SOLUBLE FIBRINOGEN-FIBRIN COMPLEXES IN PRE-ECLAMPSIA AND OTHER CLINICAL CONDITIONS WHICH MAY BE ASSOCIATED WITH INTRAVASCULAR COAGULATION

by

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VOLUME I

A thesis in two volumes submitted for the degree of Doctor of Philosophy to the University of Glasgow.
February, 1977

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VOLUME I

ACKNOWLEDGEMENTS

It is a pleasure to thank Professor E.M. McGirr for his interest and guidance during the three years spent working for this thesis. The author would also like to thank Professor McGirr for access to laboratory facilities in the University Department of Medicine at Glasgow Royal Infirmary and the Medical Research Council for financial assistance.

The author is very grateful to Dr. C.R.M. Prentice, Dr. C.D. Forbes and Dr. P.W. Howie for their help, encouragement and guidance in this project and for advice in the preparation of the text. She would also like to thank Dr. C.D. Forbes and Dr. J.H. McKillop for help in proof-reading. The advice of Dr. T. Davies of the University Department of Medicine, The General Infirmary, Leeds during the early stages of the project and of Mr. S. Cummings of the Health Services Operational Research Unit, University of Strathclyde in relation to statistical analysis, is gratefully acknowledged.

The author would like to thank Professor I. Donald and Professor M.C. Macnaughton and the staff of The Queen Mother's Hospital and The Royal Maternity Hospital in Glasgow for allowing her to study patients under their care. She is also grateful to Mr. D.W. Short (Glasgow Royal Infirmary) for referring the patients with severe peripheral arterial disease studied in Chapter IV and Dr. R.J. Weir (Gartnavel General Hospital, Glasgow) for referring the patients with suspected "pill-related hypertension" studied in Chapter VIII.

The illustrations for this thesis were prepared with great skill by Mr. F. Spiers and Mrs. B. Burn and photographed by Mr. P.M. Kent. The author is also extremely grateful to Mrs. M. Younger for typing the text with such patience and accuracy.

DECLARATION

The work described in this thesis was performed in the University Department of Medicine, Glasgow Royal Infirmary from January, 1974 until January, 1977. The detailed planning of the work and its execution was performed entirely by the author. The only exception to this was a joint study with Mr. William Edgar (University Department of Medicine, Glasgow Royal Infirmary) using polyacrylamide gel electrophoresis to study the structure of soluble fibrinogen-fibrin complexes produced by the coagulant enzyme, ancrod in vitro (Chapter III. 6) and in vivo (Chapter IV. 4 (c)). The fact that this work was not performed solely by the author is clearly acknowledged in the text. The author would like to thank Mr. Edgar for permission to include the results of this joint study.

Some of the work described in this thesis has already been published:-

(1) McKillop, C., Edgar, W., Prentice, C.R.M. & Forbes, C.D. (1975) Soluble fibrin complex production and proteolysis during ancrod therapy. Scottish Medical Journal, 20, 139-140.

(2) McKillop, C., Edgar, W., Forbes, C.D. & Prentice, C.R.M. (1975) Possible pathway for formation of fibrin degradation products during ancrod therapy. Nature, 255, 638-640.

(3) McKillop, C., Edgar, W., Forbes, C.D. & Prentice, C.R.M. (1975) In vivo production of soluble complexes containing fibrinogen-fibrin related antigen during ancrod therapy. Thrombosis Research, 7, 361-372.

(4) McKillop, C., Howie, P.W., Forbes, C.D. & Prentice, C.R.M. (1976) Soluble fibrinogen-fibrin complexes in pre-eclampsia. Lancet, i, 56-58.

Copies of these papers can be found inside the pocket in the back cover of this thesis (Volume I).

The author would like to thank the editors of the journals concerned

for permission to reproduce the following figures from these papers in this thesis:-

Figure 19	The Lancet
Figure 39a	Nature Thrombosis Research
Figure 39b	Thrombosis Research
Figure 43	Thrombosis Research
Figure 44	Nature Thrombosis Research
Figure 46	The Lancet
Figure 47	The Lancet

The following papers based on work in this thesis have been personally presented by the author:-

1. Soluble fibrin complex production and proteolysis during ancrod therapy. Scottish Society for Experimental Medicine. 1st February, 1975. Edinburgh.

2. Formation of soluble complexes of fibrinogen related material during ancrod infusion. Vth Congress of the International Society on Thrombosis and Haemostasis. 26th July, 1975. Paris.

3. Blood coagulation in pre-eclampsia. Gynaecological Visiting Society. 24th September, 1975. Glasgow.

4. Soluble fibrin complexes in pre-eclampsia. Scottish Society for Experimental Medicine. 31st October, 1975. Dundee.

5. Soluble fibrinogen-fibrin complexes in pre-eclampsia. Blair-Bell Research Society. 3rd February, 1976. London.

6. Plasma fibrinogen chromatography in disseminated intravascular coagulation. Haemostasis Club. 30th April, 1976. Oxford.

7. Plasma fibrinogen chromatography. West of Scotland Blood Club. 19th May, 1976. Glasgow.

8. Plasma fibrinogen chromatography. A new technique for the detection of thrombosis? Royal Medico-Chirurgical Society of Glasgow. 5th November, 1976.

SUMMARY

This thesis is concerned with the identification of soluble fibrinogen-fibrin complexes by plasma fibrinogen chromatography. These soluble complexes are thought to reflect intravascular coagulation in vivo. The studies, which will be described, were undertaken to investigate further the proposed association between pre-eclampsia and disseminated intravascular coagulation by searching for soluble fibrinogen-fibrin complexes in plasma samples from pre-eclamptic patients.

Pre-eclampsia remains an important obstetrical syndrome of unknown primary aetiology. There is, nevertheless, considerable evidence based on animal experimental work and on both histological and haematological studies in patients to suggest that low-grade disseminated intravascular coagulation may be associated with pre-eclampsia (Chapter V. 2). Such an association might be of importance not only in the pathogenesis and clinical management of pre-eclampsia, but might also contribute to a greater understanding of disseminated intravascular coagulation itself.

In Chapter I the biochemistry of fibrinogen, fibrinogen-fibrin degradation products and soluble fibrinogen-fibrin complexes is discussed in detail. The methods currently available for the identification of soluble complexes (and soluble fibrin) in plasma samples and the results obtained in various clinical conditions are then described (Chapter I. 6). In Chapter II the development of the technique of plasma fibrinogen chromatography is traced and details are given of the methods used for the studies to be described in the thesis.

It was confirmed in a series of in vitro experiments (Chapter III) that soluble complexes could be produced by the action of

coagulant enzymes (thrombin and ancrod) and that these complexes could be partially separated from fibrinogen by the plasma fibrinogen chromatography technique. Patients were then studied during the initial stages of intravenous infusion with the coagulant enzyme, ancrod (Chapter IV). Increased plasma soluble complex levels were found 6 hours after starting treatment, thus confirming that soluble complexes can be produced in vivo by a coagulant enzyme and identified using the plasma fibrinogen chromatography technique.

Following these preliminary studies plasma samples from pregnant patients were examined (Chapters V-VII). In order to assess the results in pre-eclampsia several other obstetrical groups were studied. Small, but statistically significant increases in plasma soluble complex concentration were found to occur in normal pregnancy (Chapter V and VII). In patients with intra-uterine growth retardation a further small but significant increase in soluble complex levels was noted (Chapter VII), while a more marked increase was found in association with pre-eclampsia (Chapter V and VI). This did not appear to be simply related to the combination of hypertension and pregnancy, as pregnant patients with essential hypertension were found to have soluble complex levels similar to those found in normal pregnancy (Chapter VII). In addition, plasma soluble complex levels were found to be slightly increased in women taking oestrogen-containing oral contraceptive drugs (Chapter VIII), a state which in some ways resembles early pregnancy.

Although soluble fibrinogen-fibrin complexes probably reflect activation of the coagulation pathway in vivo and therefore in this sense intravascular coagulation, it is not possible to distinguish between the different forms of intravascular coagulation (see the definitions in the Preface) by this technique or, indeed, to make any firm conclusions regarding insoluble fibrin deposition from the

results. Insoluble fibrin deposition occurs in local thrombosis and in local and disseminated intravascular coagulation. In each of these states increased plasma soluble fibrinogen-fibrin complex levels might be found, but it is also possible that a pure hypercoagulable state can exist in which soluble complexes are formed without insoluble fibrin deposition.

The results described in this thesis are, however, compatible with hypercoagulability in women taking oestrogen-containing drugs and in normal pregnancy, with in addition a degree of local intravascular coagulation within the maternal placental vasculature in normal pregnancy, a tendency which is increased in intrauterine growth retardation, and with disseminated intravascular coagulation in pre-eclampsia.

The soluble complex levels were most markedly increased in pre-eclampsia and during ancrod therapy. Fibrinogenolysis/fibrinolysis, however, was much more marked in the ancrod-treated patients. It may be that fibrin microclots are lysed very rapidly, perhaps even as they form, in ancrod therapy; while in pre-eclampsia they persist within the placenta, kidney, liver and other organs leading to the development of some of the clinical features of the syndrome. The theory that disseminated intravascular coagulation is an integral part of the pathology of the pre-eclamptic syndrome is therefore strongly supported by the work described in this thesis.

ABBREVIATIONS

The abbreviations used in this thesis are in the main widely accepted and are explained in the text. The less commonly accepted abbreviations are listed below:-

BP	blood pressure
SBP	systolic blood pressure
DSP	diastolic blood pressure
C-terminal	carboxy-terminal
DIC	disseminated intravascular coagulation
DVT	deep venous thrombosis
EGT	ethanol gelation test
F.V	coagulation Factor V
F.X etc.	coagulation Factor X etc.
F	fibrinogen
f	fibrin monomer produced by thrombin (deprived of fibrinopeptides A and B)
f_B	fibrin monomer produced by ancrod or Reptilase (deprived of fibrinopeptide A)
FDP/fdp	fibrinogen degradation products/fibrin degradation products
FR-antigen	fibrinogen-fibrin related antigen
FSF	fibrin stabilizing factor
GSR	generalised Shwartzman reaction
IUGR	intrauterine growth retardation
IV	intravenous
IVP	intravenous pyelogram
MAHA	microangiopathic haemolytic anaemia
N-terminal	amino-terminal
OD	optical density

PET	pre-eclampsia
SCT	staphylococcal clumping test
SD	standard deviation
SEM	standard error of estimate of mean value
SK	streptokinase
TCP	thrombin clottable protein
TCT	thrombin clotting time
TRCHII	tanned red cell haemagglutination immuno-assay
V_o	void volume
V_e	elution volume
W/V	weight per volume
X, Y, D, E	successive degradation products formed by plasmin digestion of fibrinogen (FDP)
x, y, d, e	successive degradation products formed by by plasmin digestion of fibrin (fdp)

Abbreviations in Tables

ND	not done
NS	not significant

Errata

Biogel should be spelt Bio-Gel

Biorad should be spelt Bio-Rad

PREFACE

This thesis is concerned with the identification of soluble fibrinogen-fibrin complexes in plasma using the relatively new technique of plasma fibrinogen chromatography. This technique, originally developed by Fletcher, Alkjaersig and their colleagues, is claimed to provide a sensitive method for detecting intravascular coagulation (Fletcher, Alkjaersig, O'Brien et al., 1970). The technique was set up for the project described in this thesis, with the prime aim of studying plasma samples from obstetrical patients with pre-eclampsia, a condition thought to be associated with low grade disseminated intravascular coagulation.

Definitions and nomenclature in the field of intravascular coagulation remain somewhat confused. For the purposes of this thesis the term "intravascular coagulation" will be defined as the conversion of fibrinogen to fibrin (i.e. the final stage of the coagulation pathway) within the blood vessels. As a thrombus (or microthrombus) has a definite histological structure composed of platelets and leucocytes, as well as insoluble fibrin, it is appreciated that many inter-related factors, besides coagulation, are involved in its development.

If intravascular coagulation is defined as activation of the final stage of the coagulation pathway, it is possible to classify intravascular coagulation into several types. Local thrombosis will obviously involve the formation of insoluble fibrin within the vascular compartment, but there are other forms of intravascular coagulation in which microthrombi form in small blood vessels and it may also be possible for a degree of conversion of fibrinogen into soluble fibrin to occur without insoluble fibrin deposition. The following classification of intravascular coagulation is therefore

used in this thesis:-

Types of Intravascular Coagulation (adapted from Fletcher and Alkjaersig, 1973)

(1) Hypercoagulability
(2) Local thrombosis
(3) Local intravascular coagulation
(4) Disseminated intravascular coagulation

(1) Hypercoagulability (synonyms: hypercoagulable state, thrombotic tendency and pre-thrombotic state).

This term describes a clinical state in which the patient is at increased risk of developing one of the other forms of intravascular coagulation in which insoluble fibrin deposition occurs (i.e. 2, 3 or 4), although actual thrombi or microthrombi are not present at this stage. Hypercoagulability may be associated with an increased level of coagulation factors in the blood (Nilsson, 1974a) or with the presence of activated coagulation factors in the blood (e.g. soluble fibrinogen-fibrin complexes; Graeff, von Hugo and Hafter, 1975), but precise definition is lacking. Indeed the existence of a pure hypercoagulable state is by no means generally accepted. It may be that a degree of insoluble fibrin deposition is always present in such patients (e.g. hypercoagulability and local thrombosis occurring together in the same patient).

(2) Local thrombosis

This term describes the development of a macroscopic, insoluble fibrin-platelet thrombus in a segment of an artery or vein. This often occurs secondary to local vascular disease.

(3) Local intravascular coagulation

This term describes the development of microthrombi or insoluble fibrin deposits in the small blood vessels of a single organ.

(4) Disseminated intravascular coagulation (synonyms: diffuse intravascular coagulation, often referred to as "D.I.C.").

This term may be used to describe the development of microthrombi or insoluble fibrin deposits in the small blood vessels of several organs and in portions of the vascular tree uninvolved by primary disease. This is therefore a histological diagnosis, which can usually be made only at post-mortem or occasionally on the basis of biopsy material obtained from the living patient. It is suggested that the term "histological disseminated intravascular coagulation" could be used to describe this condition.

It is clearly important to make a presumptive diagnosis of disseminated intravascular coagulation in the living patient. This is usually made on the basis of a variety of laboratory tests including a low blood platelet count and a high serum fibrinogen/fibrin degradation product (FDP/fdp) level. It is suggested that the term "haematological disseminated intravascular coagulation" could be used to describe the condition diagnosed in this way. It has to be stressed that there is no evidence of insoluble fibrin deposition in this latter group of patients. Other terms such as "consumption coagulopathy or syndrome" and "defibrination state or syndrome" have been suggested to describe such a condition although, of course, the term "defibrination" can only be used when there is definite evidence of a fall in plasma fibrinogen levels (see Nilsson, 1974b and Merskey, 1976).

The problems of nomenclature and diagnosis will be discussed throughout this thesis. Many tests of coagulation, fibrinolytic and platelet activity have been used in an attempt to diagnose the different types of intravascular coagulation. Although this may not be very difficult in the most florid cases of disseminated intravascular coagulation, the less severe forms of intravascular

coagulation may be difficult to detect. In particular, it is difficult to identify patients, who are particularly at risk of developing serious pathology (e. g. widespread disseminated intravascular coagulation) at an early stage, when some form of preventative therapy might be attempted.

One major problem in this area is lack of knowledge of the pathogenesis of the different types of intravascular coagulation leading to the development of thrombi or multiple microthrombi. It is appreciated that there is a complicated interaction of many factors including changes in blood flow and viscosity, damaged endothelium, alterations in blood platelets and leucocytes, as well as activation of the coagulation pathway with conversion of soluble fibrinogen first into soluble and then insoluble fibrin. At the same time the system of inhibitors, developed as a defense mechanism against inappropriate intravascular coagulation, has to be overcome. The relative importance of these various factors in different clinical situations is poorly understood. It is clear that intravascular coagulation with the formation of fibrin is only one aspect of this problem, yet it is the only one which will be considered in detail in this thesis. It is worth stressing at the outset that other aspects are involved, which may be of equal or greater importance in different clinical situations.

The condition studied in detail in this thesis is the obstetrical complication of pre-eclampsia, in which it is suspected that low grade disseminated intravascular coagulation occurs (Chapters V and VI). This work forms the central core of the thesis and led to the study of a variety of other obstetrical (Chapter VII) and non-obstetrical conditions (Chapters IV and VIII). The "model" of acute defibrination induced by intravenous ancrod infusion (Chapter IV) proved particularly interesting.

This thesis is concerned with the identification of soluble fibrinogen-fibrin complexes in clinical conditions. It is not concerned with the detailed biochemistry of these complexes but only with their possible use as markers of intravascular coagulation. Some in vitro experiments were, however, performed to help in setting up the technique and in the interpretation of the results (Chapter II).

In a project carried out over the same three year period in the same laboratory Mr. William Edgar has studied the biochemistry of soluble fibrinogen-fibrin complexes and soluble fibrin and inevitably there was some overlap of interest between the two projects. The results of a joint study into the structure of soluble complexes formed by the coagulant enzyme, ancrod, both in vitro and in vivo will be described in Chapters III. 6 and IV. 4(c). This joint study is important and relevant in that it resulted in an increased understanding of the possible methods of soluble complex formation (Chapter IV. 5).

Inevitably over the three years since the start of this project much has appeared in the literature which has proved relevant to it. This subject is developing rapidly and quite basic aspects of theory, methodology and interpretation are being questioned. The relevant literature on each clinical condition is discussed at the beginning of the chapter or section of the chapter dealing with that condition (e. g. the literature concerning the association between pre-eclampsia and intravascular coagulation is discussed in Chapter V. 2). The literature concerning the biochemistry of fibrinogen and soluble fibrinogen-fibrin complexes is discussed in detail in Chapter I, while the background literature concerned with agarose gel filtration and the plasma fibrinogen chromatography technique will be discussed

in Chapter II. 2 and 3.

The results obtained will be discussed in detail in the appropriate chapter. In the final chapter (Chapter IX) an attempt will be made to bring all these results together in order to make a critical assessment of the plasma fibrinogen chromatography technique and also to interpret the results in terms of the different types of intravascular coagulation.

CHAPTER I

HISTORICAL INTRODUCTION

The subject of soluble fibrinogen-fibrin complexes is both complicated and controversial. Many different types of soluble complex can be produced in vitro, but there is debate as to their presence and significance in vivo. Before discussing the literature available on the subject, it is necessary to summarise what is known about fibrinogen itself, the fibrinogen-to-fibrin conversion and the proteolysis of fibrinogen and fibrin to produce fibrinogen/fibrin degradation products.

These subjects will be discussed in detail in Chapter I. 1, I. 2 and I. 3. Fibrinogen synthesis, distribution and degradation in vivo will then be discussed in Chapter I. 4. The literature on the subject of soluble fibrinogen-fibrin complexes will be discussed in Chapter I. 5 and the techniques currently available for the identification of soluble complexes in clinical conditions together with the results of such studies will be described in Chapter I. 6.

The background literature on the clinical conditions studied in the thesis will be considered at the beginning of the appropriate chapter (e. g. the relevant literature on pre-eclampsia will be discussed in Chapter V. 1 and V. 2).

I. 1(a) Fibrinogen - Historical Aspects

Fibrinogen is a protein of considerable importance in normal blood coagulation. Malpighi (1686) demonstrated that the structural basis of blood clot was the white fibrous material, which is now called fibrin. Although the existence of a soluble fibrin precursor in plasma was suspected, it was not until the 19th century that the precursor, fibrinogen, was isolated (Denis-De-Commercy, 1859;

Hammarsten, 1879). Hammarsten observed that fibrinogen did not coagulate spontaneously but did so rapidly upon the addition of a "thrombic" enzyme. He therefore proposed that fibrin was a specific result of the interaction of thrombin and fibrinogen.

More recent work has shown that the conversion of fibrinogen to fibrin occurs in several stages (Laki and Mommaerts, 1945; Laki, 1951 and Laki, 1953). Thrombin first reacts with fibrinogen and prepares it for the polymerisation process which can then occur "spontaneously" in a suitable ionic environment. Direct evidence of the limited proteolytic activity of thrombin on fibrinogen was provided when a differences between the N-terminal amino acids of bovine fibrinogen and fibrin was demonstrated (Bailey, Bettelheim, Lorand et al., 1951).

The extensive work, which has been done in studying this reaction, will be discussed in detail in Chapter I. 2.

I. 1(b) Physicochemical Properties of Fibrinogen

There is now considerable evidence that fibrinogen prepared from blood consists of populations of slightly different molecules (Mosesson, 1970; Mosesson, 1974). These can be separated on the basis of their solubility, ranging from a type which precipitates upon standing in the cold to one which is soluble even in the presence of saturated glycine solutions. The properties of these different forms of fibrinogen have been summarised by Mosesson (1970). Following the nomencalture introduced by Cohn, Strong, Hughes and colleagues (1946), the higher the number assigned to a given fibrinogen fraction, the higher the overall solubility of that fraction. Therefore Fraction I-1 consists of the low solubility species which is said to precipitate with cold insoluble globulins. Fraction I-4 constitutes the bulk of fibrinogen in plasma and represents the species of fibrinogen purified by "classical" techniques, so called

"native" fibrinogen. Fraction I-8 is highly soluble and although)95% clottable has a molecular weight lower than that of the Fraction I-4 species. It may represent a product of limited proteolysis.

