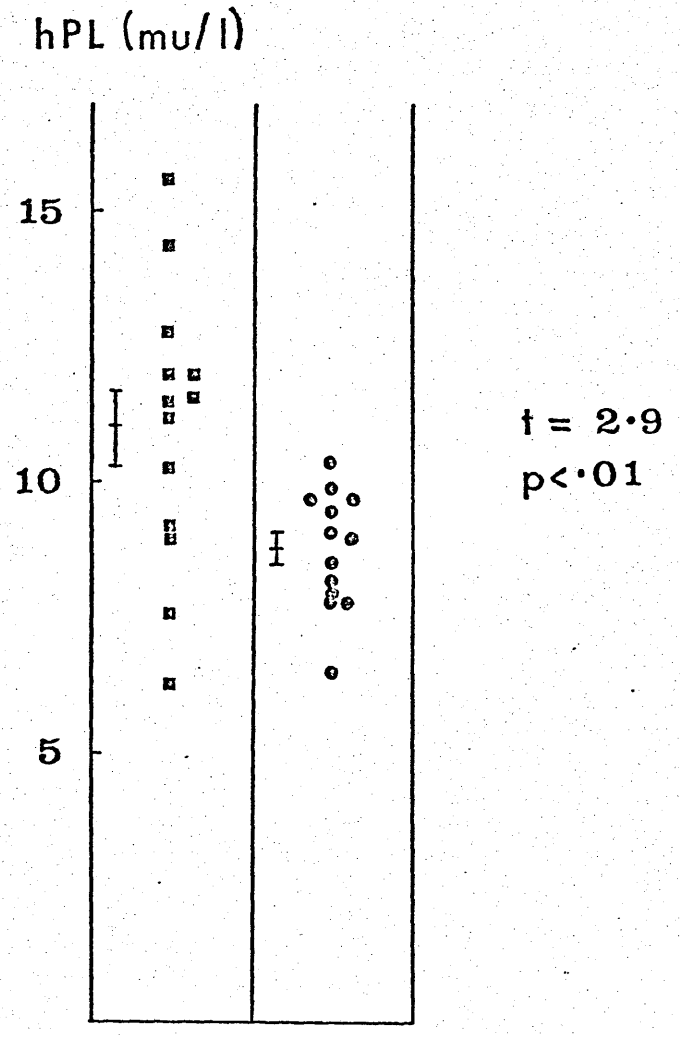

Figure 8 viii Peak hPL concentrations in diabetic mothers with placental weight > 90th centile (▪) and their non-diabetic controls (●). The bars represent mean ± SE.

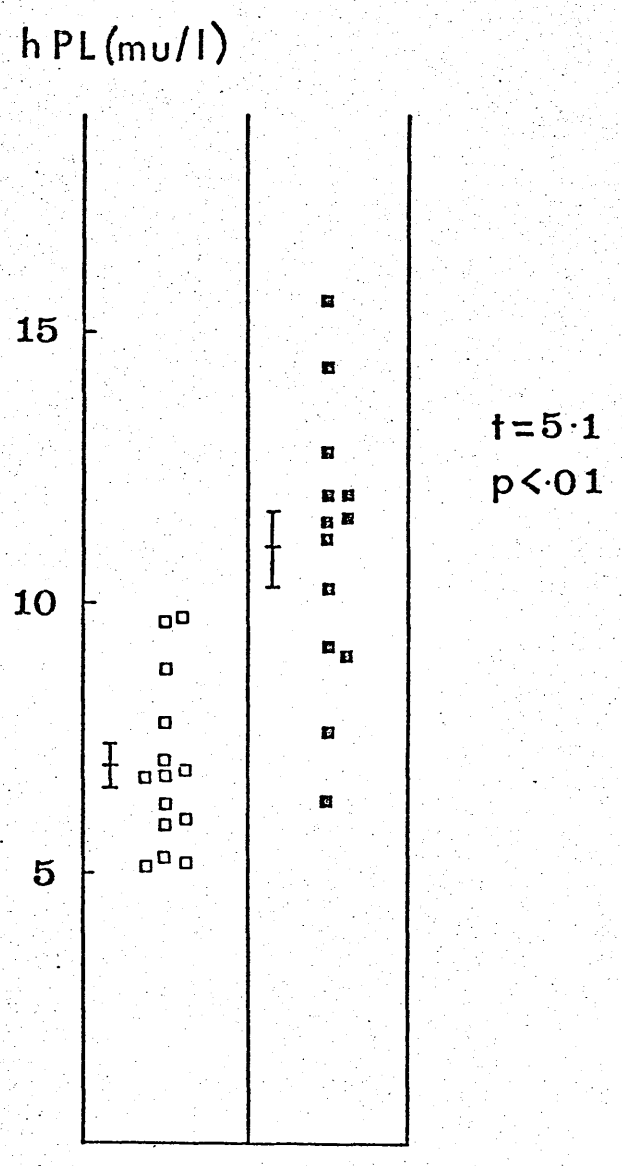

Figure 8 ix Peak hPL concentrations in diabetic mothers with placental weight < 90th centile (□) and > 90th centile (■). The bars represent mean ± SE.

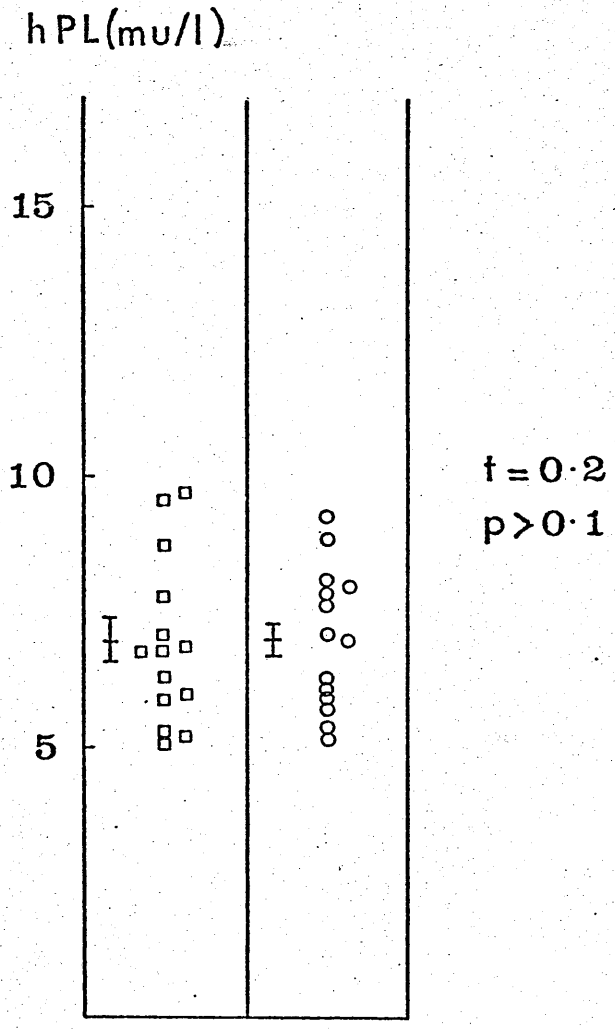

Fig 8 x Peak hPL concentrations in diabetic mothers with placental weight < 90th centile (□) and their non-diabetic controls (o). The bars represent mean ± SE.

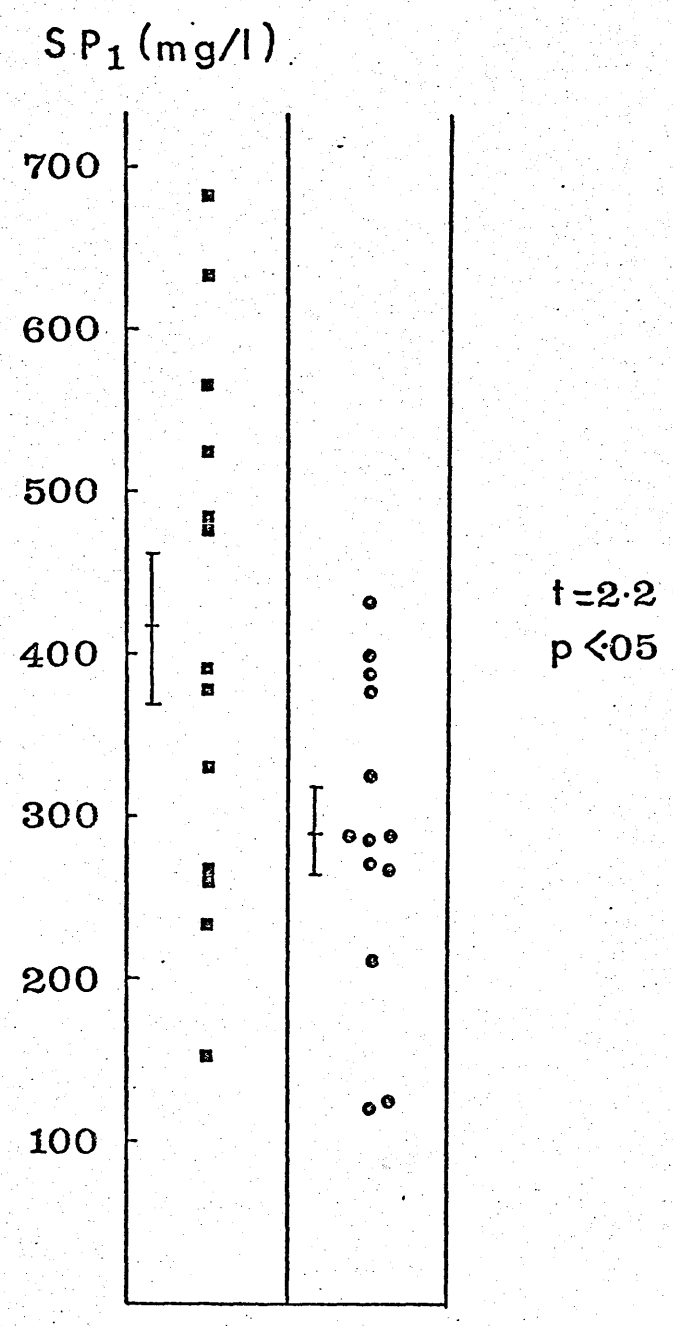

Fig 8 xi Peak SP_1 concentrations in diabetic mothers with placental weight > 90^{th} centile (■) and their non-diabetic controls (●). The bars represent mean ± SE.

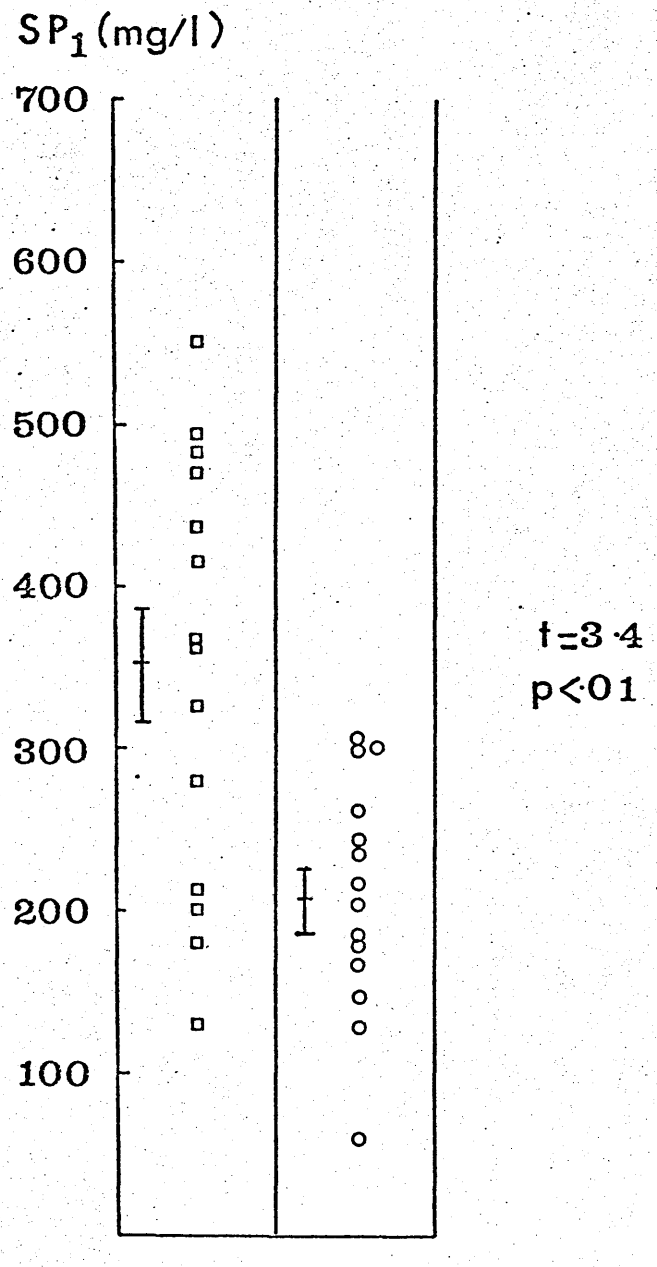

Fig 8 xii Peak SP_1 concentrations in diabetic mothers with placental weights < 90^{th} centile (□) and their non-diabetic controls (o). The bars represent mean ± SE.

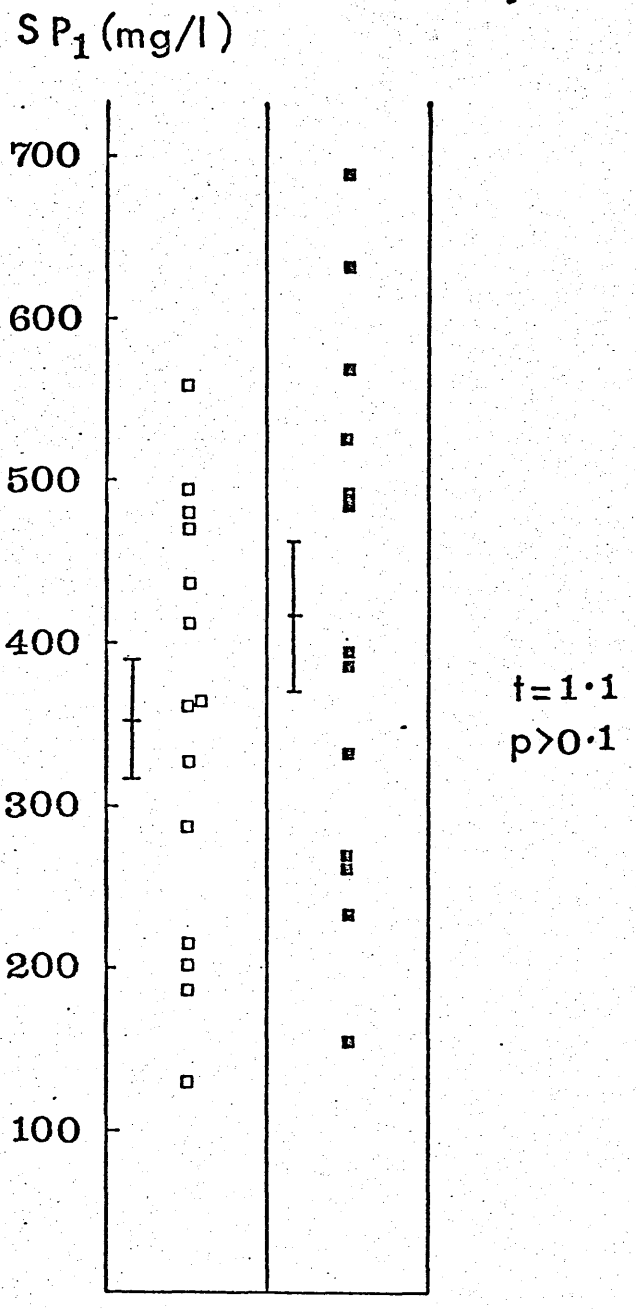

Figure 8 xiii Peak SP_1 concentrations in diabetic mothers with placental weights $< 90^{th}$ centile (□) and diabetic mothers with placental weight $> 90^{th}$ centile (■). The bars represent mean ± SE.

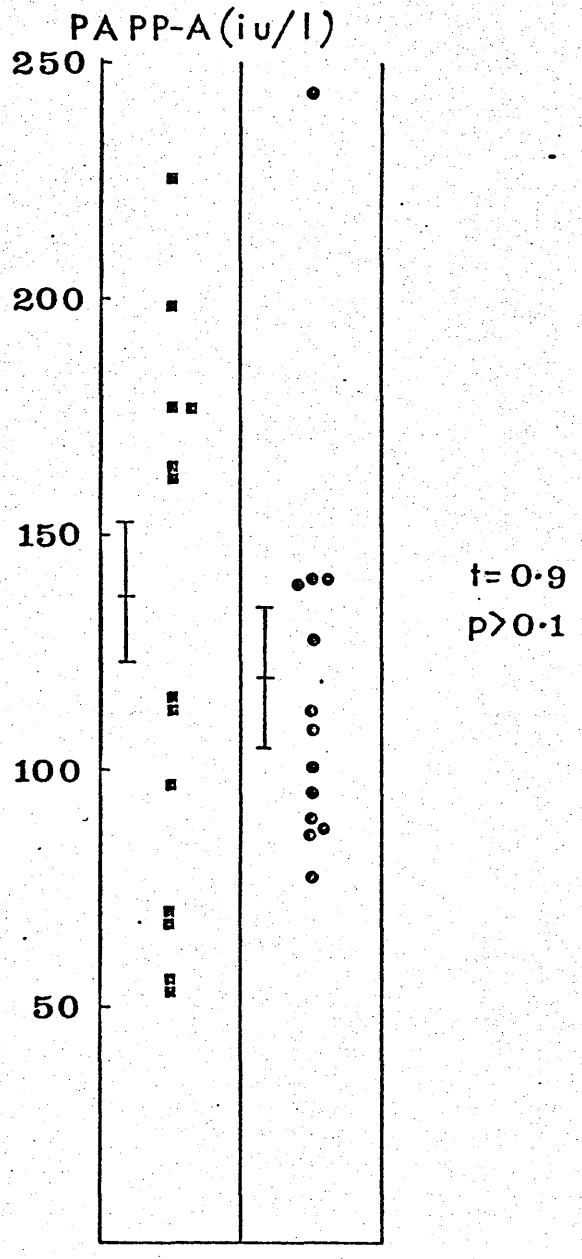

Figure 8 xiv Peak PAPP-A concentrations in diabetic mothers with placental weights > 90th centile (■) and their non-diabetic controls (●). The bars represent mean ± SE.