Due to the variety of species of fibrinogen, estimations of molecular weight vary. Mosesson (1970) suggested a molecular weight in human preparations of 269,000 for Fraction I-8 and 325,000 for Fraction I-4. The generally accepted value for "native" fibrinogen is 340,000 (Murano, 1974).

I.1(c) Structure

Fibrinogen is a symmetrical molecule with a two-fold axis of symmetry. There are three chains in each half-molecule and, according to international nomenclature, they are known as the A α , B β and γ chains (report of the Nomenclature Subcommittee of the International Committee for Thrombosis and Haemostasis, Oslo, Norway, 1971). The molecule appears to be a dimer held together by symmetrical disulphide bonds between the A α , B β and γ chains. The structure of the N-terminal region, the N-terminal disulphide knot (N-DSK), has been described in considerable detail (Blombäck, 1969; Blombäck and Blombäck, 1972). The fibrinopeptides A and B which are released by the action of thrombin are located in this region of the molecule (see Figure 1). Detailed reviews of this subject have been published by Murano (1974) and Blombäck, Hogg, Gårdlund and colleagues (1976).

The shape of the molecule is uncertain. Hall and Slayter (1959) described a triglobular structure. More recent work has challenged this and a number of different models have been suggested. One of the most recent suggests a spherical particle

22 nm in diameter folded up around the N-terminal disulphide knot (Hudry-Clergeon, Marguerie, Pouit et al., 1975). These authors suggest that marked conformational changes occur in the molecule under the action of thrombin and due to temperature, pH, ionic strength, solvent nature and protein concentration.

I. 2. Fibrinogen-Fibrin Transformation

The conversion of soluble fibrinogen into insoluble fibrin is the final reaction in the coagulation cascade (Macfarlane, 1964; Macfarlane, 1976; see Figure 2). This complicated "cascade" system of inert precursors and active enzymes has presumably evolved to prevent intravascular coagulation occurring inappropriately. As a further safeguard a correspondingly complicated system of inhibitors has also developed.

The complexity of the cascade system is increased by the realisation that even this final reaction takes place in stages. Two enzymes are important in this final reaction - thrombin (F IIa) and fibrin-stabilising factor (F XIIIa). Thrombin acts by converting fibrinogen to fibrin monomer which is then capable of spontaneous polymerisation. This reaction will be considered in detail in Chapter I. 2(a). F XIIIa acts by introducing strong covalent bonds between the units of the fibrin polymer. Its action will be considered in detail in Chapter I. 2(b).

I. 2(a) Fibrinogen-Fibrin Transformation - Thrombin

The fibrinogen-fibrin transformation occurs in a series of stages (see detailed discussion Scheraga, 1961). A simple model of this reaction is shown in Figure 3 (adapted from Laskowski, Rakowitz and Scheraga, 1952). The enzymic step induced by thrombin is limited to stage i. Thrombin first removes

the fibrinopeptide A fragments from the A α chain of fibrinogen by cleaving an arginyl-glycine bond. This reaction proceeds rapidly. The fibrinopeptide B fragments are then removed after a lag phase during which polymerisation has already occurred (Blombäck, 1958). As the fibrinopeptides carry a strong electronegative charge their removal alters the electric charge of the molecule and is also thought to produce a conformational change opening up various polymerisation sites (see Murano, 1974 and its bibliography).

The process of polymerisation is poorly understood (i. e. stages ii and iii). It would appear to involve the formation of weak, reversible bonds between the polymerising units. These are probably hydrogen bonds between tyrosyl donors and histidyl acceptors (Sturtevant, Laskowski, Donnelly et al., 1955; Endus, Ehrenpreis and Scheraga, 1966) although hydrophobic bonding or charge neutralisation may also be important (Collen, 1971).

A schematic model for the polymerisation of fibrin monomer units has been described (Blombäck et al., 1976; see Figure 4). The binding sites A and A' are located in the N-terminal disulphide knot region of each molecule. The binding sites a and a' are located in the carboxyterminal end of each molecule. The A-A' sites require activation by thrombin, while the a-a' sites are already active in the molecule as it circulates in the blood. The A or A' site interacts with the a or a' site of a neighbouring molecule. In this model a succeeding molecule covers the preceding one in a half-staggered fashion. This produces "end-to-end" alignment. "Side-to-side" alignment occurs at a second set of binding sites (B-B' and b-b') whose existence is not yet confirmed.

The conversion of soluble fibrin polymer to insoluble fibrin (stage iii) is also incompletely understood. It may be that once a critical size is reached the polymer becomes insoluble. It must be stressed that stages ii and iii are reversible under certain conditions (e. g. by altering pH). The irreversible step is produced by the formation of covalent bonds induced by the action of F XIIIa.

I. 2(b) Fibrinogen-Fibrin Transformation - Fibrin-Stabilising Factor (F XIII/F XIIIa)

In 1923 Barkan and Gaspar demonstrated that the solubility of fibrin could be altered by the presence or absence of oxalate in the initial fibrinogen preparation. Later it was shown that calcium ions increased the firmness of fibrin clot (Mellanby and Pratt, 1940). It was then demonstrated that a "serum factor" was also required for the formation of insoluble fibrin (Robbins, 1944). This "serum factor" was found to be present in higher concentrations in plasma and is now known as fibrin-stabilising factor or F XIII/F XIIIa. The reaction involving calcium ions and F XIIIa is known as cross-linking and was eventually shown to involve the formation of covalent bonds (see Finlayson, 1974 and its bibliography).

In 1966 Fuller and Doolittle showed that the ϵ -amino group of lysine took part in crosslinking and they proposed that the cross-link was ϵ (γ glutamyl) lysine. Factor XIII can be activated by a number of enzymes including thrombin, trypsin, papain and F Xa (Finlayson, 1974; McDonagh and McDonagh, 1975). Factor XIII does not appear to be activated by plasmin or chymotrypsin and is probably not activated by the most highly purified preparations of the snake venom derivatives ancrod and Reptilase, although cruder preparations may do so (Finlayson, 1974; McDonagh and McDonagh, 1975).

Characteristics of the fibrin crosslink

The α and γ chains take part in crosslinking, the γ chains crosslinking more rapidly than the α chains (McKee, Mattock and Hill, 1970). The β chains do not crosslink (Chen and Doolittle, 1969). It is therefore possible to distinguish between crosslinked and non-crosslinked fibrin by examining the chains present after cleavage of disulphide bonds before electrophoresis. (Pizzo, Schwartz, Hill et al., 1972; Gaffney and Brasher, 1973). In crosslinked fibrin γ - γ dimers and α polymers and oligomers are found.

The physiological significance of fibrin crosslinking is not understood. Duckert (1972) suggested that the consequences of fibrin crosslinking are increased mechanical strength and elasticity, decreased susceptibility to proteolysis and support for tissue repair.

It has frequently been claimed that crosslinking increases the mechanical strength of fibrin (e. g. Duckert, Jung and Schmerling, 1960), although there is no difference in the electron microscopic appearance of crosslinked and non-crosslinked fibrin (Kay and Cuddigan, 1967). The relative resistance to lysis of the two forms of fibrin has been studied using different proteolytic enzymes, activators and experimental approaches (Finlayson, 1974). Crosslinked clots appear to be more resistant to lysis induced by urokinase (McDonagh, McDonagh and Duckert, 1971) although there appears to be no difference in the susceptibility of the two kinds of fibrin to lysis induced by trypsin or plasmin (Tyler, 1972). The evidence regarding streptokinase is conflicting. Tyler (1972) found no difference between crosslinked and non-crosslinked fibrin; while Henderson and Nussbaum (1969) reported that non-crosslinked

fibrin was more rapidly lysed. It now seems possible that crosslinking of the γ chains does not increase lysis time but that crosslinkage of the α chains does (Schwartz, Pizzo, Hill et al., 1973).

It appears that crosslinkage may occur in step with polymerisation rather than occurring at the very end of the fibrinogen-to-fibrin conversion. Finlayson and Aronson (1974) induced thrombosis in the jugular vein of the rabbit and showed that even in the earliest clots γ-γ-dimerisation was essentially complete and a considerable degree of α polymer had formed. It is not known if there is a species variation in this respect.

It is recognised that F XIIIa can crosslink soluble fibrinogen-fibrin complexes (Sasaki, Page and Shainoff, 1966) and even fibrinogen itself (Kanaide and Shainoff, 1975). This group claim that soluble dissociable (uncrosslinked) fibrinogen-fibrin complexes are rapidly cleared from the circulation and that crosslinked complexes remain in the circulation for several days (Shainoff and Sasaki, 1971).

The ability of F XIIIa to crosslink fibrinogen is clearly an important observation. In vitro the initial rates of crosslinking of fibrinogen varied directly with the fibrinogen concentration. At very high concentrations (30 mg/ml) fibrinogen crosslinked more rapidly than fibrin (Kanaide and Shainoff, 1975). Experiments using thrombin-free F XIIIa have suggested that the precipitation of fibrinogen is required to produce the conformational change, which allows crosslinking to occur (Seelich, Furlan and Beck, 1976). Presumably thrombin induces a similar conformational arrangement of the sub-unit chains allowing crosslinking to occur.

The significance of the studies on crosslinking of fibrinogen have to be assessed in vivo but could provide a means of soluble

complex (i.e. a molecule larger than fibrinogen but antigenically related to it) production without the intervention of thrombin. It has been suggested that a stable dimer found regularly in patients with renal allografts and also in patients with necrotising vasculitis is formed in this way (Kanaide, Braun and Shainoff, 1973). A similar dimer has also been found in two cases of Hodgkin's disease (Shainoff and Sasaki, 1971).

I. 3. Fibrinogen and Fibrin Degradation Products

These are molecules smaller in size than fibrinogen produced by extensive proteolysis of fibrinogen, non-crosslinked fibrin or crosslinked fibrin. Although a number of proteolytic enzymes can produce such proteolysis, it is usually assumed that plasmin is the enzyme responsible in vivo. A great deal is known about fibrinogenolysis and fibrinolysis in vitro and much less about these reactions in vivo. The in vitro work will be discussed first.

I. 3(a) Fibrinogenolysis and Fibrinolysis In Vitro

In 1934 Garner and Tillett demonstrated that fibrinogen could be fragmented by a streptococcal extract into smaller molecules, which were no longer clottable by thrombin. In 1945 Seegers, Nieft and Vandenbelt clearly identified two components in a fibrinogen lysate after electrophoresis. In 1961 Nussenzweig and colleagues using DEAE-cellulose chromatography identified five products (Fragments A, B, C, D and E) in the "terminal fibrinogen lysate", Fragment D made up 50-60% of the total protein in the lysate and Fragment E 15-20% (Nussenzweig, Seligmann and Grabar, 1961; Nussenzweig, Seligmann, Pelmont and Grabar, 1961). During the 1960s and 1970s much work was done on this subject (e.g. Marder, Shulman, Carroll, 1969; Mills, 1972; Marder, Budzyński,

James, 1972; Pizzo, Schwartz, Hill et al., 1972; Budzyński, Marder and Shainoff, 1974). Despite differences in nomenclature some basic principles are established. The reaction sequence which will be described is based on the work and nomenclature of Marder's group.

Plasmin initially attacks the carboxy-terminal end of the A α chain (see Figure 5) and early Fragment X is produced. Plasmin next attacks the amino-terminal end of the B β chain, removing in the process the fibrinopeptide B fragment, forming Fragment X. The molecule is then split asymmetrically to produce Fragment Y and Fragment D. Finally, Fragment Y is itself split asymmetrically into Fragment E and a further Fragment D. Clearly, this is an over-simplification and in fact several species of Fragment X, Y, D and E exist with varying lengths of A α, B β and γ chains. This model of plasmin digestion of fibrinogen suggests that there are two Fragments D and one Fragment E contained in each molecule of fibrinogen. This has, however, been challenged (see review Marguerie, Hudry-Clergeon and Suscillon, 1975).

Non-crosslinked fibrin would seem to be degraded in a fairly similar fashion to fibrinogen (Gaffney, 1973). However, fibrin in which the γ chains are crosslinked (as γ-γ dimers) will be degraded directly to Fragment D-dimer and Fragment E (Gaffney, 1973).

It would be helpful to be able to distinguish between the degradation products of fibrinogen, non-crosslinked and crosslinked fibrin. Despite the fact that fibrinogen and non-crosslinked fibrin are degraded in a fairly similar fashion, there appear to be immunological differences between their degradation products associated with both the D and E regions of the parent molecule.

These "cleavage associated neoantigens" are thought to represent cryptic sites on the parent molecule which are revealed by plasmin digestion (Plow and Edgington, 1973; Edgington, 1975). It is claimed that these neoantigens can be used to quantitate the amount of degradation product derived from fibrinogen or from non-cross-linked fibrin (Edgington, 1975).

It is possible to distinguish between derivatives of cross-linked and non-crosslinked fibrin as only crosslinked fibrin will be degraded to Fragment D-dimer (Gaffney, 1973).

I. 3(b) The Plasminogen-Plasmin System

The basic fibrinogenolytic/fibrinolytic pathway is shown in Figure 6. There is an obvious similarity with the final stages of the coagulation pathway and indeed it seems probable that the two systems have evolved in parallel to each other, each with an appropriate system of inhibitors (see review Chesterman, 1975). Both systems can be activated by F XIIa (Kaplan and Austen, 1972) and by endothelial damage and tissue factor (Todd, 1958). Indeed the coagulation, fibrinolytic, kallikrein-kinin and complement systems are all closely related (see reviews Ratnoff, 1969 and Stormorken, 1975). These inter-relationships are probably of great importance in disease but are incompletely understood, one of the problems undoubtedly being that "the defenses of the body may be dissected by the investigator to suit his experimental convenience but that in nature they form a seamless web" (Ratnoff, 1969).

It is usually assumed that the plasminogen-plasmin system plays an important role in the removal of pathological fibrin. The reticulo-endothelial system may also be of considerable importance in this respect. There may be a complex inter-relationship between the two systems (see Chapter I. 5(c)). Although plasmin is likely to be important other fibrinogenolytic/

fibrinolytic enzymes may be involved e.g. red cell proteolytic enzymes, cathepsins or peptidases (Johnson and Merskey, 1971).

It is important to realise that there may be differences in the plasminogen-plasmin reaction depending on the means of plasminogen activation. In a recent series of publications Gurewich, Lipinski and colleagues (see below) have studied "naturally occurring plasminogen activator" and compared their results with those obtained with activator prepared for therapeutic use (i.e. streptokinase and urokinase), which had been used in the earlier work on this reaction. Natural plasminogen activator was obtained in blood samples collected after venous occlusion (Lipinski, Nowak and Gurewich, 1974), immediately after death (Gurewich, Nowak, Lipinska et al., 1974) and after strenuous physical exercise (Gurewich, Lipinska and Lipinski, 1974).

It was shown that soluble fibrinogen, soluble fibrin monomer complexes and soluble fibrin degradation products are resistant to naturally induced fibrinolytic activity. In contrast there was rapid lysis of insoluble fibrin, protamine sulphate precipitated fibrinogen and protamine sulphate and ethanol induced gels of fibrin monomer. Further work on cadaver-derived vascular plasminogen activator suggested that this is because activator-inhibitor complexes are formed which can only dissociate on the surface of insoluble fibrin or precipitated fibrinogen (Gurewich, Hyde, Lipinski, 1975). Equivalent effective concentrations of streptokinase or urokinase-activated plasmin caused extensive fibrinogenolysis. If this work is confirmed it would appear that soluble fibrinogen and its soluble derivatives have to pass through an insoluble phase before they can be lysed by plasmin induced by natural plasminogen activator.

I. 3(c) Fibrinogenolysis/Fibrinolysis In Vivo

While it is generally accepted that fibrinolysis occurs in vivo, fibrinogenolysis in vivo is at present a very controversial issue. Fibrinolysis will be considered first. It is not yet established how plasminogen activator, plasminogen and fibrin come into contact in vivo. Any model that is proposed must include the separation of the active enzymes from inhibitors.

Alkjaersig, Fletcher and Sherry (1959) suggested that as fibrin polymerises, plasminogen is adsorbed preferentially onto the fibrin and is available in large quantities within a thrombus or fibrin deposit, comparatively free of antiplasmins. When activator enters the circulation it diffuses into the clot converting plasminogen to plasmin in situ. Ambrus and Markus (1960) suggested that plasmin is bound in a plasmin-antiplasmin complex, which is reversible, and which dissociates in the presence of the preferred substrate, fibrin. Chesterman, Allington and Sharp (1972) have produced evidence that it is the activator which is adsorbed onto the fibrin and that circulating plasminogen is converted into plasmin within the thrombus.

Fibrinogenolysis in vivo is a rather controversial issue. Mosesson (1974) suggested that in the normal physiological state "fibrinogenolysis is a major catabolic pathway". He based this suggestion on the heterogenity of fibrinogen related protein found in normal blood. He claimed that human fibrinogen prepared from fresh plasma contains more than 20% high solubility material with degraded A α chains. Two major fibrinogen components (higher and lower molecular weight fibrinogen; HMW and LMW fibrinogen respectively) have also been found in plasma from healthy subjects by Lipinska, Lipinski and Gurewich (1974). HMW fibrinogen made

up about 70% of the total clottable protein.

Mosesson (1974) interpreted his findings as showing plasmin digestion of fibrinogen in vivo and suggested that α_2 macroglobulin forms a complex with plasmin, which is capable of fibrinogenolysis. In contrast Lipinska and colleagues (1974) were unable to demonstrate any digestion of fibrinogen by naturally activated fibrin(ogen)olysis. They felt some other mechanism was involved. The extensive work on naturally occurring plasminogen activator by this group already described (Chapter I. 3(b)) would tend to support this view. If soluble fibrinogen is resistant to naturally activated plasmin it is difficult to see how fibrinogenolysis can occur in vivo without the introduction of a therapeutic plasminogen activator. The only way fibrinogen could be catabolised by plasmin would be by conversion into insoluble fibrin.

This could occur in a low-grade fashion as a physiological defense mechanism. In 1956 Astrup suggested that there was a dynamic equilibrium between coagulation, with fibrin constantly being laid down to seal defects in endothelium and fibrinolysis removing such deposits after they had served their haemostatic function. In favour of this are the findings of small amounts of measurable fibrinogen-fibrin related antigen (FR-antigen) in sera from normal subjects (Merskey, Lalezari and Johnson, 1969) and low concentrations of fibrinopeptide A in normal plasma (Nossel, 1976). It is possible that these changes are occurring in vitro despite attempts taken to prevent this. A study of ^{125}I-labelled fibrinogen has suggested the formation of both cryoprofibrin and early degradation product in the healthy rabbit (Sherman, 1972). This would also be in keeping with Astrup's theory of continual low-grade coagulation and fibrinolysis in the healthy animal (Astrup, 1956).

The relative importance of fibrinolysis and fibrinogenolysis in vivo clearly requires further elucidation. This subject is, however, of considerable importance in the formation of the different types of soluble complexes (Chapter IV.4).

I.4 Fibrinogen Synthesis, Distribution and Degradation In Vivo

This complicated subject was reviewed by Reeve and Franks (1974). A summary of this review is shown as a compartmental model in Figure 7. The "fibrinogen core system" represents the normal metabolic pathway, as seen by these authors. The remaining pathways are thought to operate mainly during pathological states. Much of this theory remains highly speculative. Individual pathways are discussed in detail elsewhere in this thesis. It is useful, however, to look briefly at an overall view of fibrinogen metabolism.

I.4(a) Normal Metabolic Pathways

Fibrinogen is mainly synthesized in the liver (Miller and Bale, 1954) and like albumin appears to be secreted directly into the blood stream and only to a small extent into the hepatic lymph (Rosenoer and Rothschild, 1970; Reeve and Franks, 1974). The factors regulating fibrinogen synthesis and secretion are not yet completely understood, although it is appreciated that there may be rapid fluctuations in the synthetic rate (Atencio, Joiner and Reeve, 1969). Fibrinogen is one of the plasma proteins that shows an "acute phase response". Following surgical trauma, the injection of endotoxin or acute infections, plasma fibrinogen shows a rise in concentration to at least twice the initial level, over a period of 24 to 72 hours depending on the species of animal studied (see review Reeve and Franks, 1974).

When fibrinogen enters the blood stream, it is immediately exposed to the potential dangers of proteolysis by enzymes such

as thrombin, plasmin and possibly trypsin and elastase (Reeve and Franks, 1974). To prevent either generalised intravascular coagulation or generalised fibrinogenolysis a complicated system of enzyme precursors and inhibitors has developed (Chapter I. 2 and I. 3). The possibility that thrombin and plasmin may act on fibrinogen in the healthy animal has already been discussed (Chapter I. 3(c)). This question remains unresolved at present, although Reeve and Franks (1974) conclude that it is probable that such pathways play "a minimal role in normal fibrinogen metabolism".

Apart from attack by circulating enzymes fibrinogen is exposed to two other fates. It may pass through permeable vessels to enter the interstitial fluids or it may pass to a catabolic breakdown site. Interstitial fibrinogen is mainly distributed in the small volume rapid flow interstitial fluids of the viscera, which possess capillary beds of high permeability and which contribute to 70% of thoracic duct lymph flow (Reeve and Franks, 1974). In general, knowledge of interstitial fluid fibrinogen and its derivatives is totally lacking.

The second fate of circulating fibrinogen is catabolism. When carefully prepared labelled fibrinogen is injected into the circulation of man or experimental animals there is rapid removal of the 1 or 2% which has been altered in a good preparation (Reeve and Franks, 1974). There is evidence that the altered protein is rapidly taken up by the reticuloendothelial cells (e. g. the Kupffer cells) and rapidly catabolised by them (Barnhart and Noonan, 1973). After this phase catabolism proceeds at a regular rate without any great delay between the time the labelled fibrinogen destined for catabolism leaves the circulation and the time the catabolic products - labelled low molecular weight degradation products and free label (usually iodide) - enter the circulation (Atencio,

Bailey and Reeve, 1965 and Atencio and Reeve, 1965). Although the location of these catabolic sites is unknown, they might be in the capillary endothelium, which is rich in plasminogen activator, or in the synovium (Reeve and Franks, 1974). If significant pinocytosis of drops of plasma occurred, the plasminogen which tends to be associated with fibrinogen might be activated, leading to fibrinogen digestion by plasmin within the pinocytotic vesicles (Reeve and Franks, 1974). The subject of fibrinogenolysis/fibrinolysis in vivo is discussed in Chapter I. 3(c).

I. 4(b) Pathophysiology of the Fibrinogen System

The pathways involved include all those outwith the "fibrinogen core system" in Figure 7.

Thrombin Pathway (see also Chapter I. 2(a)).

Disseminated intravascular coagulation with microthrombi in the kidney, liver, lung and spleen can be produced by thrombin infusion in experimental animals (Margaretten, Csavossy and McKay, 1967). The subject of experimental disseminated intravascular coagulation will be discussed in detail in Chapter I. 5(c) with particular reference to soluble fibrinogen-fibrin complexes. Disseminated intravascular coagulation with reference to the obstetrical condition of pre-eclampsia will be discussed in detail in Chapter V.

Plasmin Pathway (see also Chapter I. 3(b) and 3(c))

As has already been discussed plasminogen is converted into plasmin in vitro by many different activators (e. g. streptokinase, urokinase, and various tissue activators including that produced by vascular endothelial cells). Studies using labelled plasminogen in dogs have shown the appearance of labelled plasmin in vivo after

giving streptokinase, urokinase or typhoid vaccine and the prolonged release of plasmin after venous injury (Takeda, 1972). The catabolic rate of plasmin appears to be about 1.2 plasma pools/day (Takeda, 1972), but the mechanisms controlling the amount of circulating plasmin are unknown (Reeve and Franks, 1974). Indeed present knowledge in this field is very incomplete. The relative contribution of the reticuloendothelial system and the fibrinolytic pathways in the removal of fibrinogen and its derivatives in pathological states also remains to be established.

I. 5. Soluble Fibrinogen-Fibrin Complexes

Fibrinogen, the effects of thrombin, F XIIIa and plasmin on fibrinogen and finally fibrinogen metabolism have each been considered in detail because of their importance to the subject of soluble fibrinogen-fibrin complexes. The literature on this subject will be discussed under four headings:

I. 5. Soluble Fibrinogen-Fibrin Complexes

(a) Historical and theoretical aspects
(b) In vitro experiments
(c) Animal experiments
(d) Biological properties

I. 5(a) Historical and Theoretical Aspects

A soluble fibrinogen-fibrin complex can be defined as a dimer or polymer containing antigenic material which reacts with specific fibrinogen antiserum, or which in some other way (e.g. clots on addition of thrombin or reacts with staphylococcal clumping factor) can be shown to be closely related to fibrinogen. These complexes probably represent some form of fibrinogen-fibrin intermediate, the existence of which was apparently

suggested by Hammarsten in 1876. In 1955 Donnelly, Laskowski, Notley and colleagues also suggested the existence of such an intermediate - a soluble complex of fibrinogen and fibrin monomer. The work of Shainoff and Page (1960; 1962) on "cryoprofibrin" gave further support to this suggestion. "Cryoprofibrin" was found in high concentrations in the blood of endotoxin treated rabbits. Cryoprofibrin contained 30% less fibrinopeptide A than fibrinogen, but had the same concentration of fibrinopeptide B. It was suggested that liberation of fibrinopeptides by thrombin unmasks previously unavailable polymerisation sites containing tyrosyl residues, while the histidyl residues are normally unmasked in fibrinogen. The histidyl residues of fibrinogen and fibrin monomer therefore compete to form hydrogen bonds with the unmasked tyrosyl residues on the newly formed fibrin monomer. Fibrinogen molecules by combining with fibrin monomer block further polymerisation and so in the presence of high concentrations of fibrinogen, fibrin monomer could be prevented from forming fibrin (c.f. the polymerisation scheme described in Chapter I.2(a) and Figure 8).

The original idea that soluble complexes could only be composed of units of fibrin monomer and fibrinogen was shown to be an over-simplification. Lipinski, Wegrzynowicz, Budzyński and colleagues (1967) using a radioisotopic labelling technique claimed that both early and the late fibrinogen degradation products could be incorporated into soluble complexes. Theoretically therefore many different forms of soluble complex might exist, differing in their physiochemical properties. Wegrzynowicz, Kopeć and Latallo (1971) classified soluble complexes into three types, shown in Table 1. Only dimers are shown in this scheme

but clearly polymers could also be formed in many cases and mixed forms e.g. x.F; X-f_B-F; or y-f-D could also occur. Latallo (1975) has demonstrated the various types of soluble complex in diagramatic form (see Figure 8) basing his scheme on a system of hydrogen bonding. Collen (1971) challenged the idea that complexes are formed by hydrogen bonding. He demonstrated that the application of hydrostatic pressure had an immediately depolymerising effect on intermediately polymerised or clotted solutions. This effect was completely reversible. He concluded that polymerisation was accompanied by a volume increase suggesting some form of hydrophobic bonding or charge neutralisation. Whatever form of bonding is involved, the bonds appear to be rather weak and are dependent on pH, temperature, ionic strength and concentration (Latallo, 1975). These environmental factors can lead to problems in isolating and studying soluble complexes.

It is therefore theoretically possible that many forms of soluble complex exist. Attempts have been made to demonstrate their existence in a variety of in vitro experiments.

I. 5(b) In Vitro Experiments

Bang and Chang (1974) reviewed the literature on this subject and suggested six mechanisms for the formation of soluble complexes in vitro.

Type A: (1) The direct action of thrombin on fibrinogen.

(2) The interaction of fibrin monomer and fibrinogen, in the absence of thrombin.

Type B: (3) The interaction of fibrin monomer and fibrinogen Fragments X, Y, D and E.

(4) The proteolysis of fibrinogen by plasmin.

(5) The lysis of fibrin (fibrin Fragments x, y, d and e).

Type C: (6) Cross-linking mediated by F XIIIa of fibrin monomer and fibrinogen or, alternatively, through cross-linking of fibrinogen not exposed to thrombin.

These six mechanisms can be grouped into three main types (A, B and C): complexes produced by the action of thrombin alone (mechanisms 1 and 2); complexes incorporating plasmin derivatives of fibrinogen and fibrin (mechanisms 3, 4 and 5) and complexes formed through the action of F XIIIa (mechanism 6).

Type A: Soluble Complexes Produced by the Action of Thrombin Alone (Mechanisms 1 and 2)

(1) By the direct action of thrombin on fibrinogen

This reaction has already been discussed in detail in Chapter I. 2(a) - see Figure 5. The soluble fibrin polymer in stage (ii) of this reaction is the simplest form of soluble complex. Work from many laboratories using a variety of techniques has confirmed the existence of this form of soluble complex. Smith and Bang (1972) showed that prolonged incubation of fibrinogen with low concentrations of thrombin converted almost all the fibrinogen into soluble complexes before visible gelation took place.

It is not certain whether the component units of such complexes consist solely of fibrin monomer units or whether fibrinogen units may also be involved. Von Hugo, Hafter, Stemberger and colleagues (1975) studied soluble complexes produced by incubation of fibrinogen with thrombin by adsorption chromatography. The complexes dissociated in a ratio of 1:1 into a component adsorbed by cyanogen bromide activated fibrinogen sepharose (i. e. fibrin monomer) and a component that was not adsorbed (i. e. fibrinogen). Under the conditions of their experiments, fibrinogen would seem to be incorporated into this form of soluble complex. Such complexes may also contain a cold insoluble globulin, as well as units of fibrinogen related protein (Bang and Chang, 1974).

(2) Soluble complexes produced by the interaction of fibrin monomer and fibrinogen, in the absence of thrombin

It could be argued that these complexes are very similar, if not identical, to those formed by incubation of fibrinogen with thrombin. Bang and Chang (1974), however, deal with this mechanism separately. It may be that variations in the relative concentrations of fibrin monomer and fibrinogen are important while the continuing action of thrombin on fibrinogen might also be very important. Complexes formed by the interaction of fibrin monomer and fibrinogen without continuing thrombin action correspond closely to the "cryoprofibrin" of Shainoff and Page. Later studies from this group (Sasaki, Page and Shainoff, 1966) showed that the complex formed by mixing fibrinogen and fibrin monomer could be stabilised by the addition of F XIIIa.

Jakobson, Ly and Kierulf (1974) prepared complexes by incubating ^{125}I labelled fibrinogen and unlabelled fibrin monomer,

separating the complexes by gel exclusion chromatography. The fact that fibrin monomer will adsorb on insolubilised fibrinogen fixed to cyanogen bromide activated sepharose suggests that some form of interaction between fibrinogen and fibrin monomer occurs under these conditions (Heene and Matthias, 1973).

It has, however, been suggested that intact fibrinogen does not incorporate into complexes. Smith and Bang (1972) using N-terminal analysis were unable to show the presence of fibrinogen in soluble complexes formed in a mixture of fibrinogen and fibrin monomer. Using gel exclusion chromatography Smith and Craft (1974) failed to demonstrate complex formation between ^{125}I labelled fibrinogen and unlabelled fibrin monomer; and ^{125}I labelled fibrin monomer and unlabelled fibrinogen.

These apparently conflicting results are difficult to explain but may be due to differing experimental conditions. The role of F XIIIa in this context may be important.

Type B: Soluble Complexes Incorporating Plasmin Derivatives of Fibrinogen and Fibrin (Mechanisms 3, 4 and 5)

The literature on this subject provides several conflicting results concerning which derivatives can take part in complex formation. As this thesis is concerned with the identification of soluble complexes in various clinical conditions, rather than the actual component units this literature will not be discussed in great detail.

Since the initial stages of plasmin digestion result only in the splitting off of "marginal" parts of fibrinogen and do not involve gross structural changes, these derivatives can be considered as analogues of fibrinogen and, after exposure to

thrombin, fibrin monomer (Latallo, 1975). Thus minimally degraded Fibrinogen X behaves similarly to Fibrinogen (F) and minimally degraded fibrin x behaves similarly to fibrin monomer (f; f_B). Therefore the forms of complex already discussed involving F, f and f_B should also be able to be formed by X and x. In this connection, it is interesting that minimally degraded fibrin x can form a fibrin-like network indistinguishable by electron microscopy from thrombin-induced fibrin (Niewiarowski, Stewart and Marder, 1970).

The three mechanisms suggested by Bang and Chang (1974) for the formation of soluble complexes containing degradation products will now be discussed in turn.

(3) The interaction of fibrin monomer and fibrinogen Fragments X, Y, D and E

The original work of Lipinski and colleagues (1967) suggested that fibrinogen X, Y, D and E could all complex with fibrin monomer. Kudryk, Reuterby and Blombäck (1973) showed that approximately 50% fibrinogen D was capable of complexing with fibrin monomer immobilised on cyanogen bromide activated sepharose. Fibrinogen E did not complex under these conditions. Matthias, Heine and Konradi (1973) showed that fibrinogen X and Y had greater affinity for insolubilised fibrin monomer than fibrinogen D.

In contrast the work of Smith and Bang (1972) and subsequent work from this group (summarised Bang and Chang, 1974) failed to show incorporation of ^{125}I labelled fibrinogen D and E into soluble complexes, using gel exclusion chromatography. Different experimental conditions may again be responsible for these conflicting results.

(4) The proteolysis of fibrinogen by plasmin

It is generally accepted that fibrinogen X will only form complexes if some fibrin derivative is present (Bang and Chang, 1974). There is only one published report suggesting that plasmin digestion of fibrinogen can result in polymer formation, which was demonstrated by ultracentrifugation (Fletcher, Alkjaersig, Fisher et al., 1966).

It was suggested that plasmin might cleave argine-glycine bonds, including those susceptible to thrombin, in a random fashion releasing the fibrinopeptides, and allowing complex formation. This hypothesis remains speculative as it has not been confirmed by subsequent experiments (Bang and Chang, 1974).

(5) The lysis of fibrin (fibrin x, y, d and e)

Bang, Hansen, Smith and colleagues (1973) stated that fibrin degradation products can be shown to participate in three types of soluble complex in vitro:

(a) low molecular weight complexes formed when fibrin d and e are incubated with fibrinogen and thrombin.

(b) high molecular weight complexes apparently resulting from reaggregation of components of an early fibrin digest (fibrin x). These complexes may be involved in the phenomenon of "paracoagulation" (see Chapter I. 6(a)).

(c) dimers formed when fibrin x and y are incubated with fibrinogen in the absence of thrombin.

Type C: (6) Soluble complexes arising from crosslinking produced by F XIIIa

It is recognised that F XIIIa can crosslink soluble fibrinogen-

fibrin complexes (Sasaki, Page and Shainoff, 1966) (see Chapter I. 2(b)). Ly, Kierulf, Jakobsen and Gravem (1974) added F XIIIa to complexes produced by incubating fibrinogen with thrombin. N-terminal analysis suggested that in the oligomers formed less than 30% of the component units were fibrinogen molecules. They subsequently showed γ-γ crosslinking but no α chain crosslinking in the oligomers and that F XIIIa could crosslink fibrinogen to form a fibrinogen dimer (Ly, Kierulf and Jakobsen, 1974; see also Chapter I. 2(b)). Approximately equal amounts of γ and γ-γ dimer were present suggesting that one of the two γ chains of each fibrinogen molecule was intercrosslinked (Ly, Kierulf and Jakobsen, 1974).

F XIIIa can therefore crosslink existing complexes and even form complexes. This provides a mechanism for the formation of soluble complexes without the action of thrombin, although, of course, thrombin might well act as the activator of F XIII (Finlayson, 1974). The importance of this in vivo remains to be established.

It is obvious that considerable information is available concerning the formation of soluble complexes in vitro. Despite conflicting results over certain details the important point is that stressed by Latallo (1975) - "There exists no data indicating that under "physiological" conditions of pH, temperature and ionic strength intact fibrinogen or any of its plasmic derivatives, which have not been exposed to thrombin or FSF (F XIIIa), are able to associate and form soluble polymers or complexes. Hence, it could be concluded that the removal of at least one of the fibrinopeptides is a prerequisite condition for the formation of any complexes between fibrinogen derived molecules".

In their most recent work on this subject Latallo, Mattler, Bang and colleagues (1976) suggest that under very carefully controlled experimental conditions soluble complexes formed in vitro have only "two identifiable components, fibrin monomer and clottable Fragment x monomer, although incorporation of native fibrinogen and Fragment X unreacted by thrombin into soluble fibrin complexes cannot be excluded".

I. 5(c) Soluble Fibrinogen-Fibrin Complexes - In Vivo Experimental Work

The basic aims of such experiments are:

(1) To find out how soluble complexes are metabolised in vivo.

(2) To correlate the presence of soluble complexes in plasma with definite intravascular pathology (e. g. deep venous thrombosis or disseminated intravascular coagulation).

1. Metabolism of Soluble Complexes In Vivo

This can be studied by the use of radioactive isotopes. In a series of experiments Sherman, Harwig and Lee (1975) showed that ^{131}I labelled fibrinogen had a half-life of 52 hours, when injected into rabbits. When ^{125}I labelled fibrin, consisting of a mixture of complexed and uncomplexed units, was injected a half-life of 10 hours was recorded. It therefore appeared that soluble fibrin was metabolised more rapidly than fibrinogen.

When uncrosslinked soluble fibrinogen-fibrin complexes, composed of ^{131}I labelled fibrinogen and ^{125}I labelled fibrin, were injected a half-life of 12 hours was found for the ^{125}I fibrin and 15 hours for the ^{131}I fibrinogen. The complexes were apparently stable in vivo, as they could be demonstrated by gel filtration in blood samples taken from the rabbits.

Sherman and colleagues (1975) also demonstrated that crosslinked soluble fibrinogen-fibrin complexes were handled in a similar way to non-crosslinked complexes. In this respect their results differed from those of Shainoff and Sasaki (1971) who found that crosslinked complexes remained in the circulation longer than non-crosslinked ones.

Soluble complexes consisting of early (minimally degraded) fibrin degradation products also disappeared rapidly from the circulation when injected into rabbits, with a half-life of 5 hours, high concentrations appearing in the spleen (Bang and Chang, 1974).

It therefore appears that soluble fibrin material within the circulation can be identified by the body's defense mechanisms and removed more rapidly than fibrinogen. Fibrinogen incorporated into a complex with fibrin will be catabolised more rapidly than uncomplexed fibrinogen. Gurewich, Wetmore, Nowak and colleagues (1974) showed that ^{125}I labelled fibrin monomer was progressively and rapidly deposited in the kidney, liver, lungs and spleen. The concentration of radioactivity per gram tissue was greatest in the spleen.

The probable importance of the reticuloendothelial system in removing circulating soluble fibrin material was first stressed by Lee (1962). He demonstrated that, while intravenous injections of endotoxin or infusion of thrombin rabbits did initiate a degree of intravascular coagulation in some of the animals, they do not usually result in massive fibrin deposition. However, if the reticuloendothelial system is blocked by Thorotrast, thrombin infusion will produce the classical features of the generalised Shwartzman reaction (GSR) with massive insoluble fibrin deposition. (The generalised Shwartzman reaction is produced in the experimental animal, classically by two

appropriately spaced injections of endotoxin. It results in the development of bilateral renal cortical necrosis and bleeding. These features are attributed to widespread intravascular coagulation).

Following his observations Lee (1962) suggested that "In the generalised Shwartzman reaction, the first intravenous dose of endotoxin initiates intravascular conversion of fibrinogen to fibrin, but this is then quickly cleared from the circulation by the reticuloendothelial system. Subsequently, at a time when the activity of the reticuloendothelial system is much depressed, a second injection of endotoxin again activates intravascular coagulation, but now fibrin aggregates persist and accumulate in the circulation and are progressively deposited in the terminal vascular bed".

The idea of a two stage reaction in the GSR with first the formation of soluble fibrinogen-fibrin complexes and their subsequent precipitation as insoluble fibrin, is now widely accepted. Following a single injection of endotoxin in the rabbit, soluble complexes can be demonstrated by the ethanol gelation test and the serial dilution protamine sulphate test (see Chapter I. 6(a)), a peak concentration occurring 6 hours after injection, although at this time no histological changes can be found in the kidney (Szczepanski and Lucer, 1975). This "preparative" injection presumably overloads the defense mechanisms (possibly in the reticuloendothelial system) for the removal of soluble complexes. Therefore a second (provocative) injection of endotoxin results not only in an increase in soluble complex concentration after 3-6 hours, but also in precipitation of insoluble fibrin within the kidney reaching a maximum at 6 hours (Szczepanski and Lucer, 1975).

The causes of precipitation of soluble fibrinogen-fibrin complexes (or soluble fibrin) in the second stage of this reaction are uncertain. This stage, unlike the actual formation of soluble complexes, cannot be prevented by heparin (Müller-Berghaus and Hocke, 1972; Lipinski, Gurewich, Nowak et al., 1974). The action of thrombin is therefore unlikely to be necessary at the precipitation stage. Various non-enzymic mechanisms have been suggested e.g. through basic proteins (Lipinski, Wegrzynowicz, Budzyński et al., 1967) which could be released from platelets or leucocytes. These mechanisms would have obvious similarities to the paracoagulation reaction in vitro (see Chapter I. 6(a)). Platelets and leucocytes might also play a role in the actual formation of soluble complexes through the generation of thrombin.