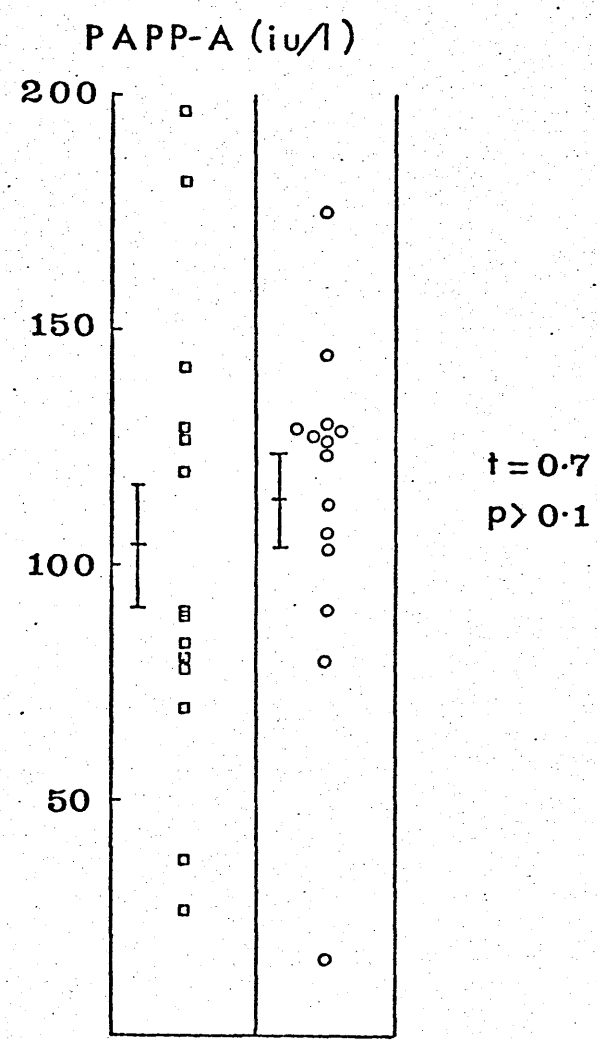

Figure 8 xv Peak PAPP-A concentrations in diabetic mothers with placental weights < 90th centile (□) and their non-diabetic controls (○). The bars represent mean ± SE.

weights for diabetics and controls with placental weight $> 90^{th}$ centile, diabetics and controls with placental weight $< 90^{th}$ centile, diabetics with placental weight $< 90^{th}$ centile and diabetics with placental weight $> 90^{th}$ centile, and non diabetic controls with placental weights $< 90^{th}$ centile compared with controls with placental weights $> 90^{th}$ centile. Only in these latter groups was a significant difference in birth weights observed.

Figure 8 viii shows hPL levels in diabetics and controls with placental weights $> 90^{th}$ centile. HPL was significantly higher in this group of diabetics compared with their non diabetic controls ($p < 0.01$).

Figure 8 ix shows hPL levels in the two diabetic groups (placental weights $< 90^{th}$ centile and $> 90^{th}$ centile). HPL concentrations were higher in the diabetics with the larger placentas ($p < 0.01$). However there was no difference in hPL concentrations between diabetics with placental weights $< 90^{th}$ centile and their non diabetic controls (Fig 8 x).

Figures 8 xi and 8 xii show SP_1 levels in diabetics with placental weights $> 90^{th}$ centile compared with those in their non diabetic controls and in diabetics with placental weights $< 90^{th}$ centile compared with those in their non diabetic controls. No difference in SP_1 concentrations existed when diabetics with placental weights $> 90^{th}$ centile were compared with diabetics with placental weights $< 90^{th}$ centile (Fig 8 xiii).

No difference in PAPP-A concentrations existed between diabetics with placental weights $> 90^{th}$ centile and their controls (Fig 8 xiv), diabetics with placental weights $< 90^{th}$ centile and their controls (Fig 8 xv) or between diabetics with placental weight $> 90^{th}$ centile and diabetics with placental weight < 90th centile.

8.2.6 Discussion

A considerable literature now exists on the use of serum hPL measurements in the investigation of pregnant diabetic patients. Letchworth in a review (1976) reported that some workers found that hPL was higher than in normal pregnancy whereas others have found normal concentrations. The value of hPL measurement in assessing placental function in the diabetic patient remains uncertain. The results obtained here show no significant differences in serum hPL concentration between diabetic and non diabetic women with placental weights $< 90^{th}$ centile. In this type of diabetic subject, low hPL concentrations such as those reported by Ursell et al (1973) may indeed be indicative of poor placental function.

When placental weights were $> 90^{th}$ centile a difference between diabetics and non diabetic subjects became apparent, the diabetics as a group having higher serum hPL concentrations. However the birth weights of babies born to diabetic patients were similar in the two groups irrespective of placental size. This is in contrast to the findings in non diabetic women where the women with placental weights $> 90^{th}$ centile gave birth to babies whose birth weights were significantly larger than those in women with smaller placentae. This is to be expected from the correlation previously found between placental and birth weight.

It seems therefore that there are fundamental functional differences between the hypertrophied placenta of the diabetic patient and the placenta of equivalent weight in the non diabetic subject.

Although clinical control of the patients as judged by blood glucose and haemoglobin A_{1c} was satisfactory and the outcome of pregnancy was satisfactory, it may be that the elevated

the elevated serum hPL levels reflect poorer control as evidenced by the hyper-placentation, rather than good placental function.

In contrast, serum SP_1 concentrations in both groups of diabetics were significantly higher than those in their respective control groups. However no significant difference was found between the serum SP_1 concentrations in the two groups of diabetics. These findings suggest that the higher SP_1 concentrations in the diabetic are associated with the diabetic process rather than placental mass.

The metabolism and function of these proteins are not understood. Both are released from the syncytiotrophoblasts but hPL has a half life of 15 minutes whereas the half life of SP_1 is about 30 hours. It is known that hPL has diabetogenic properties (Felig 1977). This and the short half life argues in favour of participation in the immediate control of carbohydrate metabolism.

The relationship between SP_1 levels and carbohydrate is equally unclear. Tatra (1976) found elevated SP_1 levels after insulin induced hypoglycaemia and Singh (1979) found SP_1 concentrations above the mean in a number of patients showing abnormal glucose tolerance tests. Grudzinskas (1979) however found SP_1 concentrations lying between the 80% confidence limits. Gemmell (1982) found a wide scatter of SP_1 concentrations 74% of results falling within the normal range.

All these studies however plotted SP_1 concentrations against the normal range. No attempt was made to match patients for placental weights against non diabetic controls. This may account for the findings in this study. It is interesting

that these differences were found in diabetics all of whom were clinically well controlled. Further investigation of SP_1 and hPL measurements in relation to carbohydrate metabolism are necessary.

Sutcliffe et al (1982) and Lin et al (1977) showed PAPP-A levels to be slightly decreased compared with non diabetic levels, although in the latter study the difference was not significant. This study does not confirm these findings. There was no significant difference in PAPP-A concentrations between either diabetic group and their non diabetic controls. Similarly there was no difference in PAPP-A levels in diabetics compared with the overall population. PAPP-A estimation is unlikely to be of use in monitoring diabetic pregnancies.

Whilst the findings relating to SP_1 levels in pregnant diabetics are of interest, a wider study is required before a use for this protein in monitoring pregnancies complicated by diabetes can be recommended. With the improvement in diabetic care it may be argued that intensive care of the pregnant diabetic is now less important. Nonetheless most obstetricians still monitor placental function closely in this condition. Providing the constraints mentioned are borne in mind, hPL is likely to remain in favour as a monitor of placental function.

CHAPTER 9

USE OF THE ONCODEVELOPMENTAL PROTEINS AS INDICATORS OF TUMOUR ACTIVITY

9.1 Introduction

The abnormal cellular function seen in neoplastic cells is often reflected in the elaboration of the onco-developmental proteins by cells which do not normally produce them (ectopic production) or in excess quantities by cells normally producing them (eutopic production).

Use has been made of a number of these proteins in diagnosis and monitoring of a variety of tumours for many years, in particular hCG for choriocarcinoma and teratoma, AFP for liver tumours and CEA for colonic tumours.

It is possible that the more recently discovered oncofoetal antigens may offer an advantage over existing markers in terms of earlier prediction of relapse. Alternatively these antigens may be effective as markers of tumours which are conventionally regarded as marker negative.

These approaches will be studied by consideration of testicular teratomas, for which conventional markers and recognized therapies exist, testicular seminomas for which no conventional markers exist but therapy is effective, and ovarian cancer for which no conventional markers exist and therapy is often ineffective.

9.2 Methods of Assay

The oncodevelopmental proteins AFP and hCG were assayed using the methods detailed in Chapter 2. CPAP, SP_1 and PAPP-A were assayed by ELISA as detailed in Chapter 3. The ELISA for SP_1 was sensitized as described and the assay for PAPP-A was sensitized in a similar manner ie by prolonging the incubation times, and omitting pre-dilution of serum samples.

Details of the assay performances over the period of this study are shown in Table 9 I.

Table 9 I

The Oncodevelopmental Proteins as Tumour Markers: Details of Assay Performance

	AFP	hCG	CPAP	SP_1	PAPP-A
Number of Assays	30	27	22	22	12
Mean QC Concentrations	50.0 iu/ml	48.1 miu/ml	1.05 iu/l	55.8 µg/l	71.6 iu/l
Overall Standard Deviation	4.5	2.4	0.16	3.26	8.5
Coefficient of Variation	9%	5%	15.5%	5.8%	11.9%
Mean Detection Limit	3.4 iu/ml	2.1 miu/ml	0.12 iu/l	5.6 µg/l	3.6 iu/l
Normal Range	0-15 iu/ml	0-5 miu/ml	0-2.0 iu/l	-	-

9.3 Investigation of Oncodevelopmental Protein Concentrations in the Normal Population

For certain of the analytes measured, knowledge of the reference range already existed either from previous work (eg for AFP) or from this study (eg for hCG, CPAP) (Table 9 I). However, when SP_1 and PAPP-A levels were assayed in sera from a group of laboratory staff, many were found to have concentrations below the limit of detection of the assays. Assignment of a reference range could therefore not be made in the usual way and discussion of concentrations of these proteins will be made by reference to the detection limits (Table 9 I).

9.4 Patients Studied

9.4.1 Specimens from Patients

As the intention was to investigate the occurence of certain oncodevelopmental proteins in neoplasia only patients on whom pre-operative specimens were available were included in this study. On some patients, serial post-operative specimens were also obtained. In some instances, insufficient sample existed for the assay of all proteins.

9.4.2 Patients with Testicular Teratoma

25 patients were investigated of which 7 were classified as stage I, 6 as stage II, 3 as stage III and 9 stage IV on histological grounds.

9.4.3 Patients with Testicular Seminoma

13 patients were investigated. All diagnoses were confirmed by histology.

9.4.4 Patients with Ovarian Carcinoma

Pre-operative specimens were obtained from 29 patients. All

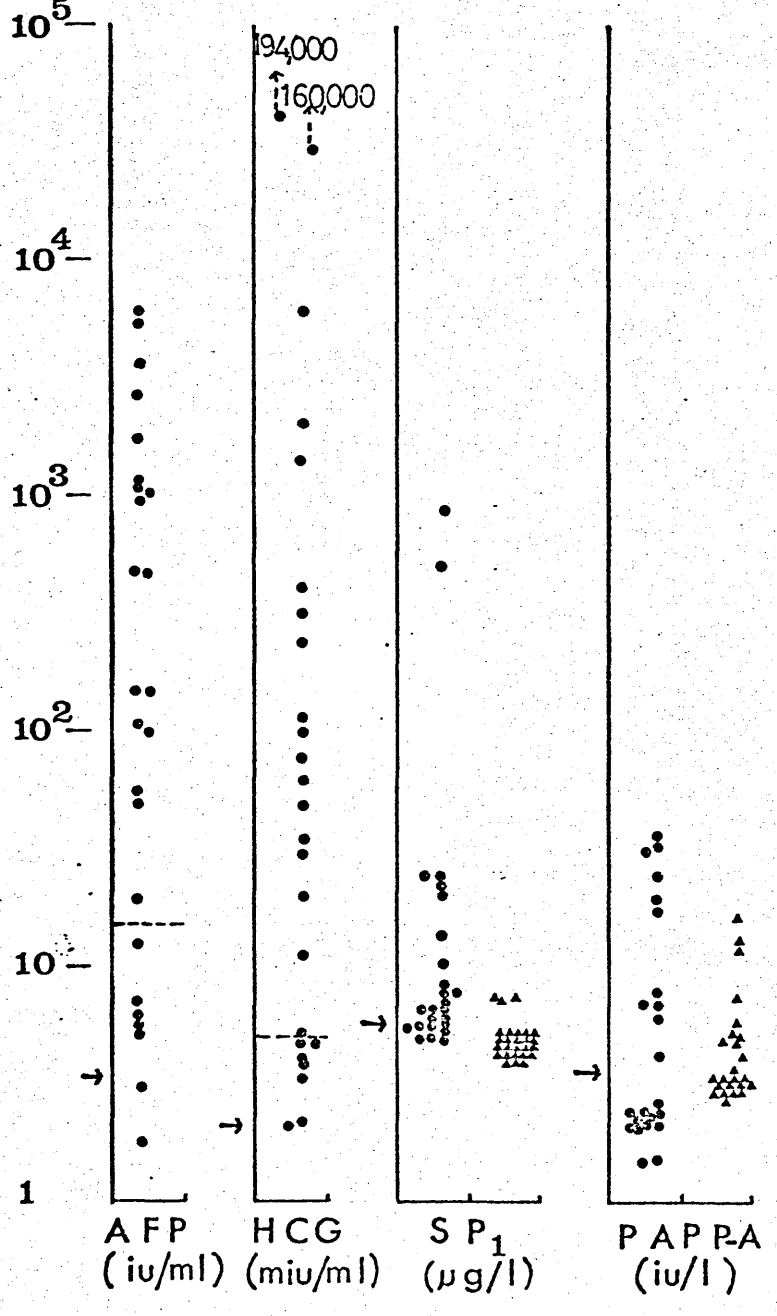

Figure 9 i Pre-operative onco-developmental protein concentrations in Patients with Testicular Teratoma.

- - - - represents the upper limit of normal

⟶ represents the mean detection limit

Table 9 II

Occurence of Pre-operative Markers in Patients with Testicular Teratoma

Patients	AFP	hCG	SP_1	PAPP-A	CPAP	Stage
PB	+	+	+	+		IV
FMcC		+	+			III (Died)
IS	+	+				IV
JSc	+	+	+	+		II
BL	+	+	+	+	+	IV
JSt		+	+			IV (Died)
GM						I
AW	+	+	+	+		IV (Died)
LM				+		I
MH	+	+		+		II
AS	+	+		+		IV
AK						I
TMcN						II
DH	+	+				I
JB			+	+		I
CS	+	+				III
JD	+	+	+			II
AA	+	+				II
TP	+		+			IV
DM	+	+				IV
DN	+	+				I
SM	+	+				IV
RC	+	+				I
DC	+					II
NG	+					III

tumours were examined by the same pathologist in order to ensure consistency of diagnosis. 11 patients were suffering from poorly differentiated adenocarcinoma, 9 had papillary cystadenocarcinoma, 3 had adenocarcinomata of endometrioid type and 2 were borderline. There were also 3 individual cases, 1 of malignant teratoma, 1 of granulosa cell tumour and 1 mucinous cystadenocarcinoma. In the final patient a full pathology report was not available.

9.5 Results

9.5.1 Normal Subjects

3/25 (12%) of normal male sera and 5/33 (15%) of normal female sera were found to contain SP_1 concentrations above the mean detection limit (5.6 µg/l).

For PAPP-A concentrations in normal sera, the figures were 11/24 (46%) and 13/24 (56%) respectively.

9.5.2 Patients with Testicular Teratoma

The concentrations of the various oncodevelopmental proteins found in this group of patients pre-operatively are shown in Figure 9 i and Table 9 II. One patient had an elevated CPAP concentration. 72% of patients had elevated AFP and 68% elevated hCG (Table 9 II). On combining these conventional markers of teratoma, 60% of patients showed elevation of both AFP and hCG. The worst detection limit achieved during SP_1 assay for this group of patients was 7.8 µg/l; 40% of patients had SP_1 concentrations in excess of this. Similarly, for PAPP-A, 30% of patients had concentrations exceeding the worst detection limit which in the present context is taken as the decision threshold. The use of serial marker assays in the

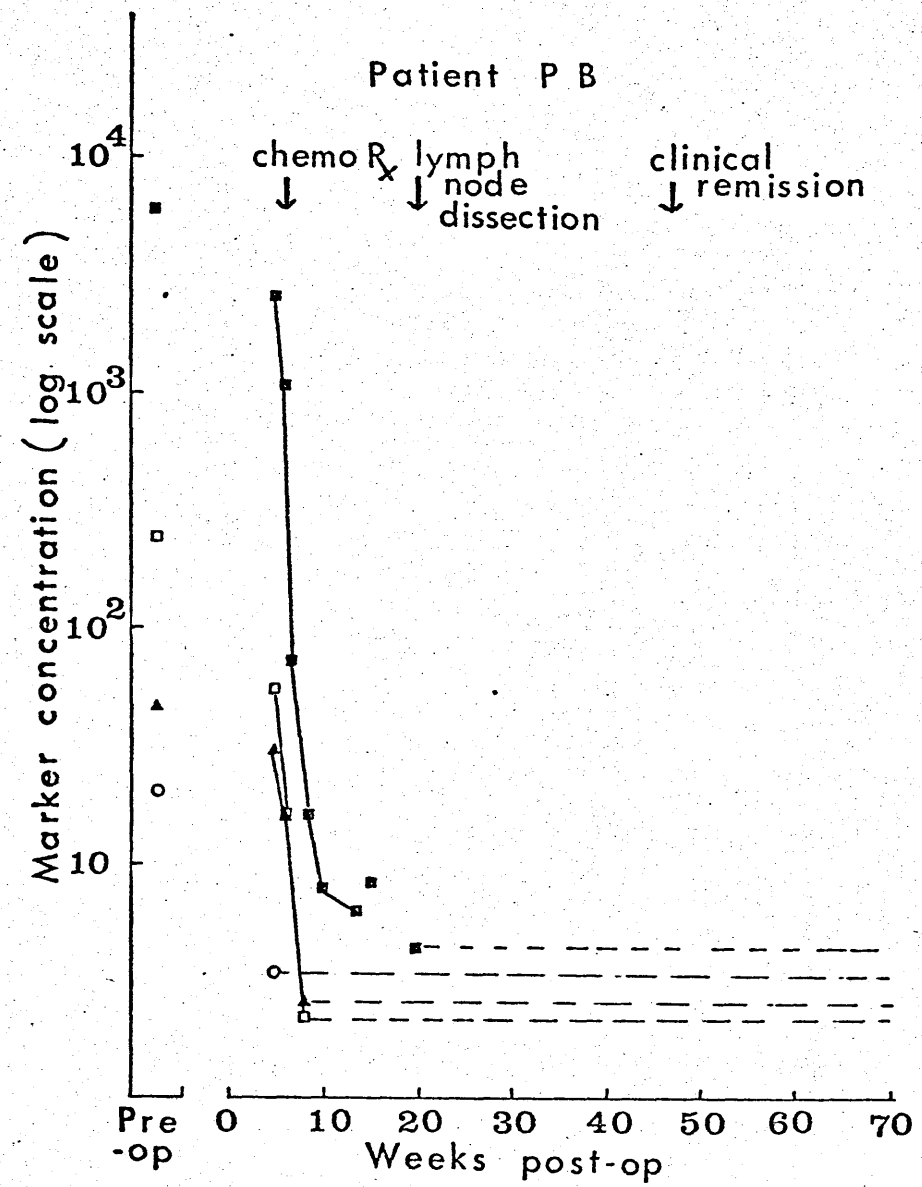

Figure 9 ii Patient PB was a 21 year old with teratoma of the Ⓡ testis. Following orchidectomy he commenced chemotherapy. He was found to have retroperitoneal metastases and underwent lymph node dissection. He is now in clinical remission and remains marker free.