The importance of leucocytes and platelets in the development of endotoxin induced intravascular coagulation remains uncertain. Although earlier work suggested that thrombocytopenia (induced by platelet antiserum) prevented micro-clot formation in the GSR (Margaretten and McKay, 1969). Lipinski and Gurewich (1976b) showed that thrombocytopenia (induced by neuraminidase) did not prevent thrombin formation demonstrated by soluble complex production following endotoxin injection. Müller-Berghaus and Kramer (1976) moreover found that thrombocytopenia (induced by platelet antiserum) did not prevent the precipitation of soluble complexes or soluble fibrin by endotoxin. It is therefore possible that platelets are not required for either of the two reactions in endotoxin induced intravascular coagulation. (Different methods of inducing thrombocytopenia with varying success may be responsible for conflicting results). Dipyridamole, however, seems to

protect against insoluble fibrin deposition in ^{125}I fibrin monomer infused rabbits (Lipinski, Gurewich and Nowak et al., 1974), although it is possible that some other action, besides that of an antiplatelet agent is involved.

The evidence linking leucocytes with the development of endotoxin-induced intravascular coagulation is rather stronger. It was originally shown by Thomas and Good (1952) that severe leucopenia prevented the development of micro-clots in the GSR. It has been shown that leucopenia protects against the formation of soluble complexes following endotoxin injection (Lipinski and Gurewich, 1976b) and also against the deposition of infused ^{125}I labelled fibrin monomer (Gurewich, Wetmore, Nowak et al., 1974). Müller-Berghaus and Eckhardt (1975), however, showed that leucopenia did not protect against the precipitation of soluble complexes (produced by initial ancrod infusion) by endotoxin. A further study, however, did show that the granulocyte count before the second injection of endotoxin (but after the first) was significantly related to the subsequent development of micro-clots (Müller-Berghaus, Bohn and Höbel, 1976). The evidence for leucocytes being involved in the precipitation step is therefore rather confusing but they may be required for the initial elaboration of thrombin by the release of procoagulant material. It may be the monocytes which are mainly responsible for endotoxin-induced procoagulant release from leucocytes (Rivers, Hathaway and Weston, 1975).

A review of this subject by Müller-Berghaus, Eckhardt and Kramer (1976) suggests that neither platelets nor leucocytes are particularly important in the precipitation and/or polymerisation of soluble fibrin by endotoxin in vivo. Instead the importance of stimulation of α-adrenergic receptor sites in the precipitation of

soluble complexes (soluble fibrin) is stressed, an observation originally made by this group in ancrod treated rabbits (Müller-Berghaus and Mann, 1973).

Other factors such as endothelial cells might be involved in a process of pinocytosis resulting in precipitation of soluble complexes, although evidence for such pathways is at present lacking. The degree to which small changes in pH, ionic concentration and stasis contribute to precipitation of soluble complexes also is unknown.

2. Correlation Between Presence of Soluble Fibrinogen-Fibrin Complexes in Plasma and Definite Intravascular Pathology

It is clear from the previous discussion that soluble complexes may be present in the blood without insoluble fibrin deposition (i. e. following the preparative injection of endotoxin in the GSR). It cannot therefore be concluded that the presence of high concentrations of soluble complexes necessarily implies the presence of thrombus or microthrombi.

If a bolus injection of thrombin is given intravenously to rabbits, thrombin sensitive, highly aggregated, soluble complexes (demonstrated by agarose gel filtration) can be found in plasma at 1, 2, 4, 6, 8 and 10 minutes after injection. Rapid clearance of these complexes must occur, as they cannot be demonstrated after 10 minutes (Bang, Hansen, Smith et al., 1973).

Chang, Wilson and Frenkel (1974) studied three experimental models in dogs:

(1) thrombi formed by stasis

(2) injection of homologous serum as a source of activated clotting factors

(3) combined infusion of homologous serum and stasis thrombosis

Soluble complexes were demonstrated by agarose gel filtration and by the serial dilution protamine sulphate test (see Chapter I. 6(a)). Serial studies demonstrated that local thrombosis in the absence of circulating activated clotting factors did not produce significant amounts of soluble complexes. The injection of serum in the absence of stasis thrombosis resulted in only occasional and transient increases in circulating soluble complexes. The infusion of serum together with the formation of stasis thrombosis consistently gave rise to substantial and sustained concentrations of soluble complexes.

The experimental design in relation to the localised thrombosis could be criticised, as the authors admitted. During the first 30 minutes two clamps were applied on either side of the developing thrombus, so that it was not in contact with circulating blood. Subsequently partially stenosing ligatures were applied and the clamps removed. This allowed contact between the thrombus and the circulating blood but growth of the thrombus at this point might have been limited. Complete removal of the ligatures allowed embolisation to occur to the lung. At this point significant elevations of soluble complex concentration were noted on the serial dilution protamine sulphate test. This could reflect propagation of the embolus within the pulmonary vasculature or the release of early fibrin degradation products due to fibrinolysis. The results do, however, suggest that pre-existing localised deep venous thrombosis might not be associated with greatly increased soluble complex concentration.

Homologous serum contains F IXa; F Xa and F XIa (Deykin, Chun, Lopez et al., 1966). The thrombus-inducing capacity of injected serum will therefore depend on the content

of these activated factors and how quickly they are removed. Traces of thrombin will probably be produced. Injection of homologous serum therefore may mimic a "hypercoagulable" state - defined as the presence of activated clotting factors within the circulation. Combining this with localised thrombosis seems to lead to high concentrations of soluble complex (Chang et al., 1974). These authors suggest that persistently increased levels of soluble complexes require a systemic "hypercoagulable state" together with "an amplifying system for thrombin generation" in a local area of stasis. This combination allows the continued build-up of soluble complexes in sufficient quantities to surpass the clearance capacity of the reticuloendothelial system.

In a differently designed set of experiments Donati, Bertoni, Mussoni and colleagues (1976) induced macroscopic liver thrombosis and "a laboratory picture of disseminated intravascular coagulation" in rats by first feeding them on a high fat diet for 10 weeks, followed by a single injection of endotoxin (Group I). Two other groups of rats received only one stimulus (Group II were fed a high fat diet for 10 weeks and Group III were given a single injection of endotoxin). In neither of these groups did macroscopic thrombosis occur, although the rats receiving an injection of endotoxin (Group III) showed "laboratory signs of moderate disseminated intravascular coagulation". All three groups showed increased soluble complex concentrations compared with an untreated control group. The results were calculated in terms of the percentage concentration of fibrinogen-like material eluted before the fibrinogen peak and were:-

Group I $55 \pm 17\%$

Group II $34 \pm 14\%$

Group III $17 \pm 4\%$

Therefore in these experiments also the combination of "hypercoagulability" and actual thrombosis gave the highest soluble complex concentration, but "hypercoagulability" alone was also associated with raised levels. The differences between Groups II and III are rather interesting - especially the rather high values obtained by feeding a fat-rich diet (Group II).

While considerable caution needs to be exercised in extrapolating these animal models to the human situation, these studies are clearly important in attempting to interpret the results to be described in this thesis. Clearly an established local thrombosis may not be associated with increased soluble complex concentrations, but different forms of "hypercoagulability" appear to result in raised levels. The combination of local thrombus (or microthrombosis) and "hypercoagulability" appears to be the most potent method of inducing sustained increased soluble complex levels. These circumstances are particularly likely to occur in association with disseminated intravascular coagulation. The animal experiments already discussed in which the GSR is induced show that soluble complexes are present in such circumstances (e.g. Szczepanski and Lucer, 1975).

I. 5(d). Biological Properties of Soluble Fibrinogen-Fibrin Complexes

Soluble complexes may play a part in the formation of insoluble fibrin deposits by precipitation (Chapter I. 5(c)). Apart from this very little is known about their biological properties.

The thrombin sensitivity of one form of soluble complex has been studied. Soluble complexes were prepared from thrombin-

treated plasma or purified fibrinogen. The purified soluble complexes showed a significantly greater sensitivity to thrombin i.e. they clotted significantly more rapidly than fibrinogen over a wide range of thrombin concentration (Bang and Chang, 1974; Hansen, Bang, Barton et al., 1975). It was also noted that soluble complexes substantially shortened the thrombin clotting time of normal plasma and enhanced the resistance of plasma to heparin action in vitro. Soluble complexes containing intact (fibrinogen/fibrin monomer) are reported to aggregate platelets (Kopeć, Wegrznowicz, Budzyński et al., 1968; Larrieu, 1971) as does polymerising fibrin (Niewiarowski, Regoeczi, Stewart et al., 1972). Soluble complexes containing degradation products only aggregate platelets if low concentrations of degradation products are present (Kopeć et al., 1968; Larrieu, 1971).

Blood and plasma viscosity may also be altered by the presence of soluble complexes. In a recent study by Jacob, Fletcher and Alkjaersig (reported by Fletcher and Alkjaersig, 1975) plasma viscosity at low shear rates was shown to be significantly and positively correlated with percentage of soluble complexes of high molecular weight. The approximate ratio of rheological activity of soluble complex: uncomplexed fibrinogen: fibrinogen:Fragment X was 10:5:1.

Blättler, Straub and Peyer (1974) studied blood viscosity in human volunteers treated with the defibrinating agent Defibrase (see Chapter IV). They found that if blood viscosity measurements were made at 22°C a significant increase in blood viscosity was noted in the Defibrase treated group compared with a control group. However, if blood viscosity measurements were performed at 37°C no difference between the two groups could

be noted. These authors therefore suggest that soluble complexes are not present at 37°C and thus do not influence blood viscosity. (They did not, however, carry out agarose gel filtration at 37°C to confirm this hypothesis).

It is therefore necessary to await further work in this field (particularly at 37°C) before accepting that soluble complexes will increase blood or plasma viscosity in vivo. If it could be confirmed that they do have this effect, soluble complexes could reduce the blood flow to an organ or tissue, producing ischaemia or even infarction, without the precipitation of insoluble fibrin. This could be of particular relevance in arterial disease.

I. 6(a) Soluble Fibrinogen-Fibrin Complexes - Techniques Available for their Identification and Assessment in Clinical Conditions

Soluble complexes or soluble fibrin can be identified by a variety of techniques, some of which have already been mentioned. These can be classified:-

(1) Paracoagulation or Gelation Tests -
   (i) ethanol gelation test (EGT)
   (ii) protamine sulphate tests e. g. serial dilution protamine sulphate test
   (iii) cryofibrinogen
(2) N-terminal analysis of a modified Cohn Fraction I of plasma
(3) ^{14}C labelled glycine ethyl ester incorporation into soluble complexes
(4) Affinity or adsorption chromatography
(5) Agarose gel filtration or plasma fibrinogen chromatography

(6) The measurement of fibrinopeptide A in plasma. (This is an indirect measurement of fibrinogen to fibrin conversion).

None of these tests are entirely satisfactory. Whatever technique is used utmost care must be taken in blood collection to prevent in vitro thrombin action.

(1) Paracoagulation or Gelation Tests

"Paracoagulation" refers to the puzzling phenomenon whereby a digestion mixture from insoluble fibrin can once again be converted into an insoluble gel or clot by various non-enzymatic means, but not by the enzyme thrombin (Derechin, 1955). Paracoagulation can be induced by among other methods the addition of protamine sulphate, platelet factor 4, dilution with distilled water, staphylococcal clumping factor and cooling to $4^{o}C$ (see review in Niewiarowski and Lipinski, 1970).

Paracoagulation may be associated with the presence of soluble complexes containing non-clottable fibrinogen-fibrin degradation products as well as fibrin monomer units (Lipinski, Wegrzynowicz, Budzyński et al., 1967). The paracoagulating agent may act by causing dissociation of these complexes into their component units and thereby allowing the fibrin monomer units to polymerise into insoluble fibrin (Lipinski et al., 1967; Niewiarowski and Lipinski, 1970). Early fibrin x units could presumably polymerise in a similar way, as it has been shown that both protamine sulphate and ethanol can produce "an extensive network" on addition to early fibrin x, which is indistinguishable on electron microscopy from that produced by the action of thrombin on fibrinogen (Niewiarowski, Stewart and Marder, 1970).

This explanation of paracoagulation has been criticised by Latallo, Mattler, Bang and colleagues (1976), who claim that soluble complexes do not contain non-clottable degradation products. Individual fractions of a thrombin-treated early fibrinogen digestion mixture were isolated by agarose gel filtration and then treated with protamine sulphate at 37°C. More than 90% of the protein in the soluble complex fractions was precipitated by the protamine sulphate, while less than 10% of the protein in the degradation product fractions was precipitated (Latallo et al., 1976). Thus it may be that protamine sulphate acts directly on soluble complexes to allow polymerisation, without first inducing dissociation of the complexes. A charge neutralisation effect may be involved.

Whatever the mechanism involved paracoagulation would seem to reflect the presence of soluble complexes or soluble fibrin in a plasma sample.

(i) Ethanol Gelation Test (EGT)

This is thought to give positive results when soluble complexes containing fibrin monomer units are present. In a series of in vitro experiments performed to evaluate the significance of a positive EGT fresh plasma was incubated with thrombin, the subsequent addition of ethanol at 20°C yielding a gel (Kierulf, 1973). When this gel was examined by ultracentrifugation a film was produced containing fibrinogen-fibrin derivatives. This material was 95% clottable and was shown by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) to contain derivatives with a molecular weight greater than the parent molecule (i.e. soluble complexes).

Oligomers with molecular weight of 700,000 (dimers); 1,000,000 (trimers); 1,300,000 (tetramers) etc. were demonstrated. If citrate was used as an anticoagulant in preparing the plasma dimers could be found in this material suggesting the action of F XIIIa. (If EDTA was used as anticoagulant, γ - γ dimers could not be demonstrated).

Kierulf (1974) then studied citrated plasmas from 5 patients with a positive ethanol gelation test using the same techniques. N-terminal analysis showed that all the fibrinogen-fibrin films contained N-terminal glycine, the amount being smaller when the plasma fibrinogen concentration was high. Polyacrylamide electrophoresis showed varying amounts of high molecular weight fibrinogen derivatives (i. e. soluble complexes) and these contained γ - γ dimers. He therefore suggested that thrombin and F XIIIa were involved in the production of soluble complexes in these cases. He also suggested that since the amount of N-terminal glycine and fibrinogen derived oligomers was smaller in samples from patients with a raised plasma fibrinogen, "it appears justified to place a greater emphasis on a positive ethanol gelation test in the presence of a normal or sub-normal fibrinogen level, than when the plasma fibrinogen level is elevated".

Conflicting results have been published regarding the usefulness of the EGT in clinical conditions. To illustrate this two studies will be described in some detail. Kierulf and Godal (1971) studied a large number of unselected patients. 11.4% demonstrated a positive EGT. In this group there was a statistically significant number of patients with malignant disease, pneumonia, pulmonary embolism, arterial and venous thrombosis. A control population of 275 healthy subjects were also studied. None of these showed a positive EGT.

In contrast Hedner and Nilsson (1972) found the EGT much less useful. They studied a group of 305 patients with malignant disease, post-operative complications, sepsis, multiple fractures, liver disease, pregnancy and various blood disorders. An overall incidence of 10% positive tests was recorded but only 5 out of 17 patients exhibiting all the classical clinical and laboratory signs of disseminated intravascular coagulation (D.I.C.) had positive tests. Other series have also produced rather conflicting results (see review Bang and Chang, 1974).

(ii) Protamine Sulphate Test

This test is thought to be sensitive to the presence of early fibrin x material, as well as fibrin monomer. A good correlation has been found between the results of the serial dilution protamine sulphate test and the soluble complex concentration using agarose gel filtration (Gurewich and Lipinski, 1976). The study examined plasma samples from thrombin and endotoxin treated rabbits, as well as samples from patients with suspected intravascular coagulation.

As with the EGT clinical studies using protamine sulphate have produced conflicting results. Most series report a high incidence of positive tests in patients with disseminated intravascular coagulation (Seaman, 1970; Niewiarowski and Gurewich, 1971; Gurewich and Hutchison, 1971). The incidence of positive tests in deep venous thrombosis varies in different series. Gurewich, Hume and Patrick (1973) found an incidence of 86% positive tests in symptomatic deep venous thrombosis patients with positive venograms. The incidence fell to 24% in asymptomatic patients with positive venograms and to 11% in patients with negative venograms. Seaman (1970) however detected only 2 positive tests in 16 deep venous thrombosis patients, while Bang and Chang (1974) found 1 positive test in 38 deep venous

thrombosis patients.

A significant incidence of positive tests has also been found in pulmonary thromboembolism (Gurewich and Hutchison, 1971; Gurewich, Hume and Patrick, 1973), in acute arterial thromboembolism (Gurewich and Hutchison, 1971), in disseminated malignant disease (Palester-Chlebowczyk, Strzyzewska, Sitkowski et al., 1971; Gurewich and Hutchison, 1971) in renal disease (Palester-Chlebowczyk et al., 1971) and in systemic hypertension, in which there appeared to be a correlation between the serial dilution protamine sulphate titre and the value of the systolic blood pressure (Żola-Śleczek and Szczepaniec, 1974).

In contrast Hedner and Nilsson (1972) only found 1 positive result out of 305 samples tested. This was a case of disseminated intravascular coagulation but 15 other similar cases gave negative tests. It is probable that the type of protamine sulphate is very important in this technique.

In the few studies in which the EGT and serial dilution protamine sulphate tests have both been used a poor correlation has been observed (Musumeci, 1971; Hedner and Nilsson, 1972). This may partly be explained on the basis of in vitro experiments which suggest that the EGT is sensitive mainly to soluble complexes containing fibrin monomer, while the protamine sulphate test is sensitive also to soluble complexes formed from lysing fibrin (Konttinen, Kemppainen and Turunen, 1972; Gurewich, Lipinski and Lipinska, 1973). The conflicting results may also be due to the different assay conditions used by different groups (see review Bang and Chang, 1974). The EGT appears to be sensitive to pH, being more specific at a more alkaline pH. The EGT may also produce a false positive result at very high fibrinogen

levels. The protamine sulphate test appears to be sensitive to the amount and preparation of protamine sulphate added, the pH, temperature and fibrinogen concentration. The optimal conditions for maximum sensitivity and specificity of these two tests has not been established but they have the undoubted advantage of being rapid and simple to perform.

(iii) Cryofibrinogen

The appearance of increased levels of cold-precipitable fibrinogen (called "cryofibrinogen") was first reported by Korst and Kratochvil (1955) in a patient with migratory thrombophlebitis associated with bronchial carcinoma. McKee, Kalbfleisch and Bird (1963) detected abnormally high levels of cryofibrinogen in 28 out of 665 patients but not in 135 healthy control subjects. This abnormality was associated with malignancy, collagen disease or thromboembolism. Pindyck, Lichtman and Kohl (1970) noted an incidence of cryofibrinogen of 26% in women taking the oral contraceptive therapy but also found an incidence of 12% in women with intrauterine contraceptive devices and 8% in healthy, normal women.

Small series and case reports have also shown cryofibrinogen to be associated with carcinoma, D.I.C., severe acute or chronic infections and major thrombo-occlusive disease (see review Bang and Chang, 1974). Cryofibrinogen has also been found in association with pre-eclampsia (Wardle and Menon, 1969; Howie, Prentice and McNicol, 1971).

Cryofibrinogen may be related to the presence of soluble fibrinogen-fibrin complexes - in particular a dimer (Alkjaersig, Roy and Fletcher, 1973) and possibly also a polymer (Kazama and Abe, 1976).

Although this is also a relatively simple test and historically the first test for "soluble fibrin" to be introduced, it has not been evaluated fully in screening for intravascular coagulation in a large series nor has it been correlated with the other available tests.

2. N-Terminal Analysis of a Modified Cohn Fraction I of Plasma

The removal of the fibrinopeptide fragments from fibrinogen by thrombin exposes previously masked glycine residues on the N-terminal end of the fibrin monomer thus formed. In this test plasma is treated with ethanol in the cold. A fibrinogen fraction of greater than 90% clottability can then be harvested. A comparison of the N-terminal glycine of the total precipitated fraction, the clottable proteins and the clot liquor can then be performed, allowing quantification of the N-terminal glycine in clottable soluble complexes (Kierulf and Abidgaard, 1971).

In a small series studied by this technique (Kierulf and Godal, 1971) N-terminal glycine was demonstrated in large amounts in samples from 10 patients with positive EGT and signs of intravascular coagulation and fibrinolysis. In addition, some patients showed increased amounts of several other N-terminal groups suggesting that plasmin or other protease as well as thrombin had been involved in the formation of these soluble complexes. The technical complexity of this technique would seem to prevent its widespread use.

(3) ^{14}C-Labelled Glycine Ethyl Ester Incorporation into Soluble Complexes

This is also a technically difficult technique and therefore

few reports have been published on its use. When ^{14}C-labelled glycine ethyl ester is incubated with thrombin-altered fibrinogen (i.e. soluble complexes) in the presence of F XIIIa and calcium, enzymic incorporation of ^{14}C-labelled glycine ethyl ester occurs. The incorporation into fibrinogen or fibrinogen degradation products is minimal (Kisker and Rush, 1971). This test was found to provide useful information for the diagnosis and treatment of two patients with meningococcal septicaemia, two patients with Rocky Mountain spotted fever and three patients in whom therapeutic abortions induced by the intrauterine injection of hypertonic saline produced D.I.C. (proven by laboratory changes). Increased levels of soluble fibrin have also been detected in women taking oestrogen-progestogen therapy using this technique (Pilgeram, Ellison and von dem Bussche, 1974).

(4) Affinity or Adsorption Chromatography

In this technique insolubilised fibrinogen is prepared by chemical fixation of purified fibrinogen to cyanogen bromide activated agarose or sepharose (Heene and Matthias, 1973). Thrombin incubated plasma containing EGT positive material (i.e. soluble complexes containing fibrin monomer) revealed absorption of a fibrinogen derivative which could subsequently be eluted at pH 4.1. The amount of material which adsorbed correlated significantly with the quantitative EGT and was therefore presumed to consist of fibrin monomer.

Raised levels of soluble fibrin have been found using this technique in samples from patients with disseminated intravascular coagulation, acute myocardial infarction and carcinoma (Matthias, Reinicke, Heene et al., 1975). It was claimed that a raised level of soluble fibrin was present before abnormalities developed in the

ethanol gelation test or in analysis of plasma coagulation factors.

(5) Agarose Gel Filtration/Plasma Fibrinogen Chromatography

(Gel Exclusion Chromatography, Column Chromatography)

In this technique plasma is separated by filtration through large pore agarose gels and the eluant fractions are analysed for fibrinogen related protein. The rate at which an individual protein flows through the column is proportional to its molecular size and in particular the individual molecular Stokes radius. The principles governing analytical gel filtration have been fully discussed by Winzor (1969) - see also Chapter II.

Soluble complexes will be eluted in advance of normal fibrinogen using this system, while fibrinogen-fibrin degradation products are eluted after the parent molecule. Fletcher, Alkjaersig and their colleagues have developed this technique (Fletcher, Alkjaersig, O'Brien et al., 1970) and using a computer programme based on chromatographic plate theory analysis are able to arrive at a semi-quantitative estimate of the relative content of soluble complex, fibrinogen and degradation products in poorly resolved chromatographic patterns (Alkjaersig, Roy and Fletcher, 1973).

This group have extensive experience of applying this technique to studying clinical conditions associated with hyper-coagulability or thrombosis.

Deep Venous Thrombosis (D. V. T.)

In a collaborative study Fletcher and colleagues studied plasma samples from patients participating in a British Medical Research Council trial of ^{125}I fibrinogen scanning in the detection of post-operative deep venous thrombosis (Fletcher and Alkjaersig, 1972). The fibrinogen scan and chromatography results correlated

well. Out of a total of 101 patients complete agreement of both positive and negative findings for the two methods was found in 72 patients - both assays were negative (i.e. normal) in 31 cases and positive (i.e. abnormal) in 41 cases. In 22 patients abnormal chromatography results were detected in patients with normal fibrinogen scans. In 15 of these cases abnormal results were present pre-operatively, using the chromatography technique. (Fibrinogen scans were not performed pre-operatively). The authors suggest that the abnormal pre-operative results were associated with underlying pathology, such as carcinoma of lung, and point out that the fibrin deposits or thrombus might have been present in some site other than the leg veins. They also suggest that the chromatography technique may be more sensitive than the scan technique in detecting small fibrin deposits. However, 7 patients had normal chromatography results and abnormal ^{125}I fibrinogen scans. A representative series of chromatography results for one patient are shown in Figure 9. This is an interesting study which does suggest that plasma fibrinogen chromatography might be useful in the early detection of deep venous thrombosis.

Hansen, Bang, Kim and Glover (reported in Bang and Chang, 1974) have carried out a similar study in the detection of post-operative D.V.T. These authors used venography to make a diagnosis rather than fibrinogen scans. They studied 38 patients. 12 developed venographic evidence of D.V.T. post-operative and all 12 showed abnormal chromatography results, although only 1 patient developed clinical signs of D.V.T. which was later complicated by pulmonary embolism. 18 patients had negative venograms and normal chromatography results. 8 patients had normal venograms and abnormal chromatography results. Out of the 20 patients with abnormal chromatography

results post-operatively 7 were abnormal pre-operatively.

This report has the advantage of confirming the diagnosis of D. V. T. by venography. The results are fairly similar to those of Fletcher's group. The usefulness of the chromatography technique is, however, limited in that it fails to localise the site of the thrombus and also (rather more importantly) there is an inevitable delay in obtaining the result, even using the modifications described by Alkjaersig and colleagues (1973).

Oestrogen-Progestogen (Oral Contraceptive) Therapy

Alkjaersig and colleagues have shown abnormal plasma fibrinogen chromatography results in women taking oestrogen-progestogen therapy (Alkjaersig, Fletcher and Burstein, 1971 and 1975). In the more recent report the results on 1,350 samples from 193 women were reported. Abnormal plasma fibrinogen chromatography results were found in 27% of women taking the oestrogen-progestogen therapy and in 6% of normal control women. The authors suggested that these results showed that women taking oestrogen-progestogen therapy develop "clinically silent thrombotic lesions" with a four to five-fold greater frequency than in control subjects. They concluded that "these women are at a four to five-fold greater risk of developing clinically overt disease, a risk factor in line with that derived by epidemiologic study".

Asbeck, Bebber and van de Loo (1974) have published results on a group of 7 healthy women studied before and during a 4-12 week period of combined oestrogen-progestogen therapy. They demonstrated increased amounts of soluble complexes (dimers) representing about 5-6% of the total amount of the fibrinogen peak using the mean results for the whole group. Similar results have been obtained by Graeff, von Hugo and Hafter (1976).

It is therefore probable that increased concentrations of soluble complexes are present in plasma from women taking oestrogen-progestogen therapy. The significance of this in terms of actual thrombotic disease remains unknown.

Cerebral Vascular Accidents due to Cerebral Thrombosis

In an initial study soluble complexes were demonstrated in 31 out of 43 patients with cerebral thrombosis (Brooks, Davis and Devivo, 1970). 7 patients had normal findings and 5 patients showed evidence of early clottable fibrinogen-fibrin degradation products. 13 of the patients were treated with urokinase. It was felt that fibrinogen chromatography helped in assessing the effects of this therapy and also in assessing the prognosis in thrombotic strokes.

Subsequently serial studies on 20 patients with acute cerebral thrombosis were reported (Alkjaersig, Laursten and Fletcher, 1972). 49% of the total plasma samples showed evidence of circulating soluble complexes. These patients also showed significantly depressed levels of antithrombin III, plasminogen, α-1-antitrypsin and α-2-macroglobulin. The suggestion was made that a "hypercoagulable state" follows acute thrombotic stroke in a high proportion of patients.

Acute Myocardial Infarction

In studies of blood samples obtained following the onset of signs and symptoms of acute myocardial infarction, Fletcher and Alkjaersig (1973) found that approximately 50% of patients had increased levels of soluble complexes which tended to fall towards normal levels over the next 2-3 days. A further 30% had increased levels of soluble complexes, which persisted unchanged for as long as 10 days, while in 20% no abnormalities were found.

Colman, Anaya-Galindo, Shattil and colleagues (1975) examined 21 patients undergoing coronary arteriography for evaluation of chest pain using the agarose gel filtration technique. 16 patients had one or more coronary arteries occluded by more than 80% and 5 patients had no angiographic abnormalities. The two groups were comparable for age, total and low density lipoprotein, cholesterol and triglyceride. There was a striking increase in soluble complex concentration measured by their technique in the patients with abnormal arteriograms (23.3 $\pm$ 3.5(S.E.M.)%; normal 4.6 $\pm$ 1.3%) compared to patients without angiographic abnormalities (10.9 $\pm$ 1.5%). Also the concentration of soluble complex showed a good correlation ($r = 0.49$; $p < 0.005$) with the degree of angiographic abnormality. They suggest that this technique could provide "a useful non-invasive screening test for assessing the extent of coronary artery occlusion".

Increased soluble complex concentrations have been found in many other medical conditions including acute glomerulonephritis (Fletcher and Alkjaersig, 1973), sickle cell crises (Chaplin, Alkjaersig and Fletcher, 1974), type II hyperlipoproteinaemia (Carvalho, Colman and Lees, 1974), thromboangitis obliterans (Kazama and Abe, 1976), disseminated intravascular coagulation due to a variety of causes (Kazama and Abe, 1976), chronic active hepatitis (Coccheri, Gasbarrini, Palaret et al., 1976) and during Defibrase therapy (Asbeck, Lecher, Martin et al., 1975).

Soluble complexes have also been identified in increased concentrations in obstetrical and gynaecological conditions. In one study Hafter, Schneebauer, Tafel and colleagues (1975) studied 67 healthy primiparous and multiparous women (6-40 weeks gestation). The amount of soluble complex (expressed as a

percentage of the total fibrinogen-related protein) increased from 2.6 ± 0.4% (mean ± S.D.) in early pregnancy to 4.9 ± 1.3% at 40 weeks. Additional increases occurred during labour. Chain analysis of the soluble complexes showed decreased amounts of α chain "indicating plasmin activity". γ-γ dimers were not observed (i.e. F XIIIa had not crosslinked fibrinogen to produce these complexes, nor had it crosslinked thrombin-produced complexes). This group have also shown a rise in the early puerperium to 6.3 ± 1.2%, with a concentration of 3.3 ± 1.3% three months after delivery (Graeff, Wiedmann, von Hugo et al., 1976).

Increased soluble complex concentrations have also been found in association with septic abortion and advanced carcinomas of the male and female genital tract (Graeff, von Hugo and Hafter, 1976). Markedly increased concentrations of soluble complex have also been observed in single case reports in eclampsia (Graeff and von Hugo, 1972); abruptio placentae (Vermylen, Donati and Verstraete, 1971; Graeff and von Hugo, 1974) and retained dead foetus syndrome treated with Reptilase (Hafter and Graeff, 1975). In addition this group have found increased soluble complex concentrations in cord blood from asphyxiated neonates (Graeff, von Hugo and Hafter, 1973) and in endotoxic shock (Graeff, von Hugo and Hafter, 1976).

Only in cases, in which florid "disseminated intravascular coagulation" was likely to have been present were γ-γ dimers found in the soluble complex material indicating intermolecular covalent bonding by F XIIIa (Graeff, von Hugo, Hafter, 1975 and 1976). These authors suggest that γ-γ dimer is only found once insoluble fibrin has been laid down and that if no γ-γ dimer is present the correct diagnosis is "hypercoagulability".

(6) The Indirect Measurement of Soluble Fibrin by a Specific Assay for Fibrinopeptide A

Plasma levels of fibrinopeptide A can be accurately measured by a specific radioimmunoassay (Nossel, Younger, Wilner et al., 1971; Nossel, 1976). Plasma samples from 100 healthy volunteers gave a mean level of 0.6 pmoles/ml, with a range of 0.1-1.5 pmoles/ml (Nossel, 1976). Grossly elevated levels ()7 pmoles/ml) were found in patients with acute pulmonary embolism, acute thrombophlebitis or acute disseminated intravascular coagulation (Nossel, 1976). Moderately elevated levels (1.5-7 pmoles/ml) were found in patients with acute infections (septicaemia, lobar pneumonia and cellulitis), in carcinomatosis without clinically evident thrombosis, systemic lupus erythematosus, renal transplant rejection and aortic aneurysm (Nossel, 1976).

Other groups have reported raised plasma fibrinopeptide A levels in deep venous thrombosis, disseminated intravascular coagulation and hypertonic saline-induced abortion (Gerrits, Flier and van der Meer, 1974), and also during ancrod therapy and in retinal vascular occlusion (Budzyński and Marder, 1975).

Potentially this would seem a useful technique for identifying intravascular coagulation. It is possible, however, that the very short half-life of fibrinopeptide A in plasma (3 mins; Nossel, Canfield and Butler, 1973) might prove a problem.

I.6(b) Soluble Fibrinogen-Fibrin Complexes - A Summary

It is clear that the literature on this subject is extensive. In some areas apparently conflicting results have been published. In this summary the information and conclusions discussed earlier are brought together. For a full discussion of each topic and references the appropriate section should be consulted.

In vitro experiments suggest that soluble complexes can be formed in a variety of ways. Fibrinogen incubated with low concentrations of thrombin provides the simplest model for their formation. Plasmin may also act on the component units of the complexes before or after their formation. F XIIIa may act by crosslinking soluble complexes, that have already been formed, or by crosslinking intact fibrinogen to form complexes. In this way soluble complexes could be formed without the action of thrombin. The relevance of these observations in vivo remains to be established.

There is good evidence that fibrin monomer and early fibrin x monomer act as component units of soluble complexes. Fibrinogen and early fibrinogen X may also be incorporated into complexes. Whether the later degradation products of fibrinogen or fibrin form complexes remains controversial.

In vivo experiments have shown that complexes can be produced by injection or infusion of thrombin, ancrod, Defibrase/Reptilase, endotoxin and the activated coagulation factors in serum. The reticuloendothelial system may be important in the clearance of these complexes. "Over-loading" or blocking the reticulo-endothelial system could result in deposition of soluble complexes as insoluble fibrin deposits.

While "hypercoagulability" can produce transient increases in soluble complex concentration, the combination of "hyper-coagulability" and insoluble fibrin deposition appears to result in higher and sustained soluble complex concentration in in vivo animal experiments. Formed thrombus (i. e. not extending or rapidly lysing) does not give rise to high levels of soluble complex in one in vivo animal model, but may under different experimental conditions.

As most work on the identification of soluble complexes is performed at 20-22°C (room temperature) care must be taken in relating the results obtained to the in vivo situation at 37°C. It is possible that at least some forms of soluble complex (e.g. in Defibrase injected human volunteers) are not present at 37°C.

A variety of techniques are available for the identification of soluble complexes (or soluble fibrin) in clinical conditions. The simpler tests (e.g. the ethanol gelation test and the protamine sulphate tests) have shown varying results in different series with appreciable numbers of false positive and negative results. The ethanol gelation test may give false positive results with high fibrinogen levels, while the protamine sulphate tests are very dependent on the type of reagent used. The more complicated tests (e.g. agarose gel filtration, ^{14}C labelled glycine ethyl ester incorporation) are too difficult and time consuming for a routine clinical laboratory but provide sensitive techniques for research use. There is good evidence relating abnormalities in the agarose gel filtration technique to clinical intravascular coagulation whether localised or diffuse. However, slightly increased values are also found in healthy pregnant women and in women taking oestrogen-progestogen therapy. In such cases the problem may be one of "hypercoagulability" rather than insoluble fibrin deposition. The assumption that the presence of soluble fibrin (or complexes) in plasma implies insoluble fibrin deposition cannot be made.

Soluble fibrinogen-fibrin complexes in plasma do, however, suggest the action of thrombin or F XIIIa, implying activation of the coagulation pathway. Examination of the chain structure of the complexes may be useful, although such work implies that this has not been altered in vitro after blood sampling. If intact $A\alpha$, B β and γ chains are found, the component units of the

complexes must be fibrinogen or fibrin monomer. Such complexes could only be formed by the action of thrombin (or a thrombin-like enzyme e. g. ancrod). If reduced amounts of intact α chain are found some other proteolytic enzyme (e. g. plasmin) has also been acting besides thrombin. If α polymers or γ - γ dimers are found, F XIIIa must have acted on these complexes - and they could have been formed without the action of thrombin. Examination of the chain structure may, but does not necessarily, allow the conclusion that the complexes have derived from insoluble fibrin deposits, as they could have been formed by the action of F XIIIa on fibrinogen or soluble complexes.

CHAPTER II

MATERIALS AND METHODS

II. 1 Introduction

In this chapter the development of the technique of "plasma fibrinogen chromatography" will be discussed. Plasma fibrinogen chromatography is based on the biochemical technique of agarose gel filtration (molecular sieve chromatography or exclusion chromatography). Agarose gel filtration will therefore be discussed first and then its application to the study of fibrinogen and its derivatives by Fletcher, Alkjaersig, O'Brien and colleagues (1970). Modifications introduced by Graeff and von Hugo (1972) and other groups will next be discussed and finally the methods employed in setting up the project described in this thesis.

II. 2 Agarose Gel Filtration - Historical and Theoretical Aspects

In 1956 Lathe and Ruthven suggested that soluble protein mixtures might be fractionated by passage through columns of swollen gel granules. Porath and Flodin (1959) introduced the use of crosslinked dextran as the gel medium and employed the term "gel filtration" to describe the technique. The terms "molecular sieve chromatography" (Hjerten and Mosbach, 1962) and "exclusion chromatography" (Pedersen, 1962) have also been used.

Gel filtration, like ultracentrifugation, is a transport process, in which migration is governed largely by the size of the solute (e. g. a protein) under investigation. The various mathematical models constructed to explain the process have been reviewed by Winzor (1969). The interaction of a specific protein and the gel

is governed by the molecular dimensions of the protein. Mathematically this is expressed as the Stokes' radius of the protein molecule (i.e. the radius of the equivalent hydrodynamic sphere). No attempt will be made to discuss the mathematical models in detail, as a non-mathematical model is easier to understand and provides adequate explanation for the application of the technique to the study of fibrinogen and its derivatives.

The following simple, non-mathematical explanation of gel filtration is adapted from that described in the Biorad Laboratory Manual on "Gel Chromatography" (1971). The particles of the gel material have a sponge-like matrix structure, containing pores of controlled dimensions. The gel is poured into a glass or plastic column under conditions which allow uniform packing of the particles to form "the bed". The sample, consisting of a mixture of soluble proteins, which differ in molecular dimensions, is then applied to the bed surface and allowed to percolate through the bed using a constant flow of eluant buffer. As elution proceeds, molecules which are too large to enter the pores of the gel matrix pass rapidly through the bed (in the space surrounding the bed particles) and are eluted in a single zone near the beginning of the elution profile (the void volume, V_o). Smaller molecules, which are capable of diffusing into the pores of the matrix are retarded in their migration through the bed. The extent of retardation is inversely correlated to the molecular dimensions of the protein. Therefore the smallest molecules are retarded to the greatest extent and are the last to emerge from the bed. In the absence of other than molecular sieve effects (e.g. ion exchange), elution of all sample solutes is complete when a volume of eluant, approximately equal to the total bed volume, has passed through

the bed. When a homogenous solute (e. g. a pure protein) is studied by agarose gel filtration, under optimal conditions, the elution profile will be a Gaussian curve.

Figure 10 shows the elution profile for a pure protein analysed by this technique. The elution volume (V_e) of a protein is the volume of eluant buffer, which issues from the gel bed from the time penetration begins until the protein begins to appear in the eluant buffer (Biorad Laboratory Manual, 1971). On the elution profile this volume is measured as the eluant volume corresponding to the half-height of the leading side of the peak (method 1). Alternatively, the leading side of the peak can be extrapolated to the base-line (method 2). In the example shown in Figure 10 the V_e of the protein by method 1 is 100 ml and by method 2 is 96 ml.

The void volume (V_o) of a gel filtration column is a measure of the total space surrounding the particles of the chromatography medium packed in the column. It is determined by measuring the elution volume of a solute, which is excluded from the pores of the gel matrix. A coloured substance which is excluded from the gel matrix is best for estimating this. Blue Dextran 2,000 (Pharmacia G.B. Ltd., 75 Uxbridge Road, London) has a molecular weight of 2×10^6, but is "polydisperse". As the gel used for this project (Biogel A5 m) has an exclusion limit of 5×10^6, Blue Dextran is not entirely satisfactory for an accurate estimation of the void volume. At a practical level, however, it gives a useful value and because it is coloured allows rapid visual checking of the bed surface (see Chapter III. 2).

Fletcher and colleagues have applied this technique to the study of fibrinogen and its derivatives. If the fibrinogen-fibrin related antigen elution profile does not conform to a perfect Gaussian curve, this is interpreted as being caused by the presence

of soluble complexes or degradation products (Fletcher et al., 1970). They introduced the term "plasma fibrinogen chromatography" to describe their technique.

II. 3(a) Plasma Fibrinogen Chromatography

Method of Fletcher's Group

This technique was developed from earlier work based on measurements of protein diffusion constants by the immunodiffusion method of Allison and Humphrey (1960). Fletcher and colleagues modified this method to determine the molecular weight of the predominant fibrinogen-fibrin derivative in plasma. During an outbreak of heat-stroke in 1967 they studied 4 patients, who subsequently died quite unexpectedly from pulmonary embolism (Alkjaersig, 1967; Fletcher and Alkjaersig, 1975). A fibrinogen-fibrin derivative with a molecular weight apparently greater than fibrinogen as determined by its diffusion characteristics (i.e. a soluble fibrinogen-fibrin complex) was found in high concentrations in the plasma of all 4 patients for some days prior to death.

This study suggested to the authors that soluble complexes might provide valuable information about "clinically silent thrombosis" (Fletcher and Alkjaersig, 1975). It established the following important principles:-

(1) the examination of plasma rather than serum for the products of enzymic action on fibrinogen

(2) the identification of these products by the use of fibrinogen antiserum

(3) the classification of these products on a molecular weight basis.

They began to experiment with the use of agarose gel filtration (using Biogel A5 m, obtained from Biorad Laboratories, 1510750) to separate the fibrinogen derivatives on a molecular weight basis.

They found that this method worked well and that they could readily analyse the eluant fractions for fibrinogen-fibrin related antigen using a modified radial immunodiffusion technique (Mancini, Carbonara and Heremans, 1965).