(■) AFP (□) HCG (▲) PAPP-A (o) SP_1

Broken lines indicate results remaining within normal range to present.

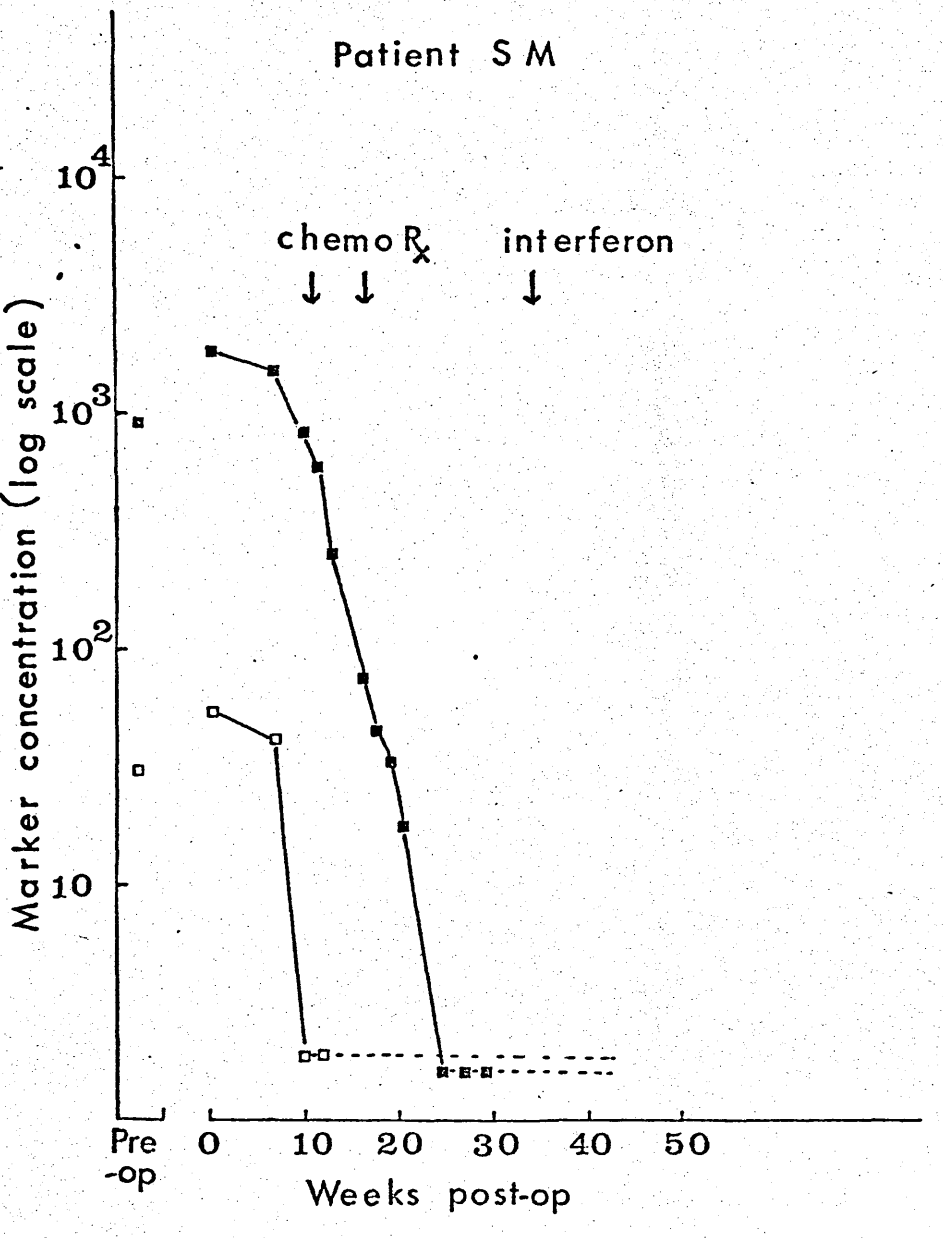

Figure 9 iii Patient SM was a 27 year old with teratoma of the (R) testis. Following orchidectomy he received two courses of chemotherapy. However he developed a differentiated adenocarcinoma (with no evidence of yolk sac elements) and received interferon. He remains alive.

(■) AFP (□) HCG Broken lines indicate results remaining within normal range to present

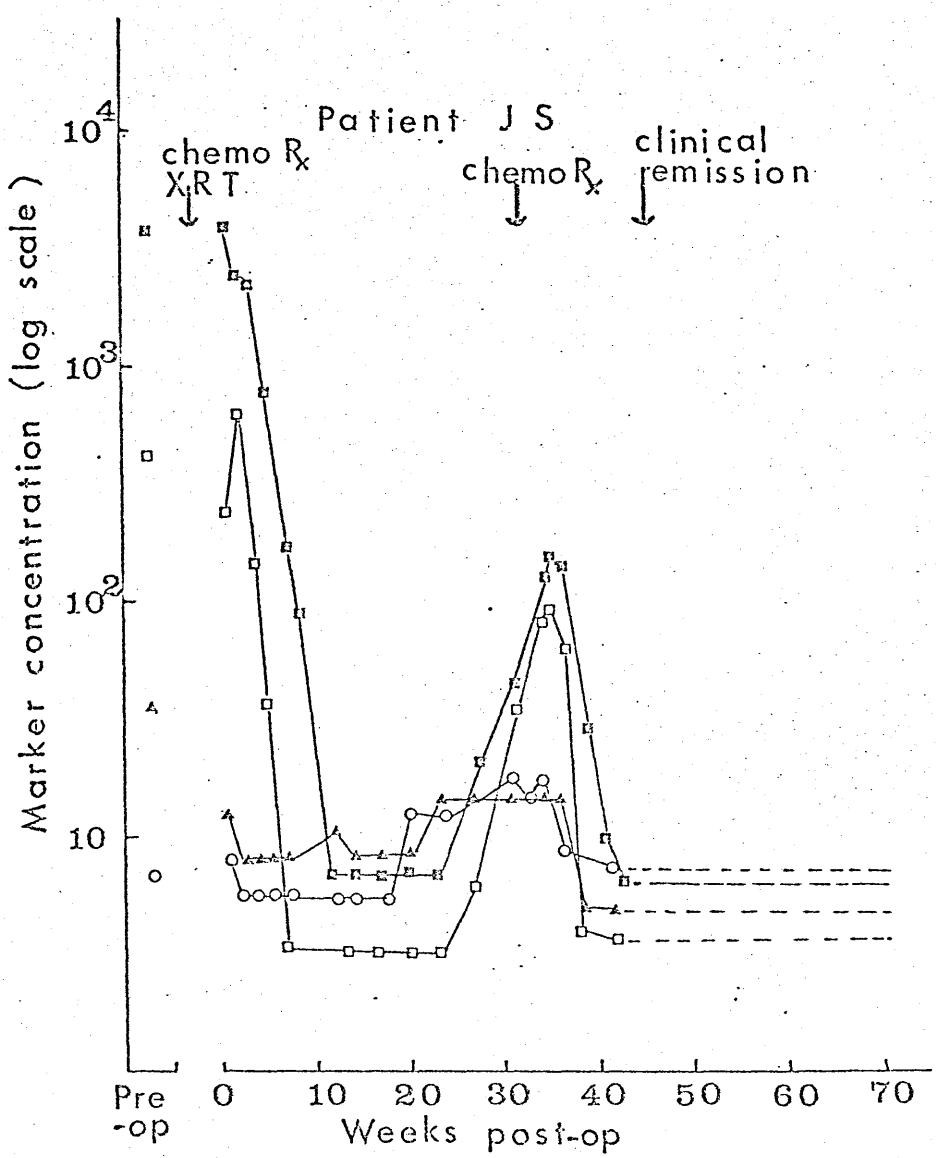

Figure 9 iv Patient JS was a 32 year old with teratoma of the Ⓡ testis and lymphatic spread. Post orchidectomy he underwent chemotherapy and XRT, followed by a further course of chemotherapy. Clinical remission was achieved and the patient remains alive and well.

(▪) AFP (□) HCG (▲) PAPP-A (o) SP_1

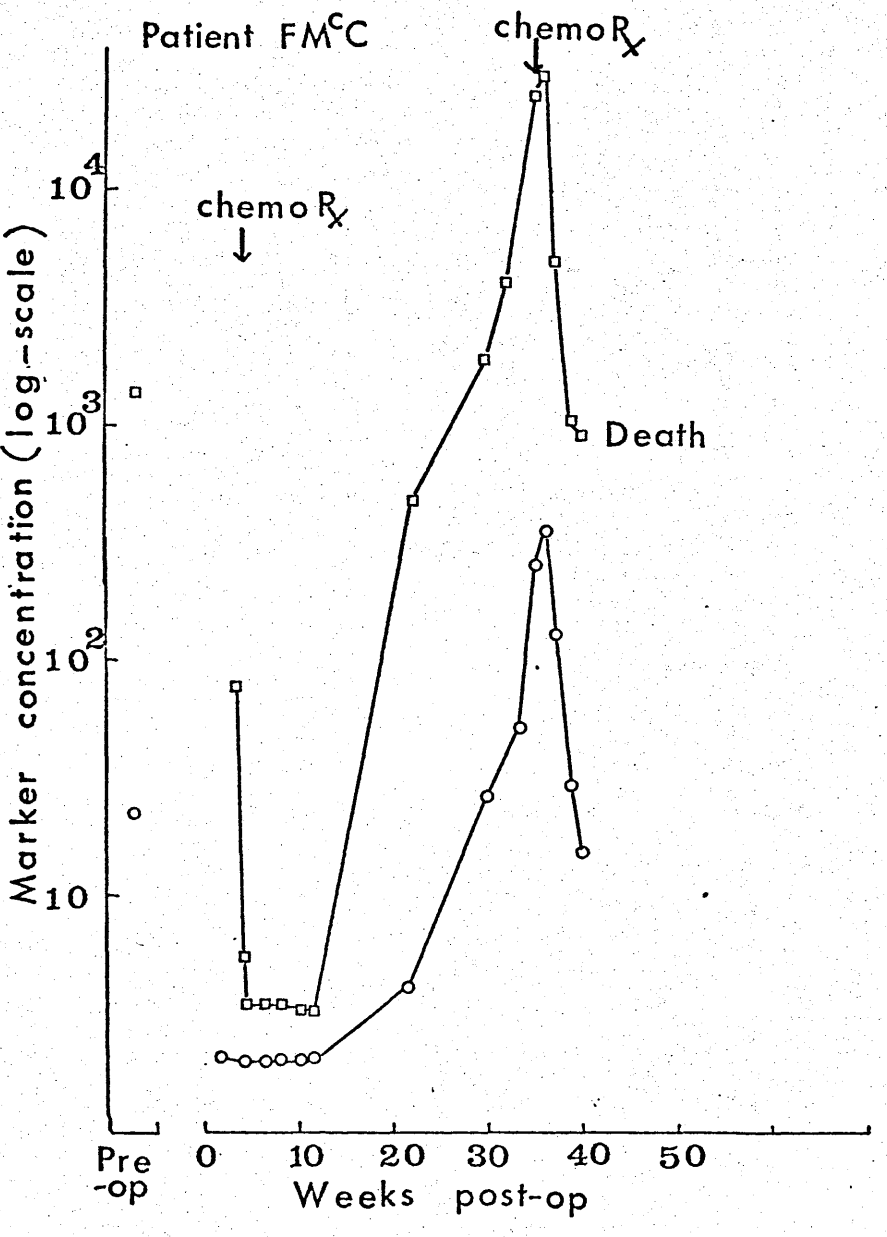

Figure 9 v Patient FMcC was a 19 year old with teratoma of the (R) testis. Following a course of chemotherapy, there was evidence of recurrence and a second course of chemotherapy was commenced. However the patient's condition deteriorated and he died 40 weeks post orchidectomy. At post-mortem examination evidence of pneumococcal meningitis was found together with lung, liver, and para-aortic node metastases.

(□) HCG (o) SP_1

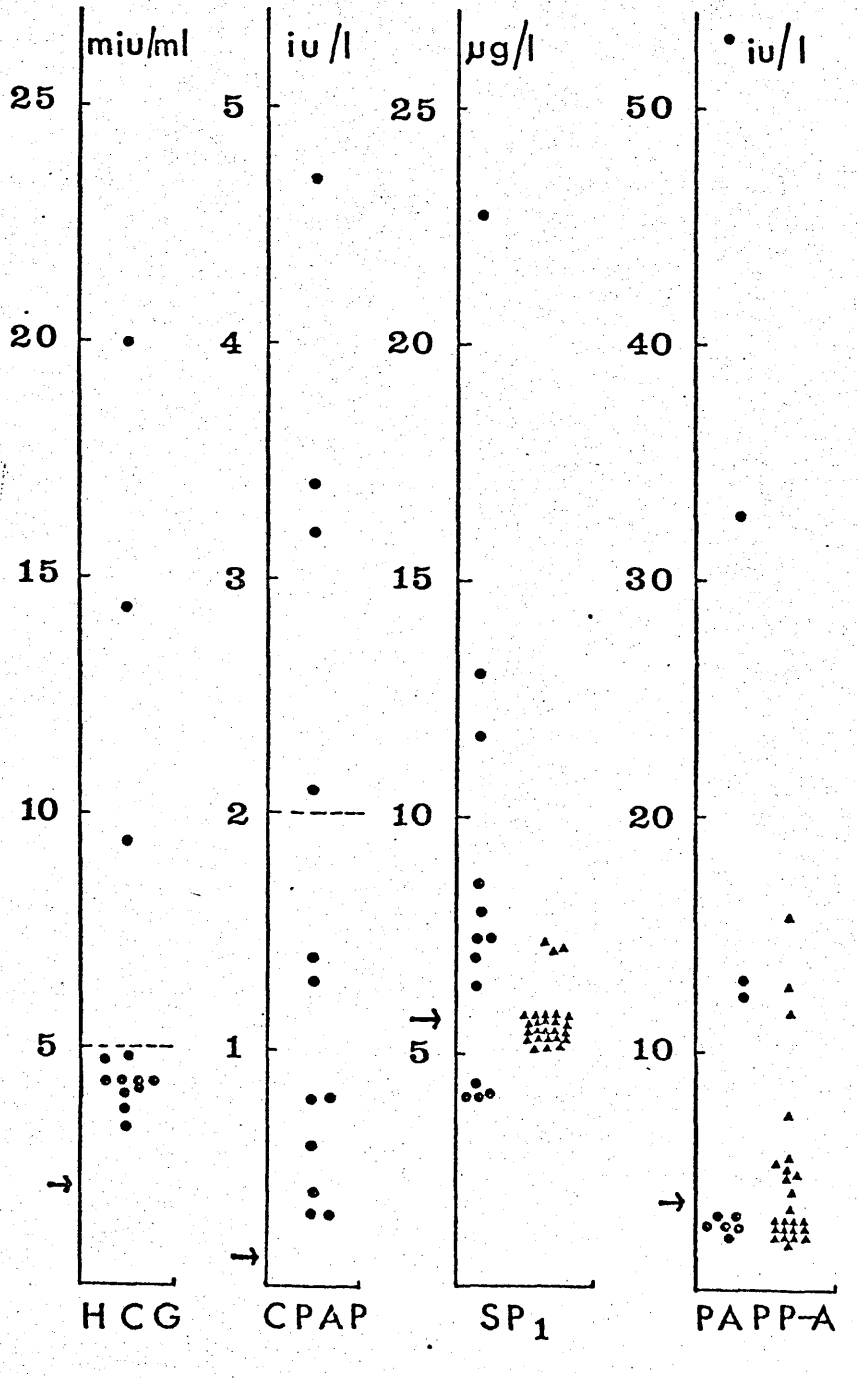

Figure 9 vi Pre-operative Oncodevelopmental Protein Concentrations in Patients with seminoma.

- - - - represents the upper limit of normal

⟶ represents the mean detection limit

Table 9 III

Occurence of Pre-operative Tumour Markers in Patients with Testicular Seminoma

Patient	AFP	hCG	CPAP	SP_1	PAPP-A
AW				+	
SC					
DP					
AG				+	
AS				+	
GW			+	+	
JC			+		+
GB		+	+	+	+
JL		+			
MP				+	
TMcG				+	+
JMcG		+	+	+	+
RMcD			+	+	

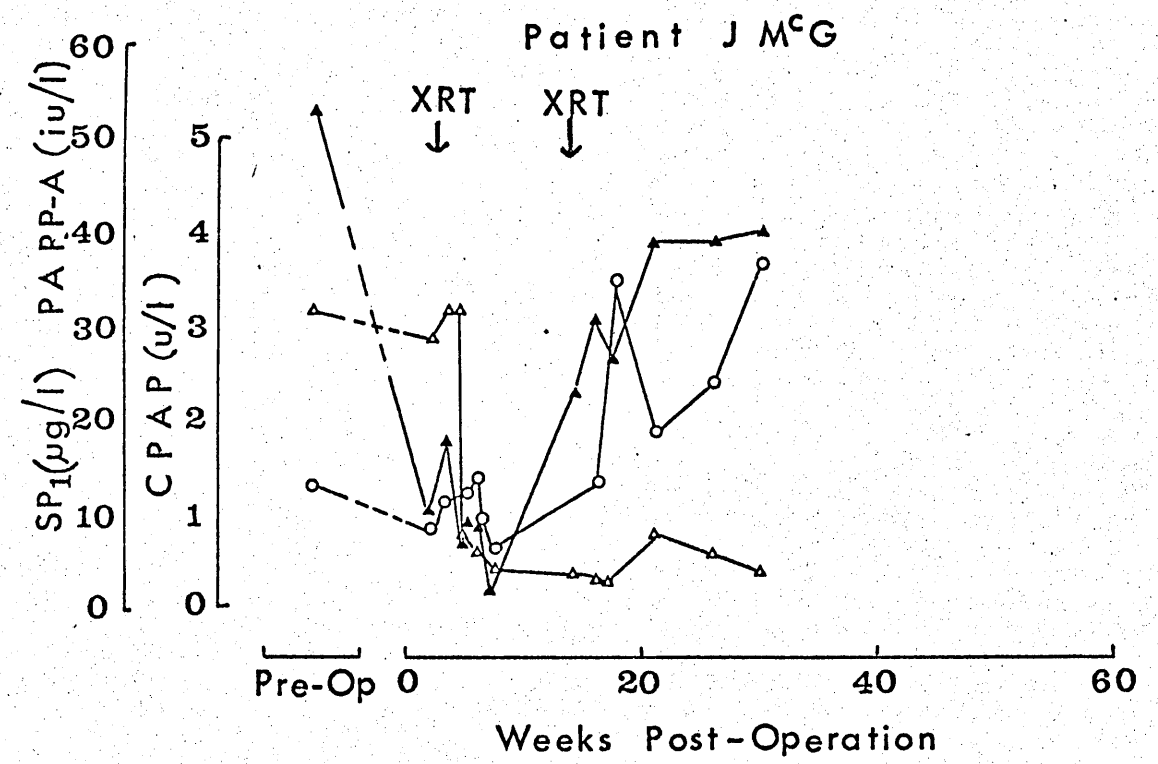

Figure 9 vii Patient JMcG was a 65 year old man with a previous history of right orchidopexy at 12 years of age. At operation he was found to have a spermocytic seminoma of the right testis, with extensive abdominal metastases. The patient underwent a right orchidectomy followed by 2 courses of radiotherapy and remains alive and well.