The theory of soluble complex formation used by Fletcher's group is shown in Figure 11 and the results of in vitro experiments supporting this theory are shown in Figure 12. The soluble complexes were produced by mixing normal plasma with the degradation products of lysed fibrin. As those complexes are larger in molecular size than fibrinogen they are eluted in advance of the normal fibrinogen peak. This is reported as a "shift to the left". Similarly early fibrinogen degradation products are eluted after the parent molecule. This is reported as a "shift to the right". In clinical conditions associated with "hypercoagulability" or thrombosis a "shift to the left" is seen, while the appearance of uncomplexed degradation products due to fibrinolysis or fibrinogenolysis causes a "shift to the right".

Examples of these patterns found in samples from a patient with a deep venous thrombosis have already been shown in Figure 9. The results of clinical series studied by this group and others have been reviewed in Chapter I. 6(a).

II. 3(b) Modifications Introduced by Other Groups

Graeff and von Hugo (1972) described several modifications to this basic technique.

(1) Fibrinogen and its derivatives were precipitated from plasma, using β-alanine, by a modification of the method of Straughn and Wagner (1966). The precipitate was redissolved before agarose gel filtration.

(2) The same gel medium (Biogel A5 m, mesh size 100-200) was used but the packed column height was 80 cms with a diameter of 2.5 cms. This is a much larger packed column than that used by Fletcher's group. Vermylen, Donati and Verstraete (1971) had also used a large column of height 96 cms and diameter 2.5 cms.

(3) Attention was paid to addition of enzyme inhibitors to both the anticoagulant in which the blood was collected and the column buffer. (Presumably this was also done by Fletcher's group, but the methodological details are difficult to obtain). This should prevent in vitro fibrinogen proteolysis.

(4) Eluant fractions were examined by

(a) optical density readings (at 280 nm) for total protein concentration

(b) thrombin time to identify fibrinogen

(c) radial immunodiffusion using Partigen-fibrinogen plates (Behringwerke, OTDP 0203) for fibrinogen-fibrin related antigen (FR-antigen).

Using this modified technique Graeff and von Hugo (1972) were able to identify elevated soluble complex levels in plasma from a patient with eclampsia. Elevated soluble complex levels were also found following in vitro lysis of insoluble fibrin (von Hugo and Graeff, 1973) and later in a variety of clinical conditions (see Chapter I. 6(a)). In their later studies they have used Biogel A15 m, mesh size 100-200 from Biorad Laboratories, 1511040 (Graeff, von Hugo and Hafter, 1973).

For the present project it was decided to adopt the preliminary β-alanine precipitation step for two reasons. Firstly, it allowed a far greater concentration of fibrinogen-related protein to be applied to the gel bed. This meant that higher concentrations were obtained in the eluant fractions, allowing easier identification of low soluble

complex levels in patient samples. Secondly, polyacrylamide gel electrophoresis could be carried out directly on the soluble complex fractions, as the amount of contaminating protein was greatly reduced.

Biogel A5 m was used as the gel medium for the present project (as recommended by Fletcher's group) because fibrinogen with a molecular weight of about 340,000 appears in the middle of the fractionation range of this gel (see Chapter III. 2, Figure 23). Biogel A15 m would improve the separation of the soluble complex fractions, but at the expense of the separation of the degradation products. It was also decided to use columns of packed height and diameter (35 x 2.6 cms), that is much larger than those used by Fletcher's group in order to improve the resolution of the elution profile. The mesh size of 200-400 was also selected to improve resolution.

Optical density, thrombin clotting time and radial immunodiffusion FR-antigen estimations were performed on the eluant fractions (cf. Graeff's group). The thrombin clotting time test had also been proved to be useful in the identification of soluble complexes by Bang, Hansen, Smith and colleagues (1973). It was decided to use the anticoagulant and column buffer suggested by Graeff's group with some slight modifications (see Chapter II. 4(h)).

Other methods have been used to identify the fibrinogen-fibrin related material in the eluant fractions. The staphylococcal clumping test (SCT) has been claimed to be better at identifying the soluble complex material than the radial immunodiffusion method (Asbeck, Lechler, Martin et al., 1975). The SCT was therefore used to obtain a semi-quantitative estimate of the amount of fibrinogen-fibrin related protein in the very high

molecular weight (void volume) fractions.

II.4 Methods Used for the Project Described in this Thesis

II.4(a) Collection of Blood Samples

Blood was collected by careful venepuncture whenever possible without venous occlusion. If venous occlusion was required the tourniquet was applied immediately before venepuncture and removed once the needle was placed in the vein. Blood was collected in sterile, plastic syringes and immediately divided into aliquots for preparation of plasma, serum and, in the ancrod-treated patients (Chapter IV), for observation of clot quality.

II.4(b) Preparation of Plasma Samples

Whole blood was collected into plastic tubes containing anticoagulant. The anticoagulant mixture (pH 7.5) contained before the addition of blood:- trisodium citrate 0.13M; N tris (hydroxymethyl) methyl 2 aminoethane sulphonic acid (T.E.S.) 0.06M; epsilon aminocaproic acid (E.A.C.A.) 0.02M; ethylene diamine tetra-acetic acid (E.D.T.A.) 0.04M; and aprotinin (Trasylol, Bayer Pharmaceuticals Ltd., Haywards Heath, Sussex, England) 10^6 K.I.U./l (pH adjusted to 7.5). In samples from patients receiving ancrod therapy 0.2 ml ancrod antiserum (snake venom antiserum, Agkistrodon rhodostoma, the Lister Institute, Elstree, England) was added to each millilitre of anticoagulant to neutralise any ancrod in the blood sample. (The E.D.T.A. was only added to the anticoagulant mixture in the autumn of 1974, therefore it was not present in the blood samples collected from the ancrod patients, but was present in the blood samples for all subsequent patient work).

This anticoagulant mixture is based on that of Graeff and von Hugo (1972). Their anticoagulant contained trisodium citrate,

T.E.S. and aprotinin. While aprotinin should directly inhibit the action of any plasmin in the blood sample, E.A.C.A. was also added to prevent activation of plasminogen. E.D.T.A. was included, not only for its anticoagulant effect, but also to prevent the crosslinking action of F XIIIa.

This anticoagulant mixture should therefore have prevented any in vitro enzymic action, although it is impossible completely to exclude this. It is also possible that a degree of in vitro proteolysis might have occurred during the actual process of venepuncture (e.g. blood within the syringe). Adding anticoagulant to the syringe would have reduced this possibility, but would have made it impossible to prepare serum.

The anticoagulated blood was cooled to 4°C immediately after collection. This should also have prevented in vitro proteolysis - but might, if cooling does cause soluble complex formation (Blättler, Straub and Peyer, 1974), have actually produced complexes. (The paper by Blättler and colleagues (1974) was not published until after this project was started. It was decided that even if the complexes were being formed by a cooling effect in vitro they did reflect changes in vivo based on the results from other groups, see Chapter I. 6(a)). The blood was centrifuged at 3,000 g for 20 mins. Plasma was separated and subsequently handled with siliconised glass-ware.

II. 4(c) Preparation of serum samples

Whole blood was collected in the containers provided for use with "Wellcome F.D.P. Kits" (Wellcome Research Laboratories, Beckenham, England, HA14). These contain thrombin, to promote complete clot formation, and a plasmin inhibitor, to prevent in vitro fibrinogen/fibrin degradation product formation. In samples collected from patients receiving ancrod therapy, ancrod antiserum

(0.05 ml) was added. Clotting was allowed to occur at room temperature for several hours (usually overnight) and serum separated and stored at -20°C.

II.4(d) Clot Quality Observation Test

This was only performed on samples taken from the ancrod group of patients (Chapter IV). 1 ml of whole blood was incubated in a glass test tube (75 x 10 mm) containing 0.05 ml ancrod antiserum at 37°C for 24 hours. The resultant clot was graded 1 to 5, a "normal clot" being graded as 1 and "no clot" as 5. The grades, 2, 3, 4 represented mild, moderate and severe defects (Reid, Chan and Thean, 1963).

II.4(e) Plasma Fibrinogen Estimation

This assay was based on the method of Ratnoff and Menzie (1951). This technique measures thrombin clottable protein (i.e. it will also measure early, clottable Fragment X and possibly also some forms of soluble complex). It was the general experience in the laboratory during the time of this project, that the Ratnoff and Menzie assay gave values lower than might have been expected. Duplicate estimations were always carried out and if these were at variance by more than 20 mg/100 ml the assay was repeated.

Because of doubts as to the accuracy of this assay, plasma fibrinogen was estimated in 28 samples from normal healthy subjects using the radial immunodiffusion method (see Chapter II.4(j)) as well as the thrombin clottable protein method. A good correlation was found between the two methods, but the immunological method consistently gave higher values than the thrombin clottable method (see Figure 13). It is likely that some loss of

fibrin occurs during the washing of the glass beads using the Ratnoff and Menzie (thrombin clottable protein) method. A discrepancy between thrombin clottable and immunoreactive fibrinogen should not, however, occur in samples from healthy subjects (Wolf, Farrell and Walton, 1972).

It has to be concluded that the thrombin clottable protein method was probably giving values which were consistently "too low", at least compared with the values obtained by the immunological method and which would have been expected from reference to the work of other groups. As relative rather than absolute values were important, it was felt that the results obtained by the Ratnoff and Menzie method could be accepted. The discrepancy in the plasma fibrinogen levels was not investigated until the project had been in progress for more than a year. Some of the samples from the early stages of the project were unsuitable for further analysis (due to age and repeated thawing-out caused by storage in a malfunctioning -20°C freezer). It was therefore decided to continue to use the Ratnoff and Menzie method for the sake of consistency.

II. 4(f) Serum Fibrinogen-Fibrin Degradation Product Estimation (Serum FDP/fdp)

This was measured by the tanned red cell haemagglutination inhibition immunoassay (T.R.C.H.I.I.) of Merskey, Kleiner and Johnson (1966), using pre-packed kits from Wellcome Research Laboratories (HA14). The result was expressed in μg/ml with reference to a standard fibrinogen preparation. The sensitivity, defined as the lowest concentration of the standard fibrinogen preparation giving a "no agglutination" reaction, was usually 1.25 μg/ml (range 0.63 - 2.5 μg/ml).

The serum FDP/fdp level was also measured by the staphylococcal clumping test (SCT) - see Chapter II. 4(l).

II.4(g) β-alanine Precipitation Technique

This was performed by the method of Graeff and von Hugo (1972), immediately following separation of the fresh plasma. 9 ml of plasma was gently mixed with β-alanine (final concentration 2.5 M) in an ice-bath (4°C) for 30 minutes. The resulting precipitate was packed by centrifugation at 9,000 g for 20 minutes. The supernatant was discarded and the precipitate redissolved in 3 ml column buffer (see below) at 20°C.

II.4(h) Agarose Gel Filtration

Biogel A5 m (mesh size 200-400) obtained from Biorad Laboratories Limited, Bromley, Kent, England (1510750) was used as the gel medium. Slight variations were found in the separation characteristics of different batches of this gel. Essentially there were two types, which will subsequently be referred to as Batches I and II. Batch I was used for the initial project work including in vitro experiments and the ancrod-treated patient group. Batch II was used for all further work including all the obstetrical groups (see Appendix I). Chromatography columns 2.6 40 cm (K26/40) from Pharmacia G.B. Limited, 75 Uxbridge Road, London were used. The gel medium was poured into the column and packed with care to ensure that the particles settled uniformly to produce an "even bed". As it is difficult to reproduce the same packed height in every gel column - any value from 34-35 cms was accepted. (The diameter of 2.6 cms remained constant). Once packed, the column bed was equilibrated overnight with buffer under standard running conditions. Before use the packing was checked using Blue Dextran 2,000 (Pharmacia). This was repeated as required to confirm that the packing was still

in good order, or to check for a suspected technical fault.

The packed gel column was always "washed-out" after use with 50-100 ml of buffer. The column buffer and gel medium were degassed before use, to prevent bubble formation within the packed gel. Sodium azide (0.015 M) was added to the column buffer to prevent bacterial contamination. Although some packed columns could be used for many protein analyses (20-30) it was found to be desirable to limit the number of analyses to 10-15 for each column. After this number of analyses it was found that technical faults tended to develop. Gel not in use was stored in buffer containing sodium azide (0.015 M) to prevent bacterial growth.

The column elution buffer (pH 7.6) contained:- tris (hydroxymethyl) methylamine 0.05M; sodium chloride 0.115 M; trisodium citrate 0.012 M; epsilon aminocaproic acid (E.A.C.A.) 0.05M; ethylene diamine tetracetic acid (E.D.T.A.) 0.02 M; aprotinin (Trasylol, Bayer), 10^5 K.I.U./l and sodium azide 0.015 M. The pH was adjusted (using 0.1 M hydrochloric acid) to 7.6.

This is based on the column buffer of Graeff and von Hugo (1972). E.A.C.A. and E.D.T.A. were added to try to prevent enzymic action on fibrinogen. Chromatography was performed at room temperature (the possibility that cooling from 37°C may induce complex formation is discussed elsewhere - Chapter I.5(d); Chapter IV.5).

The flow rate of 26 ml/hour was controlled by a constant infusion pump and 2.6 ml fractions were collected. These conditions varied slightly over the three year period of the project and so cumulative elution volumes were always recorded rather than simply the fraction number. A diagram of the apparatus is shown in Figure 14. All the equipment apart from

the column itself was supplied from L.K.B.-Producter (Box 76, Stockholm-Blomma 1, Sweden).

Constant optical density (O.D.) monitoring (at 280 nm) was performed using the Uvi-cord apparatus (spectrophotometer) and a print-out obtained on the pen-recorder. This allowed the protein containing fractions to be identified and the O.D. of these fractions was confirmed using a Beckman spectrophotometer.

II.4(i) Thrombin Clotting Time (T.C.T.) and Thrombin Clottable Protein Incubation Test (T.C.P.)

This was estimated using the method of McNicol and Douglas (1964) with minor modifications. The test system consisted of:- 0.2 ml of eluant fraction; 0.3 ml of thrombin titration mixture and 0.1 ml of thrombin (6 units/ml, Bovine Thrombin, Parke-Davis, 4-2073-1). The thrombin was added last and the clotting time recorded from that point. The thrombin titration mixture contained 6 ml of 0.9% (w/v) saline, 3 ml of 0.7% (w/v) calcium chloride and 1 ml of 0.1M tris buffer (pH 7.5). Duplicate assays were performed.

When the clotting time was in excess of 3 minutes, the test was discontinued. An identical test-system (the thrombin clottable protein incubation test or T.C.P.) was set up for each eluant fraction without mixing and incubated for 24 hours. Any evidence of clot or fibrin strand formation was regarded as a positive test.

In order to perform statistical analysis of the results two ratios were calculated. A ratio was used rather than a single elution volume in an attempt to minimise the differences between different batches of agarose gel medium.

The ratios were calculated as shown in Figure 15, using the following formulae:-

$$\text{T.C.P. ratio} = {}^{t}/_{s}$$

$$\text{T.C.T. ratio} = {}^{r}/_{s}$$

The T.C.P. ratio proved much the more useful of the two (see Chapter V.4(c)) and is the one considered in most of the thesis.

II.4(j) Radial Immunodiffusion Technique for Fibrinogen-Fibrin Related Antigen (FR-Antigen)

The FR-antigen concentration of each protein containing eluant fraction was measured using this method, adapted from that described by Mancini, Carbonara and Heremans (1965) with modifications suggested by Wolf and Walton (1965). Agar 3% w/v ("Difco" Bacto-Agar, Difco Laboratories, Central Avenue, West Molesey, Surrey, England 0141-02) was dissolved by heating in barbital-saline buffer (pH 7.2) which contained:- sodium barbitone 0.02 M; sodium chloride 0.29 M; ethylene diamine tetracetic acid (disodium salt) 0.01 M; trisodium citrate 0.012 M; epsilon aminocaproic acid 0.01 M and sodium azide 0.003 M. The pH was adjusted (using 0.01 M hydrochloric acid) to 7.2.

6 ml aliquots of this agar were used. Fibrinogen antiserum (Behringwerke-Hoechst Pharmaceuticals, Hounslow, Middlesex, England, ORCH 05) was used at a 1:120 dilution in 0.9% w/v saline (i.e. 0.05 ml antiserum in 5.95 ml saline). This was warmed to 56°C. 6 ml of dissolved agar was added to the 6 ml diluted antiserum, after cooling the agar to 60°C. The antiserum-agar mixture was then poured into a plastic petri dish (8.5 cms in diameter) placed horizontally on a levelling table and allowed to gel. Samples of eluant fractions were applied to 5 µl "holes"

punched in the gel and diffusion continued for a minimum of 48 hours at room temperature in a moist atmosphere. Assays were performed in duplicate. Clear rings were obtained by staining with 0.01% w/v tannic acid, for a few seconds, under indirect illumination. Plates were calibrated by the use of appropriate dilutions of Protein Standard Protein (P.S.P., Behringwerke-Hoechst Pharmaceuticals, OTFI, 03). Where necessary the samples were diluted in column buffer before use.

It is known that the area of the ring (once diffusion is complete) is directly proportional to the antigenic concentration (Mancini et al., 1965). Thus D^2 (where D is the diameter of the ring) will also be directly proportional to the antigenic concentration. This was confirmed in the experiment shown in Figure 16. A fibrinogen solution of known concentration (prepared from Fibrinogen, Grade L, Kabi Pharmaceuticals Ltd., Stockholm, Sweden) was diluted to give concentrations of 40, 30, 20, 10, 5 and 2.5 mg/100 ml. These dilutions were then analysed in duplicate for FR-antigen using the modified Mancini technique and D^2 measured. It can be seen that there was a very good correlation between D^2 and the known fibrinogen concentration. In this way a standard line could be drawn from which the FR-antigen concentration of each eluant fraction could be calculated. Two plates were usually set up for each gel filtration run (one for measuring range of 1-7 mg/100 ml; and one for measuring range of 7-20 mg/100 ml). The spacing of the holes and the dilution of the standards differed between the two plates, but not the antiserum concentrations.

This method did seem to work fairly well. It is possible that the concentration of FR-antigen in the soluble complex

fractions was underestimated (see Chapter III. 4 and 6). The molecules may have been too large to diffuse through the agar properly and the antigenic determinants may also have been altered (Wolf and Walton, 1965; Wolf, Farrell and Walton, 1972).

II. 4(k) Calculation of Results from Antigenic or Optical Density Elution Profiles

Fletcher's group have developed an elaborate method of interpreting their results measuring the relative concentrations of soluble complex (polymer), fibrinogen and early degradation product (first derivative). They use a computer program based on chromatographic plate theory analysis (Alkjaersig, Roy and Fletcher, 1973). This enables them to analyse even poorly resolved antigenic elution profiles and so they can use small columns which allow the analysis to be performed rapidly. A computer print-out from a sample from a patient with "post-operative thrombophlebitis" is shown in Figure 17.

In order to develop such a computer aided analysis considerable experience has to be obtained with the gel filtration technique. Such an analysis would require a high degree of technical expertise and precise knowledge of the molecular weights of the different forms of soluble complex. A simpler method of calculation was required for the present project.

A much simpler, but clearly more inaccurate method of calculating results has been suggested by Graeff's group (Hafter, Schneebauer, Tafel et al., 1975; Graeff, Wiedmann, von Hugo et al., 1976). They no longer use the radial immunodiffusion technique for the estimation of FR-antigen and now rely on optical density elution curves. They claim that optical density (OD)

readings can give an accurate estimate of the amount of FR-antigen in the soluble complex range (excluding the void volume peak, in which contaminant proteins are present) - provided the initial β-alanine precipitation step is included. The O.D. is measured at 325 nm and subtracted from the value at 280 nm. A representative example of such an elution profile is shown in Figure 18. The percentage of soluble complexes in relation to the total fibrinogen content was determined using the formula:-

$$\text{soluble complex concentration } \% = \frac{A}{A + B} \times 100$$

This type of method was adapted for this present project, but FR-antigenic curves were used because they were felt to be more accurate than O.D. curves, because of the presence of contaminating proteins even in a β-alanine precipitate. In addition much of the work to be described in this thesis had already been performed before this method of calculating results was first published (July 1975). The use of O.D. curves would, however, greatly reduce the amount of work involved in the technique and might well be worth evaluating in the future.

The method used for the present project is shown in Figure 19. The soluble complex concentration (area m) was calculated according to the formula:-

$$\frac{\text{soluble complex concentration } \%}{\text{total FR antigen}} = \frac{m}{m + n + p} \times 100$$

Similarly the degradation product concentration, mainly fibrinogen Fragment X or fibrin Fragment x (area p), is calculated according to the formula:-

$$\text{degradation product concentration \% total FR-antigen} = \frac{p}{m + n + p} \times 100$$

Clearly this method is only, at best, semi-quantitative but it was found extremely useful in separating different patient groups.