(▲) PAPP-A (△) CPAP (o) SP_1

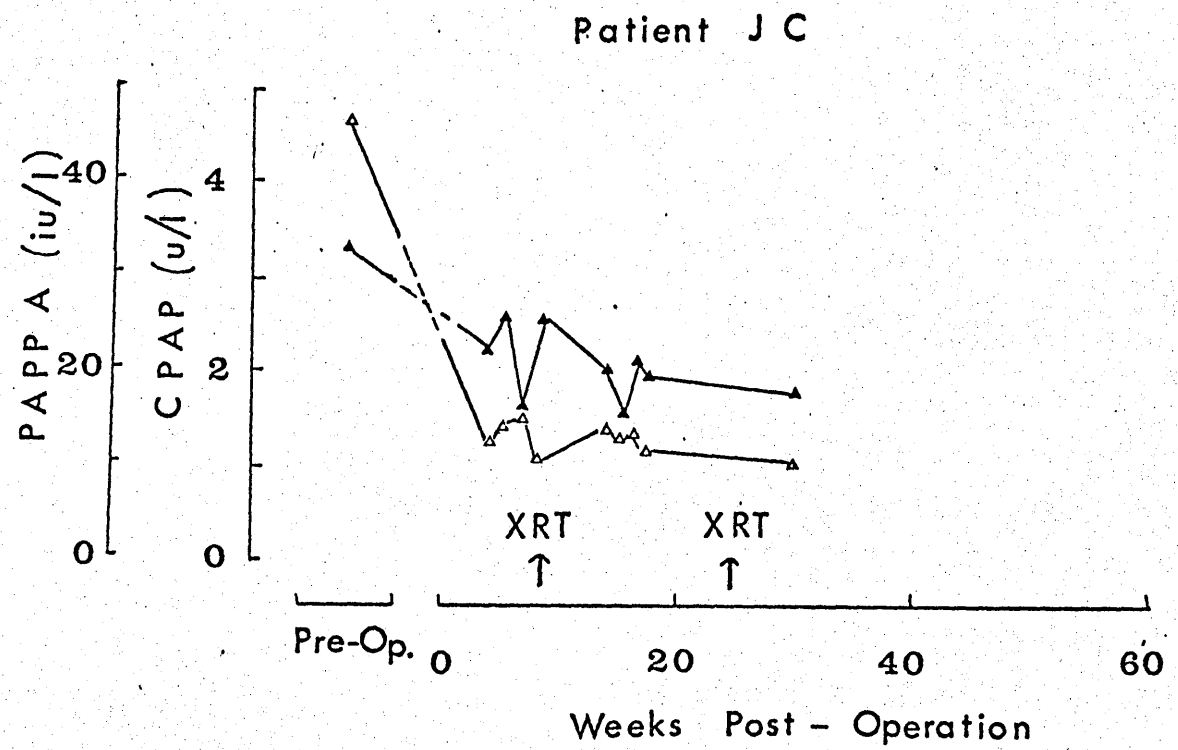

Figure 9 viii Patient JC was a 38 year old man who presented with a swelling of the right testis. He underwent right orchidectomy and was found to have a seminoma infiltrating the spermatic cord. Involvement of the para-aortic nodes was also suspected. The patient underwent two courses of radiotherapy to para-aortic and iliac nodes and remains alive and well.

(△) CPAP (▲) PAPP-A

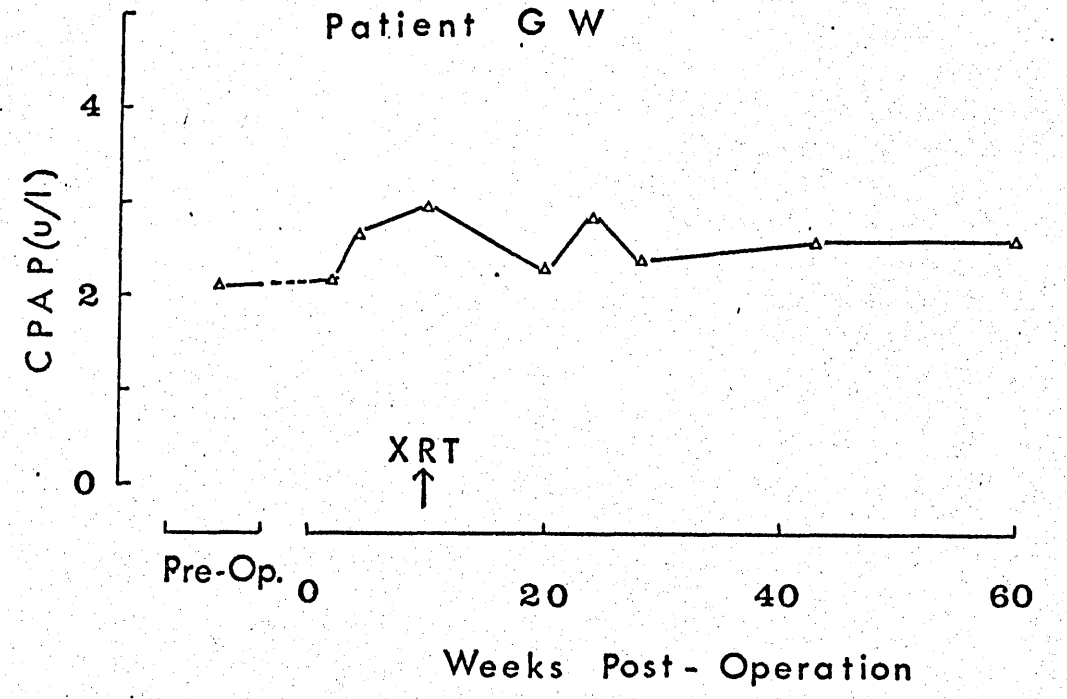

Figure 9 ix Patient GW was a 39 year old who had a localized seminoma of the right testis. Following orchidectomy he received radiotherapy to the abdominal nodes. He remains alive and well with no evidence of disease.

(△) CPAP

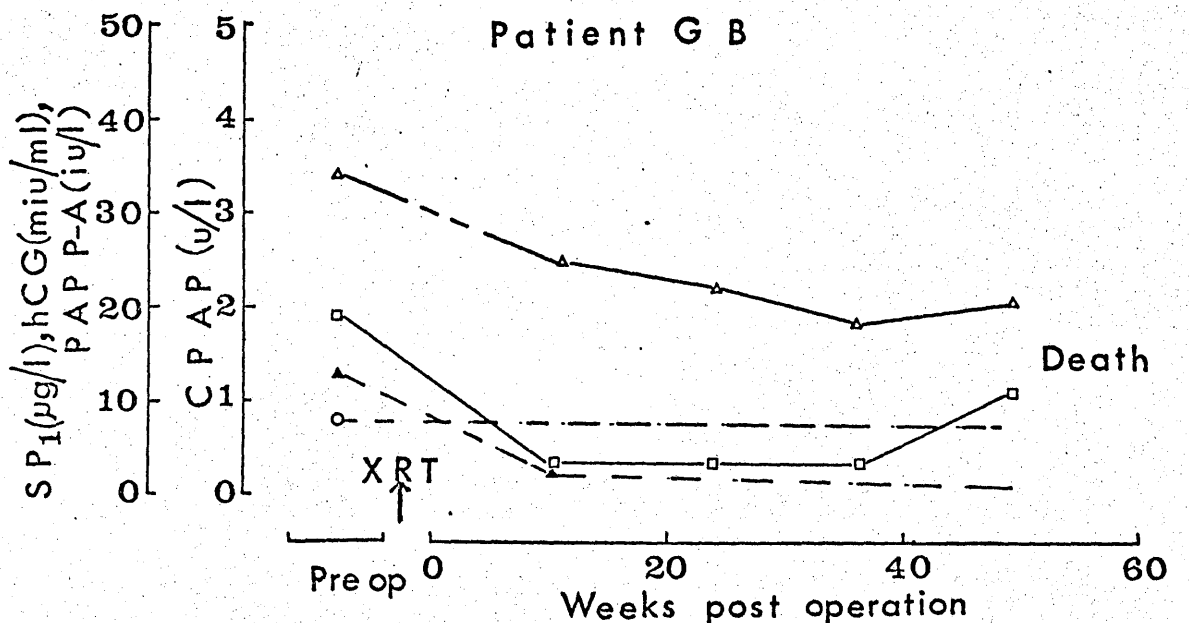

Figure 9 x Patient GB was a 43 year old man who presented with a seminoma in an undescended testes and underwent orchidectomy. Disease appeared localized and the patient had no evidence of mediastinal disease at any time. He received irradiation of the para-aortic nodes and initially appeared well. However about 12 months after surgery he presented with signs of spinal cord compression and died. Post mortem examination revealed a secondary tumour deposit in the spine.

(△) CPAP (▲) PAPP-A (○) SP_1 (□) hCG

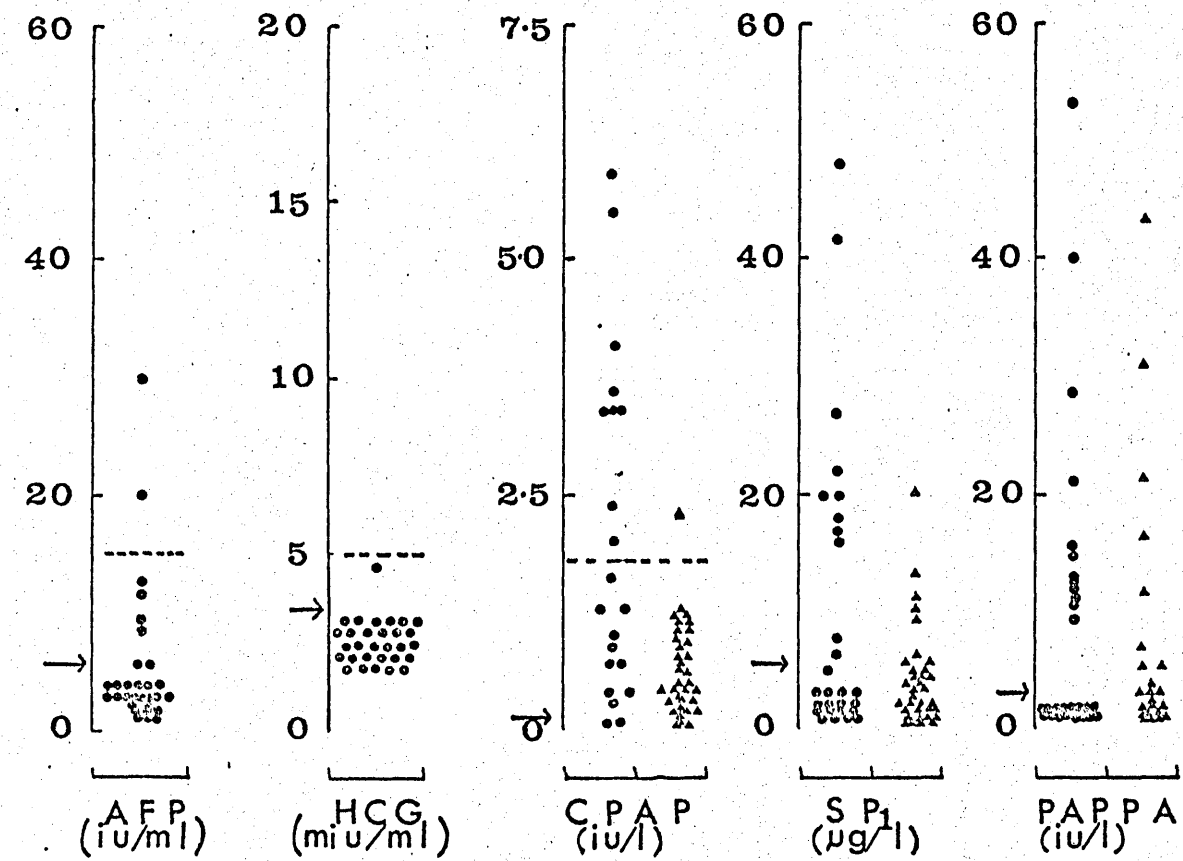

Figure 9 xi Pre-operative Oncodevelopmental Protein Concentrations in Patients with Ovarian Carcinoma.

- - - - represents the upper limit of normal

⟶ represents the mean detection limit

Table 9 IV

Occurence of Pre-operative Markers in Patients with Ovarian Cancer

Patient	AFP	hCG	CPAP	SP_1	PAPP-A	Diagnosis
JMcC				+		Cystadeno Carcinoma
MC			+	+	+	Papillary cystadenocarcinoma
ABi				+	+	Papillary cystadenocarcinoma
AM			+	+	+	Endometrioid
GMcF			+	+	+	Papillary Cystadenocarcinoma
MG			+		+	Borderline
AMcC			+		+	Adenocarcinoma
MMcD			+			Adenocarcinoma
EB						Endometrioid
AF						Papillary cystadenocarcinoma
CC						Adenocarcinoma
MSm			+			Mucinous cystadenocarcinoma
MSi						Papillary cystadenocarcinoma
MB			+			Borderline
MP				+	+	Papillary cystadenocarcinoma
JH						Poorly differentiated adenocarcinoma
BB	+					Poorly differentiated adenocarcinoma
ABr	+			+		Granulosa Cell Tumour
EM				+	+	Endometrioid
IJ				+	+	Papillary cystadenocarcinoma
MM				+		Adenocarcinoma
HB			+	+	+	Adenocarcinoma
IC						Malignant Teratoma
MF						Papillary cystadenocarcinoma
WS						Papillary cystadenocarcinoma
EHi			+			Adenocarcinoma
EHu						Adenocarcinoma
MD						Papillary cystadenocarcinoma
MG						Adenocarcinoma

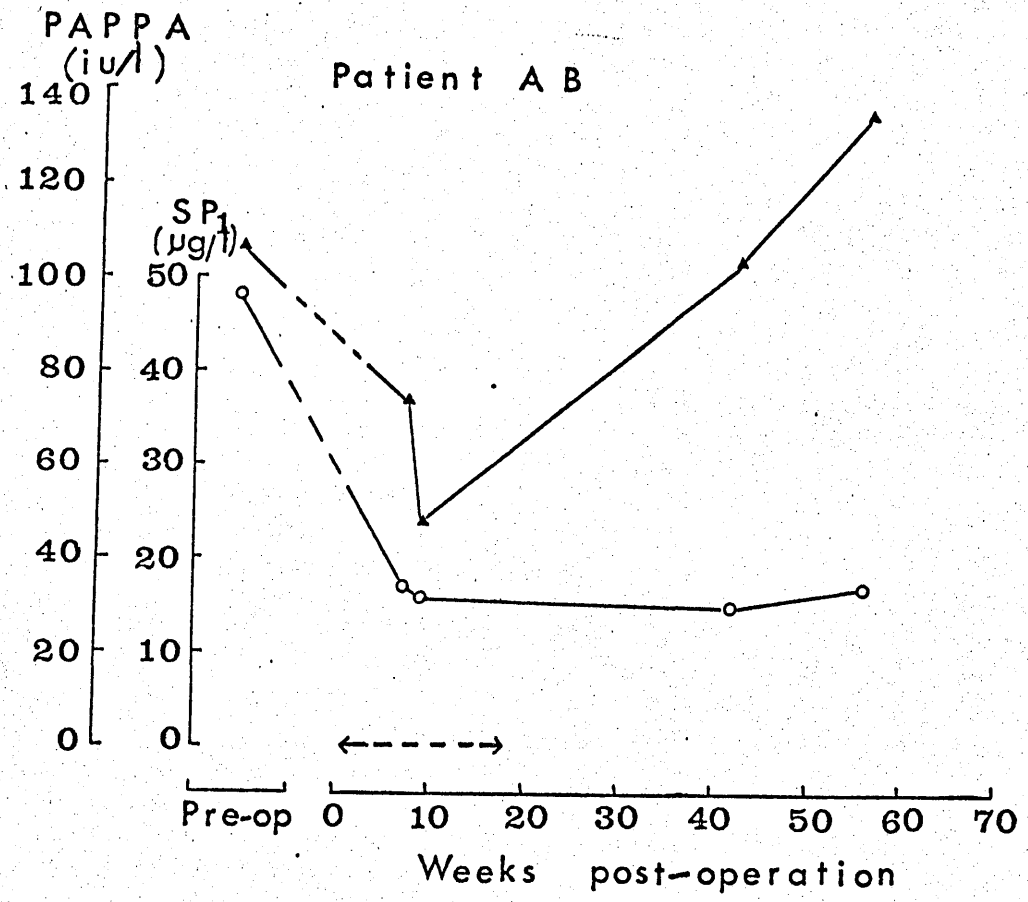

Figure 9 xii Patient AB was a 54 year old woman with previous history of colposcopy (normal result) in 1979. At laparotomy in March 1983 a mass was found in the Ⓡ iliac fossa. The omentum was clear but para-aortic node involvement was found and the patient was regarded as Stage III papillary cystadenocarcinoma. Between 31.3.83 and 10.8.83 6 pulses of CAP were given and at second look laparotomy in August 1983 there was no evidence of residual disease. The patient remains well with no evidence of recurrent disease.

(▲) PAPP-A (o) SP_1 (← – – →) Chemotherapy

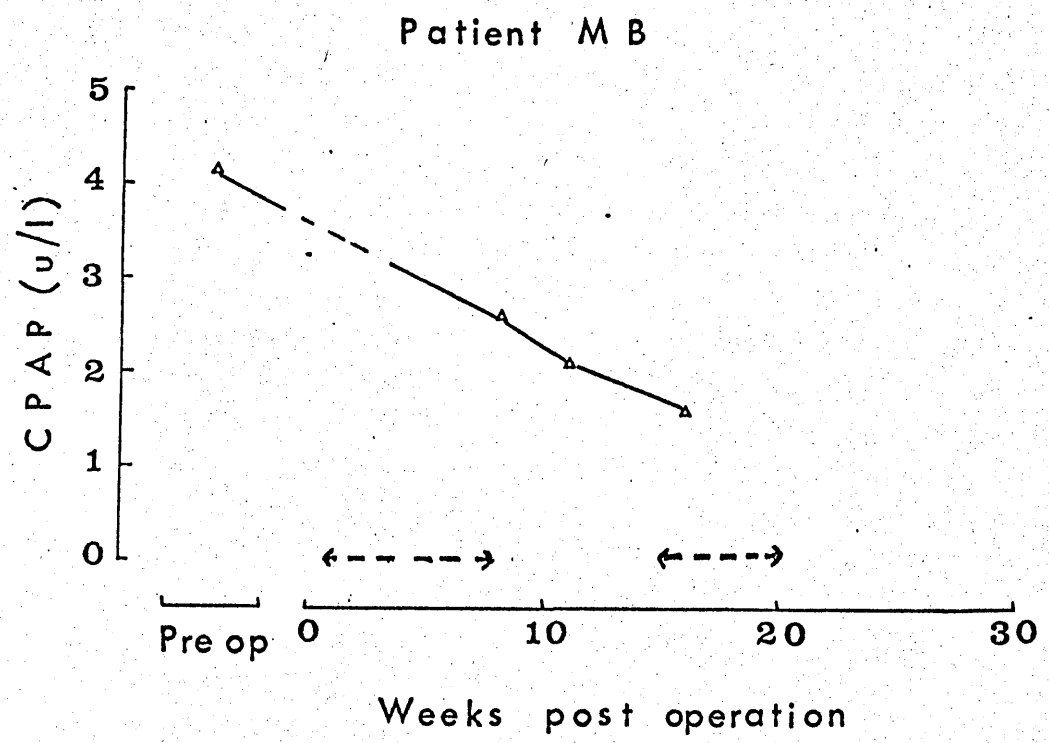

Figure 9 xiii Patient MB was a 55 year old woman with stage II mucinous cystadenocarcinoma of the left ovary which had infiltrated the left pelvic wall. Despite 4 pulses of treosulphan and 2 pulses of CB3717 the disease progressed and the patient died 9 months post-operatively.

(△) CPAP (← - - →) Chemotherapy

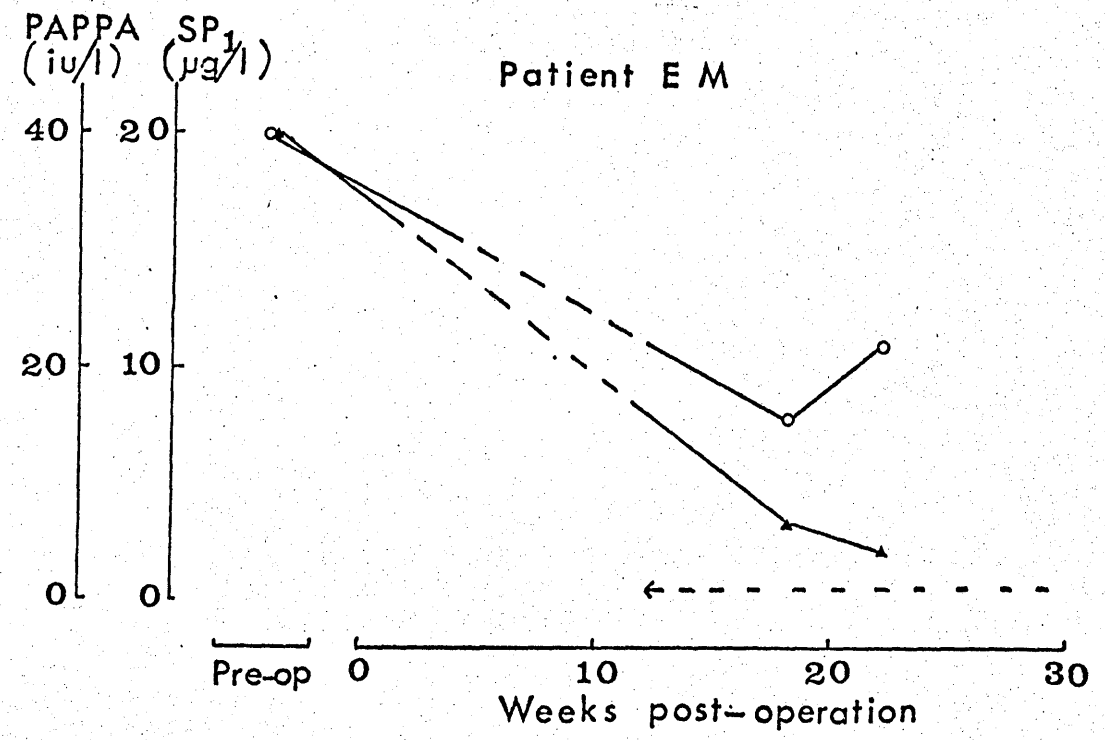

Figure 9 xiv Patient EM was a 58 year old woman with a stage IIC/III bilateral papillary adenocarcinoma. Surgery was followed by deep XRT. Three months post-operatively treosulphan/Cis Pt treatment was commenced and 6 pulses were given. A Ⓡ iliac mass remained palpable and at second-look laparotomy 11 months after initial surgery, nodes were present in the Ⓡ and Ⓛ iliac region. Treatment with chlorambucil has been commenced.

(▲) PAPP-A (o) SP_1 (← - - →) Chemotherapy

monitoring of patients during and after therapy are shown for 4 patients in Fig 9 ii - 9 v.

9.5.3 Patients with Seminoma

No patients exhibited elevated AFP concentrations prior to therapy. Concentrations of other oncodevelopmental proteins pre-operatively are shown in Fig 9 vi and Table 9 III. 23% of patients had elevated hCG concentrations, 38% had elevated CPAP concentrations, whilst 69% and 30% respectively had elevated SP_1 or PAPP-A. Few patients had elevated markers in combination (Table 9 II). Serial results from 4 patients are shown in Fig 9 vii-9 x.

9.5.4 Patients with Ovarian Carcinoma

The results obtained in this group of patients are shown in Fig 9 xi and Table 9 IV. No patients showed elevated hCG concentrations and in only 2 patients were there mild elevations of both SP_1 and PAPP-A and 21% elevation of both CPAP and PAPP-A. Only 14% had a raised CPAP in conjunction with a raised SP_1 concentration.

Serial results from 3 patients are shown in Fig 9 xii -9 xiv.

9.6 Discussion

9.6.1 The Normal Population

The detection of oncodevelopmental proteins in the sera of normal subjects is largely due to improved assay sensitivity. Whilst the presence of AFP, hCG and CPAP in the sera of normal subjects is generally accepted (Ruoslahti and Seppala 1971, Borkowski and Muquardt, Haije et al 1979), the situation is less clear regarding SP_1 and PAPP-A.

Detection of SP_1 in the non pregnant subject has been reported (Anthony et al 1980 b, Tatarinov 1982). The proportion of subjects depends upon the sensitivity of the assay. In the present study SP_1 concentrations above the mean limit of detection are found in approximately 12% of males and 15% of females.

Figures have been reported for PAPP-A in a non pregnant population. Bischof et al (1981 b) detected PAPP-A in 100% of non pregnant women. The detection rates presented here whilst not as high as those of Bischof, could be consistent with a later postulate of extra-placental production (Bischof et al 1982, Duberg et al 1982).

The fact that PAPP-A was detected in male serum suggests that a source other than decidua or endometrium is responsible for at least a proportion of the protein. It may be that PAPP-A has been fortuitously designated as pregnancy associated rather than pregnancy specific.

9.6.2 Patients with Testicular Teratoma

A teratoma is a tumour composed of various tissues chaotically arranged and usually of the most diverse types, with no relation to the site of origin (Muir 1980). Malignant teratoma of the testis accounts for 1% of all malignant tumours in males. The incidence appears to be increasing and until recently they ranked as the most common fatal cancer in men aged 25-34 (Newlands at al 1983). However new chemotherapeutic regimes have greatly improved the outlook for the patient with testicular teratoma (Hainsworth and Greco 1983).

The availability of tumour marker assays is regarded as an essential component of staging workup and follow up of these

patients (Hainsworth and Greco 1983). Furthermore multivariate analysis has shown that the strongest predictor of survival is not the clinical and radiological extent of the disease, but the initial serum concentration of hCG and AFP (Newlands et al 1983).

The occurence of elevated hCG concentrations in 68% of patients and elevated AFP concentrations in 72% of patients with teratoma found in this study are in accordance with previously published data (Braunstein et al 1973, Blacker et al 1981, Waldman and McIntire 1974). Differing positivity rates quoted by various groups are related to different patient populations and assay procedures.

Successful eradication of the disease results in decline of these markers to within the normal range (Patient PB Fig 9 ii), and they remain normal unless there is recurrence of the disease - as with patients JS and FMcC (Figs 9 iv and v). On the grounds of rising hCG concentrations (there was no clinical evidence of tumour recurrence), JS received further chemotherapy and eventually went into remission, remaining alive and well. In this case hCG estimation allowed early recommencement of treatment which would have been delayed had the clinician waited until symptoms were apparent. These conventional marker levels must however be interpreted with caution. The decline in AFP and hCG observed in patient SM (Fig 9 iii) indicated that the tumour had been successfully eradicated. However in this case the teratoma had differentiated to form an adenocarcinoma with no evidence of yolk sac elements and this later tumour was marker negative for AFP and hCG.

It is apparent that for patients with teratoma, hCG and AFP are useful markers. The question remaining is whether alternative markers will be more useful. The results show that there is a lower positivity rate for either SP_1 or PAPP-A than for hCG or AFP. Thus neither SP_1 or PAPP-A are likely to replace conventional markers for initial disease workup. In only 2 cases (patients 9 and 15) were SP_1 or PAPP-A elevated above the detection limit without concomitant rise in hCG or AFP.

Concordant elevation of hCG and SP_1 in patients with testicular teratoma has been reported previously (Blacker et al 1981, Lange et al 1980). It is further reported that SP_1 is elevated only in a small percentage of patients who do not have elevation of conventional markers (Lange et al 1980). This finding is in accordance with the results obtained in the present study.

In the patients under study who showed elevated SP_1 or PAPP-A levels prior to surgery, the possibility of improved lead-in times using these markers remains open to question. In most cases neither SP_1 or PAPP-A concentrations rose significantly in advance of conventional markers.

Occasionally, however, rising SP_1 or PAPP-A would have allowed earlier clinical intervention (patient JS). Even with later intervention, clinical remission was still achieved and it is therefore arguable whether estimation of these alternative markers offers therapeutic advantage.

Opinions on this subject vary. Lange et al (1980) in a small number of patients found SP_1 levels provided useful clinical information not given by hCG or AFP. Blacker (1981) also

suggested use of SP_1 assays in this situation but stated that elevated SP_1 levels were generally accompanied by elevated hCG, which would tend to negate the use of SP_1. It seems unlikely that the use of hCG and AFP in monitoring therapy of patients with testicular teratoma will be replaced by assay of SP_1 or PAPP-A.

9.6.3 Patients with Seminoma

Seminoma is the commonest malignant tumour of the testis (Muir 1980) comprising about 40% of testicular germ cell tumours. As these tumours are remarkably radiosensitive, radiotherapy is the treatment of choice. No patient with seminoma should exhibit raised AFP concentrations because there are no yolk sac elements, and only 5-10% of patients have raised hCG concentrations (Hainsworth and Greco 1983). There is a need for a reliable marker in patients with seminoma.

Recent evidence suggests that an alkaline phosphatase having properties of the placental isoenzyme exists in normal testicular tissue and may be useful as a marker for seminoma (Paiva et al 1983).

38% of patients in this study showed elevated CPAP pre-operatively. This occurence is lower than that reported by other groups (Jeppsson et al 1983, Javadpour 1983, Paiva et al 1983, Lange et al 1982). Strict comparison with these results is not possible as other groups have measured protein concentration rather than enzyme activity.

Two of the 5 patients in whom CPAP was raised before operation demonstrated an abrupt fall in CPAP activity after treatment to within normal limits; these patients remain alive and well

at 50 and 64 weeks after operation. No further samples have been assayed on these patients (JMcG and JC Fig 9 vii and viii). In the third patient patient GW (Fig 9 ix) there was a slight increase in CPAP after operation. Eight specimens were analysed over the following 60 weeks and all remained elevated or at the upper limit of the reference range. These findings may represent a combination of biological and analytical variability in a subject whose CPAP is normally at the upper extreme of the reference range. This patient also remains alive and well at 90 weeks post-operatively. CPAP activity in the fourth patient (GB Fig 9 x) remained elevated for many weeks after operation and radiotherapy. Although eventually CPAP activity fell to within normal limits, subsequently an increase was detected and the patient died. Secondary deposits were detected at autopsy.

No further specimens have been received on the fifth patient with pre-operatively raised CPAP.

The 8 patients with normal CPAP activity before operation remain alive and well.

Only 3 out of 7 patients had raised serum hCG concentrations 2 of which also had a raised CPAP activity. Similar results have been reported by Lange et al (1982). The significance of the raised hCG is the subject of some debate but may indicate that these patients have a mixed tumour rather than a pure seminoma (Cochran 1976, Kuber et al 1983). There is insufficient data to decide whether or not serum hCG (when present) reflects the clinical course of the disease more accurately than CPAP. Lange et al (1982) were also unable to decide upon this point.

PAPP-A concentrations were above the assay detection limit in 4/13 patients, of which 3 also had raised CPAP. In patient

JC (Figure 9 viii), PAPP-A concentrations mirrored CPAP. In patient GB, PAPP-A declined to below the detection limit and remained there despite clinical evidence of progression of disease.

Conversely, in patient JMcG (Figure 9 viii), PAPP-A levels declined initially but became elevated again. Despite this finding, the patient showed no sign of progression of disease.

In a fourth patient PAPP-A declined to below the assay detection limit post operatively; the patient showed no signs of progression.

69% (9/13) of patients had SP_1 concentrations above the mean limit of detection. However, of these, 6 were close to the mean assay limit of detection and must be interpreted with caution due to analytical noise. Only 3/13 patients showed significantly elevated SP_1 concentrations, 2 of whom also had elevated CPAP.

In patient JMcG, SP_1 levels show a similar trend to PAPP-A and do not correspond with the patients clinical condition.

No further specimens were received on the second patient with elevated SP_1 and CPAP.

SP_1 concentrations in the patient with no concomitant rise in CPAP, declined to normal post operatively and remained thus. The patient remained well with no sign of residual disease.

Lange et al (1980) in a study of patients with seminoma found elevated SP_1 in some cases usually with a corresponding elevation in hCG. Szymendera (1983) showed similar results, but neither of these authors clarified the usefulness of the test.

From the data presented here it seems that SP_1 is unlikely to be of value in monitoring patients with seminoma, as it is

elevated in only a small proportion of patients and usually in combination with CPAP. From initial investigations CPAP appears to be the preferable marker now that a simple, sensitive and specific assay is available.

9.6.4 Patients with Ovarian Carcinoma

Because of its histologic complexity, the ovary gives rise to benign and malignant tumours of epithelial, stromal and germ cell origin (Young et al 1982). In the post-menopausal woman, germ cell tumours account for only about 10% of cases.

The major problems in diagnosis and treatment of the epithelial group have been the early spread of the disease coupled with the lack of early signs and symptoms accounts for the fact that most patients already have spread throughout the peritoneal cavity when diagnosis is made (Lewis 1983).

Furthermore until recent years there has been a lack of tumour markers reported for most cases of ovarian cancer other than hCG and AFP for ovarian teratomas.

In common with other groups, (Koh and Cauchi 1983, Cauchi et al 1981, Haije et al 1979), the results obtained here show that AFP or hCG estimations are of little value in diagnosis and monitoring of ovarian cancer.

However, the other antigens investigated appear initially to offer more hope for following therapy. Elevated levels of SP_1, PAPP-A and CPAP were found in about 1/3 of cases, although not necessarily in combination.

As in previous cases, the positivity rate is too low to assist in diagnosis. This leaves the possibility of using these markers as indices of treatment and progression of disease.

Even this may not be feasible for PAPP-A because of the wide scatter of concentrations found in normal female subjects (Figure 9 v). Thus in patient AB (Figure 9 xii) rising PAPP-A concentrations might suggest recurrence of disease. All clinical evidence is against this. Similarly although the SP_1 level declined post-operation, SP_1 still remains detectable in this patient's serum.

Patient EM (Figure 9 xiv) has evidence of recurrent disease. Unfortunately no further blood specimens were received from this patient. It is possible that the elevation of SP_1 at 20 weeks post operation might have been the first evidence of recurrence. However PAPP-A and SP_1 were both elevated prior to operation, but show divergence at 20 weeks. It is equally likely that the SP_1 elevation is entirely spurious.

Similar patterns are evident in other patients not discussed in detail. It seems unlikely therefore that either PAPP-A or SP_1 reflect tumour mass or recurrence. Bischof (1982 c) reached the same conclusion regarding the use of PAPP-A in nontrophoblastic tumours.

The use of CPAP in patients with non germ cell ovarian cancer has been suggested by Kellen et al (1976). Although their data may be criticized in that the blood specimens taken were post operation, the incidence of positive sera (35%) accorded well with the indidence for pre-operative specimens presented here.

However as with SP_1 and PAPP-A, CPAP levels do not seem to correlate with the course of disease for most patients in this study eg patient MB (Figure 9 xiii).

From the results obtained so far it seems unlikely that any of these proteins will be of value in following disease in patients with ovarian cancer.

CHAPTER 10

GENERAL DISCUSSION

The discovery of an increasing number of onco-developmental proteins (Klopper 1982) poses problems for both clinician and biochemist.

Clinical interest in these proteins is diverse. Firstly the obstetrician wishes to know whether the measurement of 'placental' proteins will aid his management of the pregnant patient. Secondly the oncologist requires a marker of tumour activity both to aid in diagnosis or prognosis of disease and to act as an index of tumour regression during therapy.

None of these functions can be fulfilled until the biochemist has devised a satisfactory assay for the protein in question.

The assays of choice have been based on immunological techniques. Whilst serum concentrations of some placental proteins can be measured by relatively insensitive techniques such as immunoelectrophoresis, radial immunodiffusion or nephelometry (Schultz-Larsen et al 1979), there may be a necessity for greater sensitivity (Pledger et al 1981) and many groups have devised radioimmunoassays for these proteins. Whilst widely used, radioimmunoassay is not a perfect tool and the problems associated with it led to alternative systems being developed, such as the use of enzyme labels as described by Van Weemen and Schuurs (1971) and Engvall and Perlmann (1972).