It was also felt that the elution volume at which FR-antigen could first be identified, using the radial immunodiffusion technique, might also be helpful because of slight variation between different packed gel columns the FR-antigen ratio was calculated as shown in Figure 20.

$$\text{FR-antigen ratio} = \frac{a}{b}$$

II. 4(1) The Staphylococcal Clumping Test (S. C. T.)

This was performed according to the test-tube method (Niewiarowski and Lipinski, 1970; Hawiger, Niewiarowski, Gurewich et al., 1970). Certain strains of staphylococcal aureus produce staphylococcal clumping factor. These staphylococci will "clump" in the presence of fibrinogen, fibrin monomer and early but not late degradation products. The mechanism of staphylococcal clumping may consist of paracoagulation occurring at the surface of the bacterial wall. The bacteria may be connected by bridges formed of precipited fibrinogen and its derivatives (Niewiarowski and Lipinski, 1970).

The method is shown diagramatically in Figure 21. Doubling dilutions were performed and the result expressed in terms of the lowest dilution giving a clearly positive clumping reaction. This dilution was in turn expressed as the negative logarithm to the base 2 (i. e. $1/1 \rightarrow 0.5$; $\frac{1}{2} \rightarrow 1.0$; $\frac{1}{4} \rightarrow 2$ etc). Staphylococcal clumping factor was obtained from Sigma London

Chemical Co. Ltd., Kingston-upon-Thames, Surrey, England (850-10). The sensitivity of the method, defined as the lowest concentration of a standard fibrinogen preparation giving a positive reaction was 1.8 - 3.6 μg/ml. This is slightly different from the reported values of 0.38 - 1.5 μg/ml (Hawiger et al., 1970).

After some experience with this technique it was decided that it was useful as a "screening test" for fibrinogen-fibrin related protein in the void volume fractions of the column eluate. As it was impossible to use a "standard" soluble complex preparation to quantitate the amount of material present, the result was expressed in terms of the lowest dilution giving a clearly positive clumping reaction. Minor batch to batch variation in sensitivity probably did occur, but this was accepted. In the present project it was found that the SCT was less sensitive than the T.R.C.H.I.I. in measuring serum fibrinogen-fibrin degradation product levels (see Chapter V.4(b)).

II.4(m) Statistical Methods

Three statistical techniques were used for evaluating the results:-

(i) arithmetic mean and standard deviation (ungrouped data)

(ii) "t"-statistics for significance difference in mean

(iii) linear regression analysis

These were calculated using the Wang Model 600 Programmable Calculator. Probability levels were determined using "Scientific Tables. Documenta Geigy" edited by Diem and Lentner (1970).

CHAPTER III

PRODUCTION OF SOLUBLE FIBRINOGEN-FIBRIN COMPLEXES IN VITRO BY A VARIETY OF PROTEOLYTIC ENZYMES

III. 1 Introduction

These in vitro experiments were carried out to evaluate the technique of plasma fibrinogen chromatography, set up as described in Chapter II. 4. The following points required to be verified:-

(i) that the technique did separate solutes on a molecular weight basis

(ii) that the elution position of fibrinogen remained constant for a particular column system

(iii) that soluble complexes and fibrinogen/fibrin degradation products could be separated (at least partially) from fibrinogen using the gel filtration technique

(iv) that the β-alanine precipitation step did not alter the elution characteristics of fibrinogen and that it precipitated soluble complexes and fibrinogen-fibrin degradation products.

The background literature has already been discussed in Chapters I and II.

III. 2 The Effect of Molecular Weight of the Elution Volume (V_e) of Solutes

In this set of experiments a number of purified standards

were analysed by the gel filtration technique. The same gel column was used throughout (Column D2, Batch III gel). The different solutes, their molecular weights and elution volumes (V_e), measured by method 1 (see Chapter II. 2) are shown in Table 2. The elution curves (optical density at 280 nm) for four of these solutes are shown in Figure 22. The elution volume (V_e) measured by method 1 is plotted against the logarithm to the base 10 of the molecular weight for a given solute in Figure 23.

It can be seen that there is a good negative correlation between V_e and the logarithm to the base 10 of the molecular weight ($r = -0.962$). It can also be seen from Figure 23 that fibrinogen (M.W. 340,000) is eluted near the centre of the fractionation range of Biogel A5 m, confirming that this is a good gel medium for separating fibrinogen from derivatives of molecular weight $> 1 \times 10^6$ (soluble complexes) to $< 83,000$ (Fragment D).

Using this particular gel column the V_e of fibrinogen was 105 ml. The V_e of fibrinogen was found to vary with different gel column systems. While minor variations in height of the packed gel columns occurred, the most important factor appeared to be the batch of agarose gel. This subject is discussed in detail in Appendix I.

During the initial four months of the project a packed gel column of 2.5 cms diameter and 40.0 cms height was used (total bed volume = 196 ml). Thereafter a packed gel column of 2.6 cms diameter and 35.0 cms height was used (total bed volume = 186 ml). The results obtained during the initial stages of the project therefore varied slightly from those obtained later on. This was not important as these initial results form a

separate group, which will now be discussed. This column system was labelled Column A1 (Batch I gel) to distinguish it from subsequent column systems.

III. 3 Comparison of V_e of Fibrinogen with Samples of Purified Fibrinogen, Plasma and a β-alanine Precipitate of Plasma

Purified fibrinogen (Kabi, Grade L) was analysed using the Column A1 (Batch I gel) system. A representative result is shown in Figure 24. It was obvious from comparison of the total protein (i.e. O.D. trace) and the FR-antigen profile that contaminant proteins were present. This was consistently found and was in keeping with the clottability of 90% reported by the manufacturers. The FR-antigen profile corresponded to a Gaussian curve and there was complete identity with the T.C.T. curve. The V_e of fibrinogen for this packed gel column was 96 ml. A sample of normal plasma analysed under identical conditions is also shown in Figure 24. The FR-antigenic and T.C.T. profiles overlapped as before and appeared at an identical position. The fact that fibrinogen was present at this position was confirmed by comparing plasma and serum from the same blood sample (see Figure 25). The high concentrations of other plasma proteins besides fibrinogen was clearly apparent from this experiment.

The effects of introducing the preliminary β-alanine precipitation step is illustrated in Figure 26. A sample of plasma from a healthy volunteer, a β-alanine precipitate of plasma from the same donor and the supernatant from this preparation are shown. The precipitation step clearly did not alter the elution characteristics of the fibrinogen material and it greatly reduced the amount of non-fibrinogen protein. No FR-antigen was found in the supernatant. The V_e of fibrinogen was virtually identical

in samples of purified fibrinogen, plasma and a β -alanine precipitate of plasma (Table 3). It can therefore be concluded that the V_e of fibrinogen remains constant when using the same column system and is not altered by the β -alanine precipitation step.

III. 4 The Action of Low Concentrations of Thrombin on Fibrinogen as Demonstrated by Agarose Gel Filtration

The effect of incubation of fibrinogen with low concentrations of thrombin was studied.

Method

25 mg aliquots of fibrinogen (Kabi, Grade L) were prepared and stored at -20°C. For each experiment one aliquot (10 mg/ml) was incubated with increasing, but nevertheless low concentrations of thrombin at 37°C for 5 minutes (see Table 4). Heparin was then added in an attempt to neutralise the action of the thrombin (see Table 4). It was appreciated that this was unlikely to be very effective as heparin requires antithrombin III (or heparin cofactor) in order to neutralise thrombin. Hirudin would have been a more suitable inhibitor, but was not available. Insoluble fibrin did not appear to form during the course of these experiments, although the possibility that some insoluble fibrin formation did occur in the course of gel filtration cannot be excluded. It is now appreciated that prolonged incubation ($5\frac{1}{2}$ hours) of fibrinogen and low concentrations of thrombin can be performed without insoluble fibrin formation (Bang and Chang, 1974). This would appear to be the explanation of the lack of insoluble fibrin formation in these experiments, rather than any neutralisation of the thrombin by heparin. It should be noted that the addition of heparin to fibrinogen did not produce soluble complex formation (see below). Column A1 (Batch I gel) was used.

Results

The results of this set of experiments are shown in Figure 27 (O.D. results), Figure 28 (T.C.T. results) and Figure 29 (FR-antigen results). It can be seen that a range of soluble complexes was produced following thrombin incubation and that increased concentrations of thrombin appeared to produce increased concentrations of soluble complex. The soluble complexes were readily identified by O.D. traces and by the T.C.T. assay. It appeared that the high molecular weight complex material "clotted" more rapidly than an equivalent concentration of fibrinogen. Although it proved possible to convert most of the fibrinogen into soluble complexes without insoluble fibrin formation before and presumably during the gel filtration stage (see Figure 27h), this material tended to "clot" spontaneously on standing at room temperature overnight or, more rapidly on cooling to 4^{o}C. It proved impossible to detect this material using the radial immunodiffusion (Mancini) technique, as no antigenic material at all could be identified in the 0.2 unit thrombin experiment (Figure 29h). It is possible that complete conversion of fibrinogen to fibrin monomer had occurred in this particular experiment. The radial immunodiffusion technique is therefore less suitable than the T.C.T. assay for detecting these complexes. It was therefore decided to perform both assays on all protein containing eluant fractions when analysing patient samples. More detailed comparisons of the T.C.T. and Mancini assays are shown in Figure 30.

Conclusions

From this set of experiments it was concluded that soluble complexes could be produced by the action of low concentrations of thrombin and could be partially separated from fibrinogen by

the gel filtration technique. These complexes could be identified by changes in the O.D. and the T.C.T. elution curves. The Mancini technique was not a satisfactory method of identifying the very high molecular weight soluble complexes (polymers with V_e close to V_o).

III.5 The Action of Plasmin on Fibrinogen Demonstrated by Agarose Gel Filtration

Fibrinogen (Kabi, Grade L) was incubated with streptokinase for varying lengths of time to activate plasminogen to plasmin and the resultant mixture analysed by the gel filtration technique.

Method

10 ml of fibrinogen (10 mg/ml) was incubated with 1,000 units of streptokinase (Varidase, Lederle 5-2200) at 37°C. At 10, 20, 30, 40 and 120 minutes, a 2 ml sample was removed and pipetted into small plastic tubes containing 0.2 mgm of Soybean Trypsin Inhibitor (Sigma T. 9003) to inhibit further proteolysis. Each sample was immediately frozen in ethanol-dry ice and stored at -20°C. Samples were then analysed in turn by the gel filtration technique. Column A1 (Batch I gel) was used.

Results

The results of this set of experiments are shown in Figure 31. Only O.D. traces were obtained, as the aim of the experiment was simply to confirm that fibrinogen degradation products could be partially separated from fibrinogen using gel filtration (Marder, Shulman and Carroll, 1969). The results obtained were similar to those already published by this group. The positions of fibrinogen and fibrinogen Fragments X, Y, D and E expected from the study of Marder and colleagues (1969) are shown in Figure 31.

Conclusion

It was possible to demonstrate gradual conversion of fibrinogen into fibrinogen degradation products using the gel filtration technique.

III. 6 The Action of Coagulant and Fibrinolytic Enzymes on Plasma Fibrinogen - The β-Alanine Precipitation Step

A pool of normal plasma was collected from 8 healthy male donors. The anticoagulant on this occasion did not contain E.A.C.A. or aprotinin as digestion by plasmin was required for several experiments. The plasma was divided into 9 ml aliquots and stored until required at -20°C.

Ancrod was used instead of thrombin for this series of in vitro experiments as the results were to be compared with those obtained during ancrod infusion in vivo (Chapter IV. 4). Ancrod (Arvin, Berk Pharmaceuticals Ltd., Shalford, Sussex, England) is the thrombin-like defibrinating agent derived from the venom of the Malayan pit viper (Agkistrodon or Ancistrodon rhodostoma). A full discussion of its actions will be found in Chapter IV. 2. The aims of this set of experiments were:-

(i) to show that soluble complexes produced in plasma could be precipitated with β-alanine
(ii) to show that early fibrinogen degradation products produced in plasma could be precipitated with β-alanine
(iii) to investigate the effect of combined action of coagulant and fibrinolytic enzymes on plasma (i.e. ancrod and plasmin respectively).

Methods

These are outlined in Table 5. Incubation of plasma with enzymes was carried out in plastic test-tubes in a 37°C water-bath. Inhibitors were added after 1 minute in the case of ancrod and 20 minutes in the case of streptokinase. In experiment c the T.C.T. of the incubation mixture was prolonged by 7 seconds (from 18 to 25 seconds) demonstrating conversion of fibrinogen to early degradation products. In each case β-alanine precipitation of the fibrinogen related protein was carried out as already described (Chapter II. 4(g)) and gel filtration performed. Column B4 (Batch II gel) was used.

Results

The O.D. results are shown in Figure 32, the T.C.T. results in Figure 33 and the Mancini results in Figure 34. The overlapping T.C.T. and FR-antigen curves are shown in Figure 35. It can be seen that soluble complexes were produced in experiments b and d and probably also in experiment e. It proved difficult to obtain satisfactory data for experiment e as very low concentrations of complex were produced. It is possible that a dimer formed from the degraded material.

As in the set of experiments described in Chapter III. 4 the O.D. and the T.C.T. curves were good methods of identifying the presence of soluble complexes but the radial immunodiffusion (Mancini) technique seemed to underestimate the quantity of soluble complex present (especially in experiment b).

The structure of the component units of the soluble complexes was investigated by Mr. William Edgar (University Department of Medicine, Glasgow Royal Infirmary) using polyacrylamide gel electrophoresis in sodium dodecyl sulphate (Weber and Osborn, 1969). After cleavage of the disulphide bonds linking the chains of the

component units using mercaptoethanol, it was shown that the soluble complexes formed in experiment b had intact α chain and those produced in experiments d and e were markedly deficient in intact α chain, with some samples showing partial loss of intact α -chain.

These experiments demonstrated:-

(i) that soluble complexes and early fibrinogen degradation products formed in vitro can be precipitated by β -alanine and subsequently demonstrated by the gel filtration technique.

(ii) that soluble complexes can contain partially degraded material.

(iii) that an early fibrinogen digest can only form soluble complexes after treatment with a coagulant enzyme.

Soluble Complex Formation Following the Lysis of Insoluble Fibrin

One further experiment was performed to confirm that low concentrations of soluble complex could be formed following the lysis of insoluble fibrin.

Method

10 ml of plasma was prepared using 0.13 M trisodium citrate as anticoagulant. This was cooled to 4°C in an ice-bath and 100 units of thrombin (0.1 ml volume) added (i.e. 9.9 units/ml). As the clot started to form 25 units of partially purified plasmin (Kabi 67233, volume 0.2 ml) was added (i.e. 2.4 units/ml). The clot was then incubated at 37°C and inspected for lysis, which was complete at 45 minutes. Digestion was continued for a further 10 minutes and then

10,000 K.I.U. (1 ml volume) of aprotinin was added to inhibit further proteolysis (i.e. final concentration 885 K.I.U./ml). The reaction mixture was precipitated with β-alanine and gel filtration performed. Column B10 (Batch II gel) was used for this experiment.

Results

Very low concentrations of fibrinogen related protein were recovered (Figure 36), however, the mixture clearly contained soluble complexes and degradation products. The void volume fraction formed small wisps of "fibrin" on prolonged incubation with thrombin (24 hours). It also had a staphylococcal clumping test titre of ≥ 8.

It can therefore be concluded that low concentrations of soluble complex can be formed in an early insoluble fibrin lysate. Similar results have been reported (von Hugo and Graeff, 1973).

III.7 Conclusions

These experiments confirmed the points outlined in the introduction to this chapter and confirmed the validity of the gel filtration technique for the separation of fibrinogen and its proteolysis products.

(i) The technique separated solutes on a molecular weight basis.

(ii) The V_e of fibrinogen remained a constant for a particular column system. There was some variation between different packed gel columns (see Appendix I).

(iii) Soluble complexes and fibrinogen/fibrin degradation products were partially separated from fibrinogen using the technique.

(iv) The β-alanine precipitation step did not alter the V_e of fibrinogen. β-alanine precipitated soluble complexes and degradation products as well as fibrinogen.

The biochemistry and structure of the soluble complexes was not studied in detail, as this thesis is concerned with the identification of these complexes in clinical conditions rather than their composition. It was noted that the action of a coagulant enzyme (thrombin or ancrod) was required for the production of soluble complexes. Three methods of production were demonstrated:-

(1) the incubation of fibrinogen with low concentrations of a coagulant enzyme
(2) the incubation of early fibrinogen degradation products with a coagulant enzyme
(3) the lysis of insoluble fibrin.

Only low concentrations were produced by methods 2 and 3.

CHAPTER IV

SOLUBLE FIBRINOGEN-FIBRIN COMPLEXES DURING THERAPEUTIC DEFIBRINATION INDUCED BY ANCROD INFUSION

IV.1 Introduction

The acute defibrination state induced by infusion of ancrod (a purified fraction of venom from the Malayan Pit Viper) provides a means of studying fibrin formation in vivo. It must, however, be remembered that this form of widespread intravascular coagulation differs clinically from other forms because of its apparently benign nature.

It had already been demonstrated that soluble complexes could be produced in vitro by incubation of plasma with ancrod (Chapter III. 6). It therefore seemed probable that soluble complexes would also form in vivo during the initial stages of ancrod therapy. While the present study was in progress two papers were published showing soluble complex formation in vivo during treatment with Defibrase (Reptilase) a rather similar defibrinating agent also derived from a snake venom (Blättler, Straub and Peyer, 1974; Asbeck, Lechler and Martin et al., 1975).

A group of patients receiving ancrod (Arvin) infusion were therefore studied to confirm that the gel filtration technique could identify soluble complexes produced in vivo. It was hoped also to learn more about the formation and structure of these soluble complexes.

In order to evaluate the results that were obtained it is necessary to first discuss what is known about the actions of ancrod *in vitro* and *in vivo*.

IV.2 The Effects of Ancrod

IV.2(a) Historical Introduction

Since Fontana noted in 1787, that the blood remained fluid in animals that had died following the bite of certain species of viper, the relation of snake venoms to coagulation and haemorrhage has been studied by many workers. Mellanby (1909) suggested that viper venom defibrination was caused by *in vivo* by conversion of fibrinogen to fibrin which was then removed from the circulation.

Apart from local pain and swelling patients bitten by the Malayan pit viper (Agkistrodon or Ancistrodon rhodostoma) remain remarkably well (Reid, Chan and Thean, 1963; Reid and Chan, 1968). Serious haemorrhage does not occur even the severest cases *despite* totally incoagulable blood. These observations suggested that the purified coagulant fraction of the snake venom might be useful as an "anticoagulant" in the treatment of thrombotic disease. This fraction of the venom was isolated and studied by Esnouf and Tunnah (1967). The approved name of this fraction is "ancrod". It is manufactured under the trade name "Arvin" by Berk Pharmaceuticals Ltd. (Shalford, Surrey, England). The term "ancrod" will be used throughout this chapter.

A similar venom derivative "Defibrase" (or "Reptilase"), is manufactured by Pentapharm Ltd. (Basel, Switzerland). It is derived from the venom of Bothrops atrox and is also used therapeutically as a defibrinating agent.

IV. 2(b) The Action of Ancrod on the Coagulation Pathway

Ancrod has a thrombin-like action on fibrinogen in vitro releasing fibrinopeptide A and two small fibrinopeptide fragments AP and AY from each A α chain, but unlike thrombin ancrod does not release fibrinopeptide B (Holleman and Coen, 1970; Ewart, Hatton, Basford et al., 1970). It has been suggested that removal of fibrinopeptide A allows end-to-end polymerisation while side-to-side or lateral aggregation occurs only when fibrinopeptide B is also removed (Laurent and Blömback, 1958).

Ancrod can cause more extensive digestion of the α chain in vitro releasing a single polypeptide (or a group of polypeptide fragments) with a (total) molecular weight of 31,000, the remaining portion of the α chain having a molecular weight of 39,000 (Mattock and Esnouf, 1971; Edgar and Prentice, 1973). It is not known whether this α chain digestion occurs in vivo during therapeutic defibrination but a Cohn Fraction I preparation of pooled plasma from patients during a course of ancrod therapy did demonstrate extensive loss of intact α chain (Barlow, Lazer, Finley et al., 1973). It was suggested that this was caused by ancrod itself but could, of course, also have been caused by other proteolytic enzymes (e. g. plasmin). It is probable that the concentration of ancrod in the plasma of such patients is too low to produce extensive α chain digestion (Gaffney and Brasher, 1974).

It is possible that commercial preparations of ancrod activate other coagulation factors e. g. Factor XIII (Mattock and Esnouf, 1971). These additional effects may be due to impurities in the preparation rather than due to the ancrod itself.

IV. 2(c) The Action of Ancrod on Platelets

Ancrod therapy does not appear to alter platelet numbers, their ability to adhere to glass or to aggregate under the influence of

adenosine diphosphate (Bell, Pitney, Goodwin, 1968). A later report demonstrated a diminished response to adenosine diphosphate induced platelet aggregation during the first 24 hours of therapy (Prentice, Hassanein, Turpie et al., 1969). This was attributed to the very high levels of fibrin degradation products at this stage of therapy and not to a direct action of ancrod. It has been confirmed that the fibrin degradation products formed from ancrod-fibrin in vitro do inhibit both the first and second phases of adenosine diphosphate induced platelet aggregation (Kwaan, Barlow and Suwanwela, 1973).