ELISA made rapid inroads in certain areas of laboratory medicine notably bacteriology and virology where it was ideally suited to detection (if not quantification) of antibodies.

Clinical chemists however have been more reticent about its use and RIA still plays a major part in specific protein assay. Part of this reticence may be explained by the fact that ELISA has certain problems associated with it. As RIA was

already in use it was felt that changing assay systems (with capital expenditure involved) was not justifiable.

However, investigation of the factors involved in ELISA should make possible the more rapid development of improved assays.

Some RIA was already in use in this department. Where suitable assays existed, the associated parameters were investigated to ensure their continued suitability for use in this study (Chapter 2).

Where it was necessary to design assays for proteins not previously measured in the department, ELISA was used (Chapter 3). An assay for SP_1 was used based on that originally described by Macdonald et al (1979). This was further investigated to allow a more sensitive assay for use in monitoring SP_1 as a tumour marker. At this stage problems with the SP_1 content of normal serum became apparent, necessitating experiments to ensure removal of endogenous SP_1 from standard preparations (3.5). Close investigation of Macdonald's assay and the sensitized assay showed them to be suitable for clinical use.

Based on similar principles, an ELISA for PAPP-A was developed. Again investigation of this showed it to be suitable for measuring PAPP-A concentrations in patients' sera. In the course of assaying patient samples for PAPP-A it was noted that haemolysis caused interference. Investigation of this effect showed it to be specific to PAPP-A and it was concluded that only non-haemolyzed specimens should be measured. This was in addition to observations made previously by other groups for the collection of specimens for pregnancy associated protein

assay (Toop and Klopper 1983, Sutcliffe 1982).

Elevated PAPP-A concentrations were found in a proportion of samples from non pregnant patients as had already been noted for SP_1. The source of these proteins in non pregnant normal subjects remains uncertain, but a number of possibilities have been suggested (Bischof et al 1982 a/b, Bischof et al 1983, Duberg et al 1982). The recognition of detectable levels in normal subjects is probably a function of assay sensitivity. Whilst this is unlikely to be of importance in the application of the assays to the monitoring of placental function, where concentrations are considerably elevated, it may cause problems where they are used as tumour markers. The ELISA methods for SP_1 and PAPP-A and the immunoenzyme technique for CPAP have been shown to be suitable assays for the measurement of these proteins; all of the assays may be adjusted to tailor the detection limits and assay range to the clinical requirement.

Whilst problems specific to assays for these proteins were investigated in this thesis there are however also disadvantages common to all sandwich ELISA. The glowing description given by Hoskings (1982) would at first sight seem to imply perfection.

Are these problems sufficiently great to significantly disadvantage ELISA in favour of RIA?

The effect of differing protein matrices on performance has already been alluded to (3.5.4) but this is also common to many RIA's (Hunter 1972, Hunter and Bennie 1979).

Microtitre plates form an ideal method of dealing with a large number of specimens in a single batch and have proved to be superior to various plastic beads in terms of antibody coating

qualities. However, some variation between different types of plate has been noted. Each of these observations has implications for the sensitivity and precision of the final assay.

In common with previous observations (Engvall and Perlmann 1972, Lehtonen and Viljanen 1980) a leakage of bound antibody from the microtitre plates was found. This effect seemed to depend partially on the protein concentration in the test matrix. However, a reasonably high protein concentration was found to be advisable in order to reduce non specific binding. When designing ELISA systems investigation of the time course of reaction is essential.

The importance was emphasized by the occurence of an excess antigen effect (4.9) possibly akin to the 'hook' reported by Engvall and Perlmann (1972) and since recognized in immunoradiometric assays. The effect seen in ELISA seemed to depend only on antigen concentration. Once such problems have been observed assay design may be modified in order to circumvent them. All of the problems encountered in this study were overcome by such modifications. It therefore seems possible that ELISA can be a viable alternative to RIA for the determination of oncodevelopmental proteins. However, it is essential that some form of data reduction is available for the routine application to patient care. Investigation of several assay systems (Chapter 5) showed that methods already in use for RIA could be applied with only minor alteration to ELISA. The use of these in conjunction with the microtitre plate format of tests allows the processing of large numbers of specimens.

It is suggested that as laboratory equipment associated with RIA becomes due for renewal, biochemists should seriously consider the use of ELISA for certain analytes.

However the clinical chemistry laboratory does not exist for its own edification. Whilst ELISA and RIA have been shown to be viable test procedures, the provision of any test should be dependent on clinical need. The question must be asked as to whether analysis of the onco-developmental proteins can provide useful adjunct in diagnosis and management of patients.

What criteria must be applied in order to assess this usefulness? All too often, little thought is applied to this concept. For many years the concept of a 'normal range' has been applied although this has many limitations. Perhaps the time has come to adopt Galen and Gambino's phrase (1975) and to progress 'beyond normality'. This approach makes no prior assumptions about the distribution of data, is applicable to any type of test and more importantly provides a common baseline for meaningful comparison. However under this critical examination, test performances may not seem as perfect as we often think.

The use of multiparametric tests in the field of diagnostic oncology has been proposed by a number of authors (Schwartz 1973, Sussman et al 1975, Cooper et al 1975). Problems with the assessment of normality in this situation have been alluded to (Weldon and Mackay 1972). Applying Galen and Gambino's approach (1975) the fallacy of multiparametric diagnostic testing becomes apparent; the predictive value of a positive result in these circumstances can be so low as to be useless.

The battery test approach may be of use in order to find a positive tumour marker for following response to therapy in an individual patient. Test selection must be made carefully in this instance if the procedure is to be cost effective.

A number of biochemical tests of placental function have been proposed (Wilde and Oakey 1975). Interpretation of the results of these must be made against the background of a population similar to the one under investigation. For this reason a study of normal pregnancies was undertaken. Rather than evaluate the usefulness of the tests against a broad spectrum of problems, specific aspects only were investigated.

Application of the techniques of Galen and Gambino (1975) was used in this thesis to assess the usefulness of hPL estimation in detection of IUGR and the continued use of hPL estimation for detection of IUGR and conversely for reassurance in patients thought to have IUGR can be recommended (Chapter 7). SP_1 and PAPP-A appeared to be of little use in these situations. Whilst there is increasing use of ultrasound and CTG, as non-invasive methods of measuring placental function, it is interesting that a recent document reported "no consensus that routine ultrasound [ie screening]..... improved perinatal outcome or decreased morbidity and mortality" (Leader, Lancet 1984). The continued measurement of hPL concentrations to diagnose and assist the management of LFD pregnancies seems worthwhile.

Whilst eclampsia is seldom encountered in modern obstetric practice, pregnancy induced hypertension (pre-eclampsia) is still relatively common. As there are implications for both mother and foetus, early detection and continued monitoring are vital. The report by Hughes et al (1980) that PAPP-A

levels were elevated in women who went on to develop PIH suggested that this estimation may provide a suitable screening test. The findings in this thesis however do not support this claim. Possible reasons for this are discussed in Chapter 8, but the use of PAPP-A in this context must be regarded critically.

Again, only hPL gave levels which differed from patients with normal pregnancies and this finding may be related to the lower placental weight found in the hypertensive mothers and it is questionable whether comparison with the reference range is strictly correct.

A similar problem exists in interpretation of hPL results in diabetic pregnancies (Chapter 8).

From the data presented in this thesis a tentative link between SP_1 and carbohydrate metabolism was drawn. Since most pregnant diabetics are now well stabilized, gross abnormalities of placental function are rare and studies to further test these theories would be time consuming.

Despite the deficiencies, it seems that measurement of hPL concentrations will continue for the forseeable future. Use of other placental proteins for the monitoring of pregnancy remains uncertain.

If a biological function exists for these proteins it is unknown. Whilst hormonal effects have been proposed for hPL, involvement in the immune system for PAPP-A, definite proof has yet to be presented. The reason for the occurence of these proteins in many non pregnant subjects as well as in greater concentrations during pregnancy remains a mystery.

Are the recently discovered oncodevelopmental proteins likely to be of use in oncology? Despite the enthusiasm of some authors (Bohn 1980c), this study does not hold out any great hope for their use. The existence of detectable levels of most oncodevelopmental proteins in at least some of the population decries their use in diagnosis. Other than the use of CPAP (Chapter 9) little extra information would be gained in assaying serial samples for various oncodevelopmental proteins. There is little to warrant their routine application in oncology.

Although the proteins studied in this thesis seem to be of less value than conventional markers in the monitoring of placental function or tumour activity, it is possible that other newly discovered 'placental' proteins are worthy of study as possible monitors of placental function.

Does the ideal tumour marker exist? If it does, it has not been found among the proteins investigated here. However, the introduction of antibodies raised against tissue specific antigens (Bhattacharya and Barlow 1975, Bast et al 1983) may hold hope for the future.

The search for tumour markers will continue and time alone will reveal the usefulness of newly discovered markers.

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APPENDIX I REAGENTS

1. General Reagents

i) Phosphate Buffered Saline Tablets were obtained from Oxoid, Basingstoke, Hants and reconstituted according to the manufacturers instructions.

ii) Sephadex G25 and G150, cyanogen-bromide-activated sepharose and Sephacryl S200 were obtained from Sigma UK, Poole, Dorset.

iii) Ultragel ACA34 was obtained from LKB, Croydon, Surrey.

iv) Hydroxyl-apatite (spheroidal) was obtained from BDH, Poole, Dorset.

v) Protein calibration kits for gel filtration were obtained from Pharmacia (GB) Ltd, Hounslow, Middlesex.

vi) M partigen immunodiffusion plates for SP_1 assay (Behringwerke) were obtained from Hoechst (UK) Ltd, Hounslow, Middlesex.

vii) All other general reagents were of analytical reagent quality.

2. Reagents used in ELISA Systems

i) Carbonate/bicarbonate (coating) buffer pH 9.6 containing 0.015 mol/l sodium carbonate and 0.035 mol/l sodium bicarbonate pH 9.6.

ii) Sodium citrate (molar) solution adjusted to pH 7.4 with molar HCL.

iii) Sample diluting solution. 99 ml PBS and 1 ml sodium citrate.

iv) Wash Buffer. 10 µl Tween 20 in 100 ml PBS.

v) Diluent for antibody-enzyme. PBS containing 25% chicken

citrate.

vi) Horse radish peroxidase Type VI.Sigma London Ltd, Poole, Dorset.

vii) OPD (phosphate-citrate) buffer. 0.13 molar $Na_2HPO_4 \cdot 2H_2O$ and 0.027 molar citric acid, pH 6.0.

viii) Chromogen.Orthophenylene Diamine (OPD) was obtained from Sigma, Poole, Dorset. 20 mg of OPD was dissolved in 50 ml OPD buffer and 10 µl of H_2O_2 (30% w/v) added before use.

ix) Sulphuric acid 4 molar prepared by adding 100 ml concentrated sulphuric acid to 350 ml water.

3. Sera, Antisera and Antiserum conjugates used in ELISA systems

i) Chicken serum was obtained from Flow Laboratories, Irvine, Scotland.

ii) Antiserum to SP_1 manufactured by Dako Immunoglobulins, Copenhagen was obtained from Mercia Brocades, West Byfleet, Surrey.
Antiserum to PAPP-A and hCG were also obtained from this source.

iii) Anti SP_1 - horse radish peroxidase conjugate (Dako, Copenhagen) was obtained from Mercia Brocades, as was antiserum-peroxidase conjugate for hCG. Dilutions were made in antibody enzyme diluent as described in text.

iv) Antiserum to TSH was obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland.

v) SP_1 standard serum was obtained from Hoechst Pharmaceuticals, Hounslow, Middlesex.

vi) International Reference Preparation (IRP 78/610) was obtained from International Agency for Cancer Research, Lyon, France.

4. Solid Phases used in ELISA Systems

i) Cooke Flexible Microtitre Plates (M29A) were obtained from Dynatech Laboratories, Billingshurst, Sussex.

ii) Immulon Micro-Elisa Plates (M129A) were obtained from Dynatech Laboratories, Billingshurst, Sussex.

iii) Nunc Micro Elisa Plates (Grade I) were obtained from Gibco Europe, Paisley, Scotland.

iv) Polystyrene Beads (Matt finish) diameters 3.2, 4.8 and 6.4 mm were obtained from Euromatic Ltd, Brentford, Middlesex.

v) Nylon beads with diameters 3.2 and 6.4 mm were obtained from Northumbria Biologicals, Cramlington, Northumberland.

5. Reagents used in hPL Assay

i) Barbitone buffer pH 8.6 was prepared by mixing 1720 ml of sodium barbitone (12.37 g/l) with 270 ml of 0.06 M hydrochloric acid and adjusting the pH as necessary (hPL).

ii) hPL Assay diluent.250 mg of human albumin (Hoechst Pharmaceuticals, Hounslow, Middlesex) were dissolved in 500 ml pH 8.6 barbitone buffer.

iii) hPL Precipitant.Analar propan-2-ol (isopropanol) was obtained from BDH.

iv) Phosphate buffer pH 7.4 : 0.05 M prepared by dissolving 78 g sodium dihydrogen phosphate ($Na\ H_2PO_4 \cdot 2H_2O$) in 1 litre of distilled water (A), 90 g of disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$) in 1 litre of water (B), and mixing 19.7 ml of (A) with 80.3 ml (B) adjusting the pH if necessary.

v) Anti-hPL was obtained from RAST Allergy Unit, Benenden Chest Hospital, Kent.
Horse Serum III was obtained from Wellcome Diagnostics, Dartford, Kent.

vi) Iodination grade hPL (Code 71/166) was obtained from NIBSC. 7.33 µg in 10 µl of pH 7.4 phosphate buffer were used for iodination.

vii) hPL Standard - International Reference Preparation IRP (73/545) was obtained from the National Institute of Biological Standards and Control, Holly Hill, London. A range of standards 0-11.3 mu/l were prepared in horse serum (Wellcome III).

viii) Solid phase lactoperoxidase - gift from Dr R Chapman, Department of Biochemistry, Glasgow Royal Infirmary. 10 µl aliquots in 200 µl of 0.05 M pH 7.4 phosphate buffer containing no protein or azide were used for iodination.

6. Reagents used in AFP Assay

i) Bovine gamma globulin was obtained from Armour Pharmaceuticals, Eastbourne, England). Antiserum to AFP was obtained from SAPU, Law Hospital, Lanarkshire and used at a FD of 1:140,000.

and Gynaecology, Ninewells Hospital, Dundee. For use 20 µl were diluted in 15 ml diluent.

iii) Diluent buffer for AFP assay 0.05 M barbitone buffer pH 8.6 prepared by dissolving 36.84 g sodium barbitone in 1 L distilled water and adjusting the pH if necessary. Before use 25 mg of bovine gamma-globulin were dissolved in 50 ml of buffer.

iv) AFP Standards. British Standard (72/227) was obtained from NIBSC. Dilutions were prepared in a pooled male serum (known to be free of endogenous AFP) to cover a concentration range 0-147 u/ml.

v) PEG. 5.6 g PEG 6000 were dissolved in 50 ml barbitone buffer without protein.

7. Reagents used in CPAP Assay

i) Tris Buffered Saline (TBS) was prepared from saline tablets obtained from Oxoid Ltd and buffered to pH 7.4 with 10 mmol/l Tris (hydroxymethyl/methylamine and hydrochloric acid).

ii) Diluent was prepared from TBS to which had been added 75 g/l of human albumin (obtained from Hoechst UK Ltd, Hounslow).

iii) Chromogen reagents were obtained as a kit (Cat No 415286) for alkaline phosphatase analysis from Boehringer Corporation Ltd, Lewes, England. This comprised 10 mmol/l p-nitrophenyl phosphate in 1 mol/l diethanolamine pH 9.8 containing 0.5 mmol/l magnesium ions.

iv) Placental alkaline phosphatase was obtained from Miles Laboratories Ltd, UK, Cat No 36-495-2.

APPENDIX II COMPUTER PROGRAMS

PROGRAM FOR CALCULATION OF ENZYME KINETIC DATA (Ch. 5)

```
EADY.

5 PRINT""
10 PRINT"ABEL"
20 PRINT"CALCULATES KM ,VM ,AND STD ERRORS FOR HYPERBOLIC CURVES OBEYING TH";
21 PRINT"E MICHAELIS MENTEN EQN"
25 PRINT"PRESS  TO CONTINUE"
30 GETC$:IF C$<>"C" THEN30
50 DIMS(20),V(20)
100 PRINT""
101 N=0
110 PRINT"INPUT NUMBER OF POINTS":INPUTN
130 PRINT"INPUT DATA IN THE FORMAT.....SUBSTRATE,VELOCITY
135 X=0:Y=0:A=0:B=0:G=0:D=0:E=0:DE=0:KM=0:VM=0:V0=0
140 FOR I=1TON
150 INPUTS,V:S(I)=S:V(I)=V
160 X=V↑2
170 Y=X/S
180 A=A+V*X
190 B=B+X↑2
200 G=G+V*Y
210 D=D+X*Y
220 E=E+Y↑2
230 NEXT
240 DE=(A*E)-(G*D)
250 KM=(B*G-A*D)/DE
260 VM=(B*E-(D↑2))/DE
270 VO=VM
450 A1=0:G1=0:D1=0:B1=0:E1=0
500 FOR I=1TON
510 F=(VM*S(I))/(S(I)+KM)
530 F1=-(VM*S(I))/((S(I)+KM)↑2)
550 A1=A1+(F↑2)
560 G1=G1+(F*F1)
570 D1=D1+V(I)*F
580 B1=B1+(F1↑2)
590 E1=E1+V(I)*F1
600 NEXT
610 DE1=A1*B1-(G1↑2)
620 B8=(B1*D1-G1*E1)/DE1
630 B9=(A1*E1-G1*D1)/DE1
640 VM=VM*B8
650 KM=KM+(B9/B8)
660 C=C+1:IFC<10 THEN450
700 FOR I=1TON:V1=V1+(V(I)↑2):NEXT
710 S2=(V1-(B8*D1)-(B9*E1))/(N-2)
720 S=SQR(S2)
730 KE=(S/B8)*SQR((A1/DE1))
740 VE=(VO*S)*SQR((B1/DE1))
750 OPEN4,4:CMD4
760 PRINT:PRINT
770 PRINT"KM= "KM
780 PRINT"VM= "VM
790 PRINT"STD ERROR KM= "KE
800 PRINT"STD ERROR VM= "VE
810 FOR K=1TO10:PRINT:NEXT
820 PRINT#4:CLOSE4
860 STOP
```

```
2010 A(1,2)=A(1,2)+X↑3
2020 A(1,3)=A(1,3)+X↑4
2030 A(1,4)=A(1,4)+X↑3
2040 A(2,1)=A(2,1)+X↑5
2050 A(2,2)=A(2,2)+X↑4
2060 A(2,3)=A(2,3)+X↑3
2070 A(2,4)=A(2,4)+X↑2
2080 A(3,1)=A(3,1)+X↑4
2090 A(3,2)=A(3,2)+X↑3
2100 A(3,3)=A(3,3)+X↑2
2110 A(3,4)=A(3,4)+X
2120 A(4,1)=A(4,1)+X↑3
2130 A(4,2)=A(4,2)+X↑2
2140 A(4,3)=A(4,3)+X
2150 G(1)=G(1)+(X↑3)*Y
2160 G(2)=G(2)+(X↑2)*Y
2170 G(3)=G(3)+X*Y
2180 G(4)=G(4)+Y
2190 RETURN
3000 S(1,1)=SQR(A(1,1))
3010 S(1,2)=A(1,2)/S(1,1)
3020 S(1,3)=A(1,3)/S(1,1)
3030 S(1,4)=A(1,4)/S(1,1)
3040 S(2,2)= SQR(A(2,2)-(S(1,2)↑2))
3050 S(2,3)=(A(2,3)-(S(1,2)*S(1,3)))/S(2,2)
3060 S(2,4)=(A(2,4)-S(1,2)*S(1,4))/S(2,2)
3070 S(3,3)=SQR(A(3,3)-(S(1,3)↑2)-(S(2,3)↑2))
3080 S(3,4)=(A(3,4)-S(1,3)*S(1,4)-S(2,3)*S(2,4))/S(3,3)
3090 S(4,4)=SQR(A(4,4)-S(1,4)↑2-S(2,4)↑2-S(3,4)↑2)
4010 K(1)=G(1)/S(1,1)
4020 K(2)=(G(2)-S(1,2)*K(1))/S(2,2)
4030 K(3)=(G(3)-S(1,3)*K(1)-S(2,3)*K(2))/S(3,3)
4040 K(4)=(G(4)-S(1,4)*K(1)-S(2,4)*K(2)-S(3,4)*K(3))/S(4,4)
4050 U(4)=K(4)/S(4,4)
4060 U(3)=(K(3)-S(3,4)*U(4))/S(3,3)
4070 U(2)=(K(2)-S(2,3)*U(3)-S(2,4)*U(4))/S(2,2)
4080 U(1)=(K(1)-S(1,2)*U(2)-S(1,3)*U(3)-S(1,4)*U(4))/S(1,1)
4090 PRINTU(1),U(2),U(3),U(4)
4100 RETURN
```

CUBIC FIT OF ABSORBANCE / CONCENTRATION

EADY.

```
10 PRINT"■□■"
20 PRINT"■ CUBIC SOLUTION■"
30 PRINT""
40 PRINT"FOR SOLVING EQNS OF THE TYPE.. Y=AX↑3+BX↑2+CX+D
50 PRINT"PRESS C TO CONTINUE"
60 GET A$:IF A$<>"C" THEN60
100 DIM A(4,4):DIMS(4,4):DIMU(4):DIMG(4):DIMK(4)
101 FOR I=1TO4:FOR J=1TO4:A(I,J)=0:S(I,J)=0:G(I)=0:U(I)=0:K(I)=0
102 NEXT:NEXT
200 PRINT"■□■"
210 PRINT"ENTER DUPLICATE BLANKS"
220 INPUT B1,B2
230 B=(B1+B2)/2
240 PRINT""
250 PRINT"ENTER NUMBER OF STDS IN STD CURVE"
260 INPUTN
261 A(4,4)=N
270 FOR I=1TON
280 PRINT"■□■"
290 PRINT"■POINT "I"ENTER CONC":PRINT""
295 INPUT Y
296 Y(I)=Y
300 PRINT"ENTER DUPLICATE ODS"
310 INPUT O1,O2
320 X=((O1+O2)/2)-B
330 Z(I)=X
340 GOSUB2000
350 NEXT
360 GOSUB 3000
370 PRINT"■□■"
380 PRINT"EQN SOLUTION IS"
390 PRINTU(1)"X↑3+"U(2)"X↑2+"U(3)"X+"U(4)
400 OPEN4,4:CMD4
410 PRINTCHR$(1),"CUBIC FIT"
420 PRINT"EQN SOLUTION IS...
430 PRINT"Y="U(1)"X↑3+"U(2)"X↑2+"U(3)"X+"U(4):PRINT""
440 PRINT"STD CURVE DATA"
450 PRINT#4:CLOSE4
460 FOR I=1TON
470 C=0:X=0:Y=0
480 OPEN4,4:CMD4
490 PRINT"ACTUAL VALUE="Y(I)
495 X=Z(I)
500 PRINT"CALCULATED VALUE="U(1)*X↑3+U(2)*X↑2+U(3)*X+U(4)
510 PRINT#4:CLOSE4
511 C=U(1)*X↑3+U(2)*X↑2+U(3)*X+U(4)
520 R9=R9+(Y(I)-C)↑2
530 R7=R7+(Y(I)-C)↑2/Y(I)
540 NEXT
550 OPEN4,4:CMD4
560 PRINT"RESIDUAL SQUARES="R9
570 PRINT"MEAN RESIDUAL="R9/N
580 PRINT"CHI SQUARED="R7
590 PRINT#4:CLOSE4
600 STOP
```

QUADRATIC FIT OF ABSORBANCE / CONCENTRATION

```
10 REM PROGRAM FOR CURVE FITTING USING A TRIANGULAR MATRIX
15 REM UNKNOWNS STORED IN MATRIX U
16 PRINT"■□■"
17 PRINT"CURVE FITTING PROG SUITABLE FOR PARABOLIC CURVES SOLVED BY QUADRATIC"
18 PRINT"EQUATION"
19 PRINT"IN THIS FORMAT THE PROG IS SET UP FOR ""■ELISA"
20 PRINT"E.G. PAPP-A AND CEA CURVES"
21 PRINT""
22 PRINT""
23 PRINT"■NOTE THAT STD CURVE DATA IS IN AU/L■"
24 PRINT"■TEST CONCS ARE PRINTED IN IU/L BASED ON IRP 78/610■"
50 DIM A(3,3):DIM S(3,3)
60 DIM G(3):DIMK(3):DIMU(3)
70 FOR I=1TO3
80 FOR J=1TO3
90 A(I,J)=0
100 S(I,J)=0
110 NEXT:NEXT
120 FOR I=1 TO3
130 G(I)=0:K(I)=0:U(I)=0
140 NEXT
141 PRINT""
142 PRINT""
150 PRINT"INPUT BLANK ODS..BL1,BL2"
160 INPUT B1,B2
170 B=(B1+B2)/2
171 PRINT""
200 PRINT "HOW MANY STDS IN STD CURVE EXCLUDING BLANK?"
210 INPUT N
220 A(3,3)=N
230 FOR M=1TON
240 PRINT"■□■"
250 PRINT "POINT"M"ENTER DUPLICATE ODS IN THE FORMAT..OD1,OD2"
260 INPUT O1,O2
261 X=((O1+O2)/2)-B
262 X(M)=X:Z(M)=X
270 PRINT"ENTER STD CONC IN AU/L"
280 INPUT Y
281 Y(M)=Y :
290 A(1,1)=A(1,1)+X↑4
300 A(1,2)=A(1,2)+X↑3
310 A(1,3)=A(1,3)+X↑2
320 A(2,1)=A(2,1)+X↑3
330 A(2,2)=A(2,2)+X↑2
340 A(2,3)=A(2,3)+X
350 A(3,1)=A(3,1)+X↑2
360 A(3,2)=A(3,2)+X
370 G(1)=G(1)+(X↑2)*Y
380 G(2)=G(2)+X*Y
390 G(3)=G(3)+Y
400 NEXT
410 PRINT "DATA LOADED"
460 S(1,1)=SQR(A(1,1))
470 S(1,2)=A(1,2)/S(1,1)
480 S(1,3)=A(1,3)/S(1,1)
490 S(2,2)=SQR(A(2,2)-(S(1,2)↑2))
500 S(2,3)=(A(2,3)-(S(1,2)*S(1,3)))/S(2,2)
510 S(3,3)=SQR(A(3,3)-(S(1,3)↑2)-(S(2,3)↑2))
520 K(1)=G(1)/S(1,1)
530 K(2)=(G(2)-S(1,2)*K(1))/S(2,2)
```

```
560 U(2)=(K(2)-(S(2,3)*U(3)))/S(2,2)
570 U(1)=(K(1)-(S(1,2)*U(2))-(S(1,3)*U(3)))/S(1,1)
580 PRINT "■□"
590 PRINT "■UNKNOWN VALUES ARE....■"
600 PRINT
610 PRINT TAB20,"U1="U(1)
620 PRINT TAB20,"U2="U(2)
630 PRINT TAB20,"U3="U(3)
631 PRINT"ENTER ASSAY NAME":INPUTA$
632 PRINT"ENTER DATE":INPUTY$
640 OPEN4,4:CMD4
641 PRINT CHR$(1),A$
642 PRINT CHR$(1),Y$
650 PRINT "EQN SOLUTION IS ..."U(1)"X↑2+"U(2)"X+"U(3)
660 PRINT#4:CLOSE4
661 GOTO1000
670 PRINT" TEST DATA INPUT"
680 PRINT"PRESS C TO CONTINUE"
685 INPUTC$:IF C$<>"C" THEN685
690 PRINT"■□■"
730 PRINT"ENTER DUPLICATE ODS IN THE FORMAT...OD1,OD2"
735 PRINT"TERMINATOR=100,0"
740 INPUT T1,T2
741 IF T1>99 THEN STOP
745 V=V+1
750 T=(T1+T2)/2
770 Y=(U(1)*T↑2+U(2)*T+U(3))
900 OPEN4,4:CMD4
920 PRINT"TEST "V "CONC="Y*9"IU/L"
930 PRINT#4:CLOSE4
960 GOTO730
1000 OPEN4,4:CMD4
1010 PRINT CHR$(1)"STD CURVE DATA"
1011 PRINT#4:CLOSE4
1020 FOR M=1TON
1025 OPEN4,4:CMD4
1030 PRINT"ACTUAL VALUE"Y(M)
1040 PRINT "CALCULATED VALUE="(U(1)*Z(M)↑2+U(2)*Z(M)+U(3))
1041 C=(U(1)*Z(M)↑2+U(2)*Z(M)+U(3))
1042 R1=R1+(C-Y(M))↑2
1043 R2=R1/N:R3=(C-Y(M))↑2/Y(M)
1045 PRINT#4:CLOSE4
1050 NEXT
1051 OPEN4,4:CMD4
1052 PRINT"SUM OF RESIDUAL SQUARES="R1
1053 PRINT"MEAN RESIDUAL="R2
1054 PRINT"CHI SQUARED ="R3
1055 PRINT#4:CLOSE4
1060 PRINT"DO YOU WISH TO ALTER DATA (Y OR N)"
1070 INPUT Y$
1080 IF Y$<>"Y" GOTO670
1090 IF Y$="Y" THEN GOTO70
```

LOG-LOGIT TRANSFORM OF ABSORBANCE / CONCENTRATION

EADY.

```
10 REM LOG LOGIT TRANSFORM FITTED ON LINEAR REGRESSION EQN
20 PRINT"■□■"
30 PRINT"■LOG-LOGIT TRANSFORM■"
40 PRINT""
50 PRINT"ACCEPTS +VE AND -VE LOGIT POINTS"
60 PRINT"FITS CURVE BY LINEAR REGRESSION AND PROVIDES HARD COPY OUTPUT"
70 PRINT"TEST DATA ENTRY PROVIDED AFTER CALCULATION ROUTINE"
80 PRINT"PRESS C TO CONTINUE"
90 GET A$:IF A$<>"C" THEN 90:
100 PRINT"■□■"
110 PRINT "STD CURVE DATA INPUT"
120 PRINT
121 PRINT""
130 X=0:X2=X:XY=X:Y2=X:D1=X:D2=X:B=X:A=X:D=X:E=X:F=X:G=X:H=X:U1=X:U3=X
140 N=X:M=X:C=X:R=X:SG=X:P=X:Q=X:SI=X:T=X
150 PRINT"ENTER DUPLICATE BLANKS"
160 INPUT B1,B2
170 B3=(B1+B2)/2
180 PRINT"INPUT NUMBER OF DATA POINTS"
190 INPUT K
200 FOR I=1TOK
210 PRINT"■□■"
220 PRINT"POINT "I
230 PRINT"ENTER DUPLICATE OD VALUES"
240 INPUTY8,Y9
250 Y3=(Y8+Y9)/2-B3
260 YI=LOG(Y3/(1-Y3))
270 Y(I)=YI
280 PRINT""
290 PRINT"ENTER CONC"
300 INPUTXK
301 XI=LOG(XK)/2.30259
310 X(I)=XI
400 N=N+1
410 X=X+XI
420 Y=Y+YI
430 X2=X2+XI*XI
440 Y2=Y2+YI*YI
450 XY=XY+XI*YI
460 D1=D1+(XI-YI)
470 D2=D2+(XI-YI)↑2
480 NEXT
490 A=XY-X*Y/N
500 B=X2-X*X/N
510 M=A/B
520 D=Y2-Y*Y/N
530 C=(Y-M*X)/N
540 E=B*D
550 F=SQR(E)
560 R=A/F
570 G=D/B
580 H=(1-R↑2)/(N-2)
590 SG=SQR(G*H)
600 P=X/N
610 Q=Y/N
620 U1=D1/N
```

```
650 OPEN4,4:CMD4
660 PRINT CHR$(1)"STD CURVE DATA"
670 PRINT"NUMBER OF PAIRS = ";N
680 PRINT"MEAN OF LOGIT ODS =";P
690 PRINT"MEAN OF LOG CONC = ";Q
700 PRINT"GRADIENT          = ";M
710 PRINT"STD ERROR OF GRADIENT=";SG
720 PRINT"Y AXIS INTERCEPT =";C
730 PRINT"STD ERROR OF INTERCEPT=";SI
740 PRINT"CORRELATION COEFF.  =";R
750 PRINT"STUDENT'S T VALUE =";T
760 PRINT#4:CLOSE4
770 PRINT"■"
780 FOR I=1TOK
790 Z=X(I)
800 Y=Y(I)
810 OPEN4,4:CMD4
820 PRINT"ACTUAL CONC="EXP(Z*2.30259)
831 PRINT"CALC CONC="EXP(((Y-C)/M)*2.30259)
832 E1=E1+(EXP(Z*2.30259)-EXP(((Y-C)/M)*2.30259))↑2
833 R9=R9+(EXP(Z*2.30259)-EXP(((Y-C)/M)*2.30259))↑2/(EXP(Z*2.30259))
840 PRINT#4:CLOSE4
848 PRINT"RESIDUAL SUM OF SQUARES="E1
849 PRINT"MEAN RESIDUAL SUM OF SQUARES="E1/K
850 NEXT
851 OPEN4,4:CMD4
852 PRINT"RESIDUAL SUM OF SQUARES="E1
853 PRINT"MEAN RESIDUAL SUM OF SQUARES="E1/K:PRINT"CHI SQUARED="R9
854 PRINT#4:CLOSE4
860 PRINT"TEST DATA?(Y OR N)"
870 GET A$:IF A$<>"Y"ANDA$<>"N" THEN870
880 IF A$="N" THEN STOP
881 PRINT"■"
882 Z9=0
890 PRINT"ENTER TEST ODS(-999,0 TO STOP)"
891 Z9=Z9+1
900 INPUTY8,Y9
901 IF Y8=-999 THEN STOP
910 Y3=(Y8+Y9)/2-B3
920 YI=LOG(Y3/(1-Y3))
930 OPEN4,4:CMD4
939 PRINT "TEST "Z9
940 PRINT"CONCENTRATION= "EXP(((YI-C)/M)*2.30259)
960 PRINT#4:CLOSE4
970 GOTO890
```

DATA CAPTURE PROGRAM AND HEALY FIT

OF DATA — AUTOMATED FOR USE ON MULTISKAN

EADY.

```
5 PRINT"■□■"
50 PRINT"**********************************************":PRINT""
51 PRINT"■AUTO-HEALY PROGRAMME"
57 PRINT"":PRINT"**********************************************"
70 PRINT"ENTER YOUR NAME"
71 INPUT N$
73 PRINT"INPUT ASSAY NAME":INPUTZ$
75 PRINT"INPUT DATE":INPUTD$
80 OPEN4,4:CMD4
81 PRINT CHR$(1),"ASSAY..."Z$
82 PRINT CHR$(1),"OPERATOR..."N$
83 PRINT CHR$(1)"DATE..."D$
84 PRINT#4:CLOSE4
90 DIMA(4,4):DIMS(4,4)
91 DIM G(4):DIMK(4):DIMU(4)
100 GOTO210
110 CLOSE1:OPEN1,7:FORI=1TO10:GET#1,A$:NEXT
120 PRINT"□■READ PLATE ON MULTISKAN.":L=1
130 GET#C1,A$:IFA$=""THEN130
140 T=TI
150 GET#C1,CH$(L)
160 IFTI>T+C4GOTO190
170 IFCH$(L)=""GOTO150
180 L=L+C1:GOTO140
190 PRINT"■DATA READ IN.■"
200 GOTO250
210 C1=1:C2=2:C3=10:C4=30:C5=600
220 DIMCH$(615),OD(8,12)
230 MO$=" "
240 GOTO110
250 FORI=1TO9
260 IFCH$(I)="."THENX=I-2:FI=VAL(CH$(I-4)+CH$(I-3))
270 NEXT
280 I=0
290 N=INT((L-5)/51)+FI
300 FORK=FITON
310 FORJ=1TO8
320 A$=""
330 FORM=1TO5
340 A$=A$+CH$(X+I+M)
350 NEXT
360 A=VAL(A$)
370 IFA>8THENA=(INT((A-10)*1000+0.5))/1000
380 OD(J,K)=A
390 I=I+6
400 NEXT
410 I=I+3
420 NEXT
430 A$="ABCDEFGH":FORI=1TO8:AL$(I)=MID$(A$,I,1):NEXT
440 L$="¯"
450 IFM$="M"THENPRINT"□      MC MODE ";MO$;"      ■COLUMN■":GOTO470
460 PRINT"□      MULTISKAN      ■COLUMN■"
470 P$(2)="■ROW■¯"
480 P$(3)="     |     ■1■       ■2■       ■3■       ■4■       ■5■       ■6■"
```

```
510 K=1
520 FORJ=0TOINT((N-1)/6)
530 IFJ=1THENPRINT"■":PRINTP$(4)
540 FORL=1TO8
550 FORM=1TO6
560 IFM=1THENPRINT" ■";AL$(L);"■ | ";
570 IF(M+6*J)>NOR(M+6*J)<FITHENPRINT"    -   ";:GOTO600
580 AB=OD(L,M+6*J)
590 GOSUB680
600 NEXT
610 NEXT
620 NEXT
621 PRINT"■■HARDCOPY?"
622 GETA$:IFA$=""THEN622
623 IFA$="H"THENGOSUB1000
624 PRINT"■FILE ON ■TAPE OR ■DISK OR ■NO FILE?"
625 GETA$:IFA$=""THEN625
626 IFA$="T"THENGOSUB1440:GOTO624
627 IFA$="D"THENGOSUB1540:GOTO624
628 PRINT"CALCULATE RESULTS (Y OR N)"
629 INPUT Y$:IF Y$="Y" GOTO2000
630 PRINT"■MORE PLATES?"
640 GETA$
650 IFA$="N"THENSTOP
660 IFA$="Y"ORA$=" "THEN800
670 GOTO640
680 P=0
690 IFAB<0THENAB=AB*-1:P=1
700 A=INT(AB*1000+0.5)+2000
710 B$=STR$(A)
720 FORR=2TO5
730 A$(R)=MID$(B$,R,1)
740 NEXT
750 A=VAL(A$(2))-2
760 A$(2)=STR$(A)
770 IFP=1THENA$(2)=" -"
780 PRINTA$(2);".";A$(3);A$(4);A$(5);
790 RETURN
800 PRINT"■■PLEASE WAIT."
810 A=FRE(0)
820 FORI=1TO615:CH$(L)="":NEXT
840 GOTO110
1000 A$="ABCDEFGH":FORI=1TO8:AL$(I)=MID$(A$,I,1):NEXT
1010 CLOSE4
1020 OPEN4,4
1030 PRINT#4,CHR$(1)"  MULTISKAN"CHR$(129)
1040 L$="¯"
1050 PRINT#4,TAB(34);CHR$(1)"COLUMN"CHR$(129)
1060 PRINT#4,CHR$(1)"ROW"CHR$(129);:FORI=1TO70:PRINT#4,L$;:NEXT:PRINT#4
1070 PRINT#4,CHR$(1)"     1  2  3  4  5  6  7  8  9 10 11 12"H$
1080 FORL=1TO8
1090 FORM=1TO12
1100 IFM>NTHENPRINT#4,"    -   ";:GOTO1150
1110 ZZ=OD(L,M):ZM=1:ZN=3
1120 IFM=1THENPRINT#4:PRINT#4:PRINT#4,CHR$(1)" "AL$(L)" "CHR$(129);" ";
1130 GOSUB1190
1140 PRINT#4,ZZ$;
1150 NEXT
1160 NEXT
1170 PRINT#4
1180 RETURN
1190 IFZM<0THENZM=0
1200 IFZN<0THENZN=0
1210 SI=SGN(ZZ)
```

```
1240 IFZA<>0THENZL=INT(LOG(ZA)/LOG(10)+1):IFZM<ZLTHENZM=ZL
1250 ZA=ZA/10↑ZM
1260 SI$=" ":IFSI=-1THENSI$="-"
1270 NU$="":DI=0
1280 IFZM=0THENNU$=" ":GOTO1360
1290 FORZI=1TOZM
1300 ZA=ZA*10:ZB=INT(ZA):ZA=ZA-ZB
1310 IFDI<>0THEN1340
1320 IFZB=0ANDZI<>ZMTHENNU$=NU$+" ":GOTO1350
1330 DI=1:NU$=NU$+SI$
1340 NU$=NU$+CHR$(ZB+48)
1350 NEXT
1360 IFZN=0THEN1420
1370 NU$=NU$+"."
1380 FORZI=1TOZN
1390 ZA=ZA*10:ZB=INT(ZA):ZA=ZA-ZB
1400 NU$=NU$+CHR$(ZB+48)
1410 NEXT
1420 ZZ$=NU$
1430 RETURN
1440 PRINT"■PREPARE TAPE■"
1450 INPUT"■FILE NAME";A$
1460 CLOSE2:OPEN2,1,1,A$
1470 FORI=1TON
1480 FORJ=1TO8
1490 PRINT#2,OD(J,I)
1500 NEXT
1510 NEXT
1520 CLOSE2
1530 RETURN
1540 INPUT"■DRIVE NUMBER (■0■ OR ■1■)■■■■■■■■";B$
1550 INPUT"■FILE NAME";A$
1560 A$=B$+":"+A$+",SEQ"+",W"
1570 CLOSE8:OPEN8,8,8,A$
1580 FORI=1TON
1590 FORJ=1TO8
1600 PRINT#8,OD(J,I);CHR$(13);
1610 NEXT
1620 NEXT
1630 IFDS<>0THENPRINT"■DISK ERROR":PRINTDS$:GOTO1540
1640 CLOSE8
1650 RETURN
2000 PRINT"■■■"
2010 PRINT"CALCULATION ROUTINE COMMENCES"
2100 FOR I=1TO4:FOR J=1TO4
2130 A(I,J)=0:S(I,J)=0
2140 U(I)=0:K(I)=0:G(I)=0
2150 NEXT:NEXT
2160 X1=0:X2=0:X3=0:X4=0
2170 REM CONSTANT VALUES ARE...
2180 A=0.01223
2190 B=0.74635
2200 C=-5.75063
2210 D=-1.08382
2220 PRINT TAB30"ENTER NUMBER OF STDS IN STD CURVE EXCLUDING BLANKS"
2230 INPUT N
2240 PRINT"■■■"
2260 FOR M=1TON
2270 X(M)=0
2280 NEXT
2290 PRINT"■■■"
2300 FOR M=1TON
2301 PRINT"■POINT "M "ENTER CONC"
2310 INPUT X(M)
```

```
2350 PRINT"STD VALUES ENTERED ARE..."
2360 FOR M=1TON
2370 PRINT "POINT"M"    "X(M)
2380 NEXT
2381 PRINT""
2382 PRINT"DO YOU WISH TO ALTER THESE VALUES?(A=ALTER OR N=NOT ALTER)"
2383 INPUTY$:IF Y$="N" THEN2420
2390 IF Y$="A"THEN PRINT"INPUT POINT TO BE CHANGED(1TO"N")"
2391 INPUTC1:X(C1)=0:PRINT"INPUT CORRECTVALUE":INPUTX(C1):GOTO2350
2420 O3=((OD(2,3)+OD(2,4))/2)
2430 PRINT"BLANK1="OD(2,3),"BLANK2="OD(2,4),"MEAN="O3
2440 IFOD(2,3)>OD(2,4)+0.3*OD(2,4) THEN GOSUB 6000
2450 IF OD(2,3)<OD(2,4)-0.3*OD(2,4) THEN GOSUB6000
2460 FOR K9=5TO15 STEP2:K8=INT(K9/2)-1
2470 IF K9<=11 THEN K6=2:K7=K9
2480 IF K9>11 THEN K6=3:K7=K9-12
2490 Y(K8)=((OD(K6,K7)+OD(K6,K7+1))/2)-O3
2495 PRINT"OD VALUE  -BLANK POINT"K8:PRINT Y(K8)
2500 IF OD(K6,K7)>OD(K6,K7+1)+0.2*OD(K6,K7+1) THEN GOSUB6200
2510 IF OD(K6,K7)<OD(K6,K7+1)-0.2*OD(K6,K7+1) THEN GOSUB 6200
2520 NEXT K9
2590 PRINT"CHECK THAT ALL STD MEAN ODS ARE POSITIVE"
2600 PRINT" DO YOU WISH TO ALTER OD DATA AT ANY POINT INCLUDING BLANK?(Y OR N)"
2610 INPUTY$:IF Y$="Y" THEN GOSUB6500
2630 PRINT"■□■"
2640 PRINT"CALCULATION SUBROUTINE ...PAUSE OF 80 SECONDS"
2641 FORM=1TON:X=X(M):Y=Y(M)
2642 GOSUB7000
2643 NEXTM
2644 GOSUB7270
2650 FOR T=1TO10
2660 A=A+U(1):B=B+U(2):C=C+U(3):D=D+U(4)
2670 FOR I=1TO4:FORJ=1TO4:A(I,J)=0:S(I,J)=0:U(I)=0:K(I)=0:G(I)=0
2680 NEXT:NEXT
2690 FOR M=1TON
2710 X=X(M)
2715 Y=Y(M)
2716 GOSUB7000
2720 NEXT
2725 GOSUB7270
2730 NEXT T
2770 PRINT"■□■"
2780 PRINT "CONSTANT A  %DIFF="(A2-A)*100
2790 PRINT "CONSTANT B  %DIFF="(B2-B)*100
2800 PRINT "CONSTANT C  %DIFF="(C2-C)*100
2810 PRINT "CONSTANT D  %DIFF="(D2-D)*100
2820 PRINT""
2830 PRINT"IF %DIFF IS OF THE ORDER E-05 PRESS C TO CONTINUE"
2840 GETA$:IFA$<>"C" THEN2840
3020 X=0:Y=0
3029 CLOSE4
3030 OPEN4,4:CMD4
3031 PRINT""
3040 PRINT CHR$(1)"STD CURVE DATA"
3041 PRINT#4:CLOSE4
3050 FOR M=1TON
3051 OPEN4,4:CMD4
3060 Z=X(M)
3070 Y=Y(M)
3080 X=EXP((C/D)-((1/D)*LOG((Y-A)/(A+B-Y))))
3090 R9=R9+(Z-X)↑2:R8=R8+((Z-X)↑2)/N
3091 R7=R7+((Z-X)↑2)/Z
3110 PRINT"ACTUAL VALUE"Z
3120 PRINT"CALCULATED VALUE"X
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```
3160 OPEN4,4:CMD4
3170 PRINT"A="A2
3180 PRINT"B="B2
3190 PRINT"C="C2
3200 PRINT"D="D2
3210 PRINT"MEAN RESIDUAL SQUARE="R8:PRINT"CHI SQUARED ="R7
3220 PRINT#4:CLOSE4
3230 PRINT"MANUAL OR AUTO INPUT OF TEST DATA?(M OR A)"
3231 INPUTA$:IF A$="M"THEN GOSUB 7600
3232 OPEN4,4:CMD4
3233 PRINT CHR$(1),"TEST RESULTS"
3234 PRINT#4:CLOSE4
3245 PRINT"■□■"
3250 PRINT"ENTER NUMBER OF UNKNOWNS"
3260 INPUT U1
3270 X=0:Y=0
3271 U2=0
3280 FOR J=5TO11 STEP2
3290 Y=(OD(3,J)+OD(3,J+1))/2-O3
3300 U2=U2+1
3302 IF (Y-A)<0 THEN 3320
3310 X=EXP((C/D)-((1/D)*LOG((Y-A)/(A+B-Y))))
3320 OPEN4,4:CMD4
3330 IFY-A<0THEN PRINT"TEST "U2"OD < BLANK ":IFY-A<0THEN3350
3340 PRINT"TEST"U2"="X"MG/L"
3341 IF OD(3,J)>OD(3,J+1)+0.2*OD(3,J+1) THEN PRINTCHR$(1)"BAD REPLICATES"
3342 IF OD(3,J)<OD(3,J+1)-0.2*OD(3,J+1) THEN PRINT CHR$(1)"BAD REPLICATES"
3350 PRINT#4:CLOSE4
3360 IF U2=U1 THEN GOTO4000
3370 NEXT J
3380 FOR I=4TO7
3390 FOR J=1TO11 STEP2
3400 Y=(OD(I,J)+OD(I,J+1))/2-O3
3405 U2=U2+1
3407 IFY-A<0THEN 3420
3410 X=EXP((C/D)-((1/D)*LOG((Y-A)/(A+B-Y))))
3420 OPEN4,4:CMD4
3430 IFY-A<0THEN PRINT"TEST "U2" OD<BLANK":IFY-A<0THEN3450
3440 PRINT "TEST"U2"="X"MG/L"
3441 IF OD(I,J)>OD(I,J+1)+0.2*OD(I,J+1) THEN PRINT CHR$(1)"BAD REPLICATES"
3442 IF OD(I,J)<OD(I,J+1)-0.2*OD(I,J+1) THEN PRINT CHR$(1)"BAD REPLICATES"
3450 PRINT#4:CLOSE4
3460 IF U2=U1 THEN GOTO4000
3470 NEXT:NEXT
4000 FOR I=1TO25
4010 PRINT"■TEST DATA COMPLETE■"
4020 NEXT
4030 PRINT"MORE PLATES?(Y OR N)"
4031 INPUTY$:IF Y$="N"THEN STOP
4032 IF Y$="Y" THEN PRINT"■NOW TYPE RUN<CR>":STOP
6000 O3=0
6010 PRINT"■□■"
6020 PRINT"BAD BLANK REPLICATES...ENTER MANUALLY IN THE FORMAT OD1,OD2"
6030 INPUT O1,O2
6040 O3=(O1+O2)/2
6050 PRINT"BLANK1="O1,"BLANK2="O2,"OK TO CONTINUE?(Y OR N)
6060 INPUT Y$:IF Y$="N" THEN GOTO6000
6070 RETURN
6200 PRINT" BAD REPLICATES IN STD CURVE...ENTER OD DATA MANUALLY? (Y OR N)"
6205 INPUTY$:IF Y$="N" THEN STOP
6215 PRINT"■□■"
6230 PRINT" POINT"K8"ENTER DUPLICATE OD VALUES IN THE FORMAT OD1,OD2"
6240 INPUTY1,Y2
6250 Y(M)=(Y1+Y2)/2-O3
```

```
6510 IF Y$="N" THEN6540:O3=0:PRINT"ENTER NEW BLANK ODS"
6511 PRINT"ENTER NEW BLANK ODS"
6520 INPUT O1,O2:O3=(O1+O2)/2
6530 PRINT"OK TO CONTINUE?(Y OR N)":INPUTY$:IF Y$<>"Y" THEN6500
6540 PRINT"DO YOU WISH TO ALTER A STD OD VALUE?(Y OR N)":INPUTY$
6550 IF Y$="N" THEN2630
6560 FOR M=1TON:PRINT"POINT "M"  "Y(M):NEXT
6570 PRINT"INPUT POINT TO BE ALTERED":INPUT C1:Y(C1)=0
6580 PRINT"INPUT NEW DUPLICATE ODS":INPUT Y1,Y2:Y(C1)=(Y1+Y2)/2-O3
6590 FOR M=1TON:PRINT"POINT "M"  "Y(M):NEXT:GOTO6540
6650 FOR M=1TON:PRINTY(M):NEXT
6660 PRINT "OK TO CONTINUE (Y OR N)"
6670 INPUTY$:IF Y$="N" THEN GOTO6500
6680 RETURN
7000 Y3=A+B*(EXP(C-D*LOG(X))/(1+EXP(C-D*LOG(X))))
7010 REM SUB ROUTINE FOR DATA STORAGE..RETURNS TO 2720(NEXT X/Y)
7020 X1=1
7030 X2=(EXP(C-D*LOG(X))/(1+EXP(C-D*LOG(X))))
7040 X3=B*(EXP(C-D*LOG(X))/(1+EXP(C-D*LOG(X)))↑2)
7050 X4=-(B*LOG(X)*EXP(C-D*LOG(X))/(1+EXP(C-D*LOG(X)))↑2)
7060 R=Y-Y3
7070 A(1,1)=A(1,1)+X1↑2
7080 A(1,2)=A(1,2)+X1*X2
7090 A(1,3)=A(1,3)+X1*X3
7100 A(1,4)=A(1,4)+X1*X4
7110 A(2,1)=A(2,1)+X1*X2
7120 A(2,2)=A(2,2)+X2↑2
7130 A(2,3)=A(2,3)+X2*X3
7140 A(2,4)=A(2,4)+X2*X4
7150 A(3,1)=A(3,1)+X1*X3
7160 A(3,2)=A(3,2)+X2*X3
7170 A(3,3)=A(3,3)+X3↑2
7180 A(3,4)=A(3,4)+X3*X4
7190 A(4,1)=A(4,1)+X1*X4
7200 A(4,2)=A(4,2)+X2*X4
7210 A(4,3)=A(4,3)+X3*X4
7220 A(4,4)=A(4,4)+X4↑2
7230 G(1)=G(1)+X1*R
7240 G(2)=G(2)+X2*R
7250 G(3)=G(3)+X3*R
7260 G(4)=G(4)+X4*R
7265 RETURN
7270 S(1,1)=SQR(A(1,1))
7280 S(1,2)=A(1,2)/S(1,1)
7290 S(1,3)=A(1,3)/S(1,1)
7300 S(1,4)=A(1,4)/S(1,1)
7310 S(2,2)=SQR(A(2,2)-(S(1,2)↑2))
7320 S(2,3)=(A(2,3)-(S(1,2)*S(1,3)))/S(2,2)
7330 S(2,4)=(A(2,4)-S(1,2)*S(1,4))/S(2,2)
7340 S(3,3)=SQR(A(3,3)-(S(1,3)↑2)-(S(2,3)↑2))
7350 S(3,4)=(A(3,4)-S(1,3)*S(1,4)-S(2,3)*S(2,4))/S(3,3)
7360 S(4,4)=SQR(A(4,4)-(S(1,4)↑2)-(S(2,4)↑2)-(S(3,4)↑2))
7370 K(1)=G(1)/S(1,1)
7380 K(2)=(G(2)-S(1,2)*K(1))/S(2,2)
7390 K(3)=(G(3)-S(1,3)*K(1)-S(2,3)*K(2))/S(3,3)
7400 K(4)=(G(4)-S(1,4)*K(1)-S(2,4)*K(2)-S(3,4)*K(3))/S(4,4)
7410 U(4)=K(4)/S(4,4)
7420 U(3)=(K(3)-S(3,4)*U(4))/S(3,3)
7430 U(2)=(K(2)-S(2,3)*U(3)-S(2,4)*U(4))/S(2,2)
7440 U(1)=(K(1)-S(1,2)*U(2)-S(1,3)*U(3)-S(1,4)*U(4))/S(1,1)
7450 A2=A+U(1)
7460 B2=B+U(2)
7470 C2=C+U(3)
7480 D2=D+U(4)
```

```
7610 PRINT"ENTER NUMBER OF UNKNOWNS"
7620 INPUT U1
7630 PRINT"NOW ENTER DUPLICATE OD VALUES IN THE FORMAT OD1,OD2
7640 FOR I=1TOU1
7650 U2=U2+1
7660 PRINT"■TEST"U2"INPUT ODS"
7670 INPUT T1,T2
7675 X=0:Y=0
7680 Y=(T1+T2)/2-03
7690 X=EXP((C/D)-((1/D)*LOG((Y-A)/(A+B-Y))))
7700 OPEN4,4:CMD4
7710 PRINT""
7720 PRINT"TEST"U2"="X"MG/L"
7730 PRINT""
7740 PRINT#4:CLOSE4
7750 NEXT I:PRINT"MORE RESULTS?(Y OR N)":INPUT Y$
7751 IF Y$="N" THEN STOP
7760 RETURN
```