IV. 2(d) The Action of Ancrod on the Fibrinolytic Pathway

In vitro studies have shown that ancrod does not activate plasminogen directly or enhance the activator effect of streptokinase on plasminogen, in addition, ancrod has no direct lytic activity on fibrin plates and no significant lytic effect on experimental thrombi in the Chandler tube (Turpie, Prentice, McNicol et al., 1971).

However, when ancrod is administered to patients there is a rapid fall in plasma plasminogen concentration (Bell et al., 1968). There is also a minimal, but inconstant increase in the circulating levels of plasminogen activator but circulating plasmin cannot be detected (Pitney, Bell and Bolton, 1969). Increased tissue fibrinolytic activity in the lung and liver has been observed using histochemical techniques following ancrod injection in mice (Silberman, Potter and Kwaan, 1971). It has been suggested that both plasminogen and activator are adsorbed onto the surface of fibrin micro-clots produced by ancrod with local release of plasmin causing lysis of the fibrin and the appearance of fibrin degradation products (Pitney et al., 1969).

The insoluble fibrin produced by ancrod may be more susceptible to plasmin induced lysis than the insoluble fibrin produced by thrombin (Kwaan and Barlow, 1971; Kwaan et al., 1973). The lower molecular weight fibrin degradation products are produced more rapidly from ancrod-fibrin than thrombin-fibrin. The increased susceptibility to lysis was shown not to be related to the fact that the ancrod-fibrin was non-crosslinked and it therefore was suggested that the lack of side-to-side polymerisation in the ancrod-fibrin (due to the presence of fibrinopeptide B) left the bonds susceptible to plasmin lysis more readily available for cleavage (Kwaan et al., 1973). In addition ancrod itself might attack the α chain if present in high enough concentration, although this is unlikely in the therapeutic situation (Gaffney and Brasher, 1974). The actual structure of the fibrin degradation products formed during ancrod therapy is similar to those produced by plasmin on fibrinogen (Prentice, Edgar and McNicol, 1974).

The relevance of these observations to fibrinolysis in patients undergoing therapeutic defibrination remains to be assessed. Fibrinolysis is presumably very important in such patients, as animals treated with E.A.C.A. or soybean trypsin inhibitor prior to ancrod injection show extensive intravascular clot formation at post-mortem (Regoeczi, Gergely and McFarlane, 1966) and if they survive long enough develop microangiopathic haemolytic anaemia (Rubenberg, Regoeczi, Bull et al., 1968).

IV.2(e) The Action of Ancrod on the Reticuloendothelial System

Ancrod also appears to cause a rapid, pronounced stimulation of the reticuloendothelial system in rabbits (Ashford and Bunn, 1970). This appears to be unrelated to its coagulant action on fibrinogen and might involve another venom derivative besides ancrod. This stimulant effect may involve a direct action on phagocytic cells

and an effect on colloidal particles via serum opsonins. It is not known whether a similar action occurs in man but there may be a species variation in this respect, as conflicting results have been published using crude venom in the dog (Esnouf and Marshall, 1968).

IV. 2(f) Comparison of Ancrod and Defibrase (Reptilase)

The action of Defibrase (Reptilase) on fibrinogen is very similar to that of ancrod, in that it also removes fibrinopeptides A, AP and Y but not fibrinopeptide B (Blömback, 1958). It seems to have a similar in vivo action to ancrod in experimental animals (Egberg and Nordström, 1970) and when administered therapeutically to human patients (Egberg, 1973). F XIII concentrations fall during Defibrase therapy (Egberg, 1973), and it would appear that activation of F XIII is more pronounced than during ancrod therapy.

IV. 2(g) The Action of Ancrod in Producing Therapeutic Defibrination

On the basis of studies in rabbits using crude venom and radioactivity labelled fibrinogen, Regoeczi and colleagues (1966) suggested that the venom rapidly converted intravascular fibrinogen into insoluble fibrin micro-clots, which temporarily were trapped in the smaller vessels of various organs. These micro-clots were demonstrated both by conventional histology and by autoradiography. It was thought that they were rapidly lysed by the fibrinolytic system.

It has been generally accepted that a similar sequence of events occurs during therapeutic defibrination induced by a slow intravenous infusion of ancrod (Pitney et al., 1969; Prentice et al., 1974). The slow rate of ancrod infusion is thought to result in the formation of micro-clots at a rate, which is well within the capacity of the defense mechanisms to remove them from the circulation.

The relative and interdependent roles of the reticuloendothelial and fibrinolytic systems in this respect are incompletely understood.

Local release of plasminogen activator from the vascular endothelium would seem to be important (Pitney et al., 1969; Silberman et al., 1971). The ease with which ancrod micro-clots are cleared from the circulation probably reflects the instability of fibrin which retains fibrinopeptide B (Kwaan et al., 1973). The lack of a direct action of ancrod on platelets and the impaired platelet aggregation during ancrod therapy may prevent incorporation of platelets into forming micro-clots contributing to their instability (Prentice et al., 1969).

More recently it has been suggested that, in the therapeutic situation, ancrod does not cause the deposition of insoluble fibrin (Gaffney and Brasher, 1974). It was instead suggested that the fibrinolytic system attacked the fibrin while it was still in the soluble form. Gaffney and Brasher (1974) produced indirect evidence to suggest that ancrod in therapeutic concentrations converts prothrombin to thrombin in vitro and that the thrombin in its turn activates Factor XIIIa. These authors have never observed D-dimer or γ-γ dimer in the plasma of patients treated with ancrod and they therefore suggested that the fibrin formed by ancrod is digested by the fibrinolytic system while it is still soluble before there is time for crosslinking and insoluble fibrin deposition to occur (Figure 37).

Much of this theory remains unproven but it presents an interesting alternative pathway for the formation of fibrin degradation products during ancrod therapy and may have wider implications. A number of animal experiments support this theory. Ancrod infusion, in a dose of 1.5 U/Kg, does not appear to generate histologically demonstrable glomerular micro-clots in rabbits (Muller-Berghaus and Hocke, 1972). Detailed studies of the microcirculation in the mesentery, intestinal wall and thin abdominal muscles, in rabbits,

cats and rats (Hauck, 1975) failed to show any disturbance during ancrod infusion in "therapeutic" doses (2-3 units/Kg). Only when the dose of ancrod was increased (4-8 U/Kg) did micro-clot formation occur.

Therefore it would seem possible that micro-clots do not form when low doses of ancrod are administered. This would help to explain why the acute defibrination state induced by ancrod infusion is so "benign". Whether the micro-clots fail to form because of selective rapid fibrinolysis of soluble fibrin (complexes) according to Gaffney and Brasher's theory or because of some other defense mechanism (e.g. the reticuloendothelial system) remains uncertain. The work of Konttinen, Lalla and Turunen (1973) does suggest that the fibrinolytic system has an essentially higher affinity for fibrin monomer than fibrinogen. Streptokinase was used as the plasminogen activator in these experiments and extrapolation of their conclusions to the in vivo situation has been criticised by Lipinski and Gurewich (1976a) because of the differences between natural and artificial plasminogen activators (see discussion Chapter I. 3(b)). The work of Gurewich, Lipinski and colleagues is certainly opposed to the rapid selective lysis of soluble fibrin suggested by Gaffney and Brasher (1974).

The initial stages of defibrination induced by ancrod infusion therefore remain incompletely understood. It is not certain whether insoluble fibrin micro-clots form in this situation but, nevertheless, it provides a clinical model of widespread intravascular coagulation according to the definition used for this thesis. The opportunity was therefore taken to study 7 such patients using the plasma fibrinogen chromatography technique.

IV. 3 Design of Clinical Study

The clinical details for the seven patients studied are summarised in Table 6. There were five males and two females in the group with

an age range of 22-72 years. Four patients were suffering from established local venous thrombosis and three from severe peripheral arterial disease. Ancrod (Arvin, Berk Pharmaceuticals Limited, Shalford, Sussex, England) was administered as a constant intravenous infusion in a dose of 2 units/Kg body weight/12 hours. No anticoagulant or fibrinolytic agent was given during the period of study. Blood samples were collected from all patients before starting treatment and after 6 and 24 hours ancrod infusion. In some cases additional blood samples were collected. Columns B1 (Batch I gel), B2 (Batch I gel) and B3 (Batch I gel) were used for this study.

IV.4 Results

IV.4(a) Clot Quality Observation Test (Chapter II.4(d))

In all of the patients the pre-treatment samples were normal (Grade 1 clot). After 6 hours ancrod infusion the clot remained Grade 1 or 2. After 24 hours infusion the blood was completely incoagulable (Grade 5).

IV.4(b) Plasma Fibrinogen and Serum FDP/fdp levels

The plasma fibrinogen levels fell sharply following the start of ancrod infusion (Table 7). Significantly lower levels were found after 6 hours therapy. After 24 hours therapy the assay recorded virtually zero levels. There was, however, a degree of individual variation in the rate at which the patients reached very low fibrinogen levels e.g. Patients 3 and 7 were almost completely "defibrinated" after 6 hours therapy.

The serum FDP/fdp levels, as measured by both the tanned red cell haemagglutination inhibition immunoassay (Table 8a) and the staphylococcal clumping test (Table 8b), showed a highly significant rise 6 hours after the start of ancrod infusion. There was no significant difference in the results recorded after 6 and 24 hours ancrod therapy. There was a highly significant positive correlation

($r = 0.964$; $2 \propto < 0.001$) between the two methods for measuring serum FDP/fdp.

IV.4(c) Plasma Fibrinogen Chromatography

The optical density tracings for a typical patient (W. McG. No. 7) are shown in Figure 38. It can be seen that as the amount of protein in the elution position of fibrinogen decreased there was a corresponding increase in protein in the elution position of soluble complexes. It was necessary, however, to confirm that this material was fibrinogen-related.

The FR-antigen elution profiles from two typical patients are shown in Figure 39a and b. It can be seen that after 6 hours ancrod infusion a range of soluble complexes and degradation products had formed. After 24 hours ancrod infusion very little fibrinogen-related protein remained but soluble complexes and degradation products were still present.

Overlapping T.C.T. and FR-antigen curves for two further patients after 6 hours treatment are shown in Figure 40. The fractions showing clot formation after 24 hours incubation with thrombin (T.C.P.) are also indicated. It can be seen that, as in the in vitro experiments, the soluble complexes will clot with thrombin (Chapter III.4 and 6) although the very short times observed in vitro were not found in vivo. A double peak was invariably noted in the T.C.T. curve. After 24 hours ancrod infusion no fibrinogen-related protein with a recordable T.C.T. was identified.

It is probable that the radial immunodiffusion (Mancini) technique underestimated the amount of soluble complex present to some extent, but comparison with the O.D. traces suggested that this was less marked than in the case of the in vitro experiments (Chapter III.4 and 6). The FR-antigen concentration on the void volume (V_o) fractions in samples from 4 patients measured by the radial immunodiffusion (Mancini) technique and by the SCT titre

(Chapter II. 4(l)) are shown in Tables 9a and b. There was a significant positive correlation ($r = 0.804$; $2 \propto \langle 0.01$) between the results measured by the two methods. It can be seen that the FR-antigen concentration, measured by both methods rose significantly after 6 hours ancrod infusion, but that there was no significant difference between the 6 and 24 hour values. It will also be noticed that one patient (No. 6) had a high pre-treatment value.

Areas m, n and p (Chapter II. 4(k)) were measured in samples from 5 patients. (The remaining two patients were studied very early in the project and the separation was not good enough for analysis). It can be seen from Tables 10, 11 and 12 that, after 6 hours ancrod infusion, there were significant increases in area m and area p values and a significant decrease in area n values. There were no significant differences between the 6 and 24 hour measurements. This was because percentage rather than absolute values were used for, clearly, the absolute values had fallen after 24 hours treatment (see Figure 39). There was a degree of individual variation in the pattern of changes found in the five patients. This becomes more obvious on looking at the scatter of results for areas m and p shown in Figures 41 and 42.

The FR-antigen ratio (Chapter II. 4(k)), the T. C. P. ratio (Chapter II. 4(i)) and the T. C. T. ratio (Chapter II. 4(i)) were also calculated and the results are shown in Tables 13, 14 and 15 respectively. The FR-antigen ratio fell significantly after 6 hours ancrod infusion, but there was no significant difference between the 6 and 24 hour results (Table 13). The T. C. P. ratio did not alter significantly after 6 hours treatment (Table 14) but the T. C. T. ratio did show a highly significant decrease (Table 15). (It was not possible to calculate results for the T. C. P. or T. C. T.

ratios on the 24 hour results as no recordable T.C.T. was found in any fractions).

It can therefore be concluded that a range of soluble complexes are produced during ancrod infusion. Additional information about these soluble complexes was obtained by Mr. William Edgar, who studied their component units using polyacrylamide gel electrophoresis in sodium dodecyl sulphate (see Figures 43 and 44). The component units had a molecular weight slightly less than that of fibrinogen, similar to a minimally degraded early fibrinogen Fragment X or fibrin Fragment x (Figure 43). After reduction of the disulphide bonds with mercaptoethanol it was found that the units in the soluble complexes lacked intact α chain (Figure 44). These soluble complexes were therefore similar to those produced in vitro by the action of both ancrod and plasmin on the fibrinogen of plasma (Chapter III. 6). It is worth also noting that no α chain fragment of molecular weight 39,000 was identified, suggesting that ancrod was not responsible for the α chain digestion. In addition no γ-γ dimer or α polymer was found, suggesting that no crosslinking by F XIIIa had occurred.

IV. 5 Discussion

This study demonstrated that ancrod infusion results in the formation of a range of soluble complexes in vivo. These complexes were readily demonstrated using the plasma fibrinogen chromatography technique. This was encouraging, as it suggested that soluble complexes formed in other clinical conditions might also be demonstrated using this method.

The possibility that these soluble complexes were formed in vitro has to be seriously considered. A low concentration of ancrod would be present in the blood sample. Thrombin or plasmin

might also have been formed due to activation of precursors at the time of blood sampling. Inhibitory concentrations of ancrod antiserum, E.A.C.A. and aprotinin were added with the anti-coagulant used in preparing the plasma, but these may not have neutralised active enzyme immediately for even a delay of a few seconds might have been sufficient to allow some enzyme action in vitro.

It is also possible that cooling from 37°C was responsible for the soluble complex formation as suggested by Blättler and colleagues (1974) with reference to soluble complexes formed during Defibrase therapy. It is true, however, that soluble complexes were not found in high concentrations in plasma samples from normal individuals (Chapter V.4(c)). In the in vitro experiments soluble complexes were only formed following the action of a coagulant enzyme (Chapter III.7). These observations would suggest that the presence of soluble complexes in plasma does reflect the action of a coagulant enzyme in conversion of fibrinogen to soluble fibrin monomer. Whether this soluble fibrin monomer takes part in soluble complex production in vivo at 37°C remains unproven, but it is clearly important to appreciate that it may not do so.

Soluble complexes deficient in intact α chain could theoretically be produced in a number of ways:

(i) Digestion of α chain by ancrod itself

High concentrations of ancrod progressively attack the α chain of fibrinogen in vitro (Mattock and Esnouff, 1971; Edgar and Prentice, 1973), however, the concentrations of ancrod required to produce this digestion are much higher than those likely to be present therapeutically (Gaffney and Brasher, 1974). In addition, if ancrod were responsible for the α chain digestion the α chain fragment of M.W. 39,000 should be present (Edgar and Prentice,

1973) and this was not identified in the present study.

It would therefore seem more probable that the α chain digestion was caused by some other proteolytic enzyme. In view of the marked plasminogen depletion which occurs during the early stages of ancrod infusion (Pitney et al., 1969) it seemed likely that plasmin was responsible for the α chain loss.

(ii) Digestion of the α chain by plasmin

The method of plasminogen activation during ancrod therapy is unknown. While plasmin will attack fibrinogen, soluble fibrin monomer and insoluble fibrin in vitro, it is uncertain whether it will also attack all these substrates in vivo (see discussion on fibrinogenolysis/fibrinolysis in vivo, Chapter I. 3(c)). The component units in the soluble complexes were similar to early fibrinogen Fragment X or fibrin Fragment x. Presumably at least some of the units lacked fibrinopeptide A (due to ancrod action) or they would not have been able to form complexes. Early fibrin Fragment x could be formed in such circumstances by:

(a) the action of ancrod on early fibrinogen Fragment X, which was formed by the action of plasmin on fibrinogen.

(b) the action of plasmin on insoluble fibrin micro-clots, which were formed by the action of ancrod on fibrinogen.

(c) the action of plasmin on soluble fibrin monomer (either uncomplexed or complexed), which was formed by the action of ancrod on fibrinogen.

The early fibrin Fragment x could then circulate in complex form until further digestion by plasmin destroyed the remaining polymerisation sites (which must be on the β chain). The complex would then break up into uncomplexed fibrin degradation products (Figure 45).

The results of the experiments described in this chapter cannot distinguish between these pathways. All three pathways can be shown to operate in vitro (Chapter III. 6). Although (a)

and (b) only caused small amounts of soluble complex in these *in vitro* experiments it is possible that *in vivo* with a constant supply of native fibrinogen, this would enter the complexes and might increase their concentration (Fletcher and Alkjaersig, 1972).

IV. 6 Conclusions

It can be concluded that the gel filtration technique (set up as described in Chapter II. 4) will demonstrate soluble complexes in plasma samples from patients in whom intravascular coagulation was induced by ancrod infusion. These soluble complexes might not be present *in vivo* but appear to reflect enzymic action by a coagulant enzyme. Steps were taken to try to prevent any *in vitro* enzymic action although this cannot be completely excluded.

The soluble complexes, which were formed, could have derived from either soluble or insoluble non-crosslinked fibrin. They certainly could have formed without the production of insoluble micro-clots according to the theory of Gaffney and Brasher (1974). It is not yet certain whether F XIII activation occurs during ancrod therapy and, if it does, whether F XIIIa will only crosslink *insoluble* fibrin as suggested by these authors. If these suppositions are correct, the results described in this chapter would be in favour of their theory that micro-clots do not form during therapeutic ancrod infusion. The theory remains highly speculative.

CHAPTER V

SOLUBLE FIBRINOGEN-FIBRIN COMPLEXES IN NORMAL PREGNANCY AND PRE-ECLAMPSIA

V.1 Introduction

The central aim of the fibrinogen chromatography project was to study plasma samples from women with pre-eclampsia and this work will be described in the next two chapters. It has been suggested that intravascular coagulation plays a part in the pathogenesis of pre-eclampsia and the background literature in support of this idea will be reviewed in the first part of this chapter (V. 2). In the second part of this chapter (V. 3 and 4) the initial study in the present project will be described. In this study plasma samples from 10 pre-eclamptic patients are compared with those from 10 age, parity and gestation matched normal pregnant women and 10 age matched non-pregnant women.

Pre-eclampsia is a useful clinical model because patients can be followed through their illness to the phase of recovery during the puerperium. The results of sequential studies in six pre-eclamptic patients will be discussed in detail in Chapter VI. 2.

In Chapter VII. 2 the plasma fibrinogen chromatography findings in normal pregnancy will be presented in more detail. The results from samples from patients suffering from conditions closely related to pre-eclampsia will also be described. These related

conditions include intrauterine growth retardation (Chapter VII. 3), essential hypertension in pregnancy (Chapter VII. 4) and also chronic renal disease in pregnancy (Chapter VII. 5). Conclusions from the obstetrical studies and from a further study in women taking oestrogen-containing oral contraceptive therapy (Chapter VIII. 2) will be discussed together in Chapter IX.

Pre-eclampsia is an interesting obstetrical syndrome, which, although it has engaged the attention of research workers with widely varying interests for many years, nevertheless remains a "disease of theories" of unknown primary aetiology (Jeffcoate, 1966). Confusion surrounds even the nomenclature and definition of this syndrome. The old term "toxaemia of pregnancy" was criticised by Strauss (1939) as "a diagnostic waste basket" and the same criticism could probably be raised to the use of the term "pre-eclampsia" today.

Hippocrates in the 5th century B.C. noted that "drowsiness, fits and coma" were of serious prognostic importance in a pregnant woman, but the actual term "eclampsia" was first introduced by Varandeus in 1619. It is derived from the Greek word for a "flash" because many such patients complain of seeing "flashing lights" before the onset of a seizure. In 1843 Lever reported that proteinuria was present in patients with eclampsia and this led to the recognition of a "pre-eclamptic state".

It was believed for many years that the placenta manufactured a specific toxin, which was responsible for both pre-eclampsia and eclampsia (Holland, 1909) and this concept led to the use of the terms "toxaemia of pregnancy" or "pre-eclamptic toxaemia (P.E.T.)". A toxin has never been discovered, so it is preferable to use the term "pre-eclampsia" to describe a clinical state which can potentially progress to eclampsia.

A precise, generally acceptable definition of pre-eclampsia has not yet been reached and different clinical criteria have been used (Campbell, 1976). The main features of this syndrome are systemic hypertension, proteinuria and oedema, which develop during pregnancy and resolve following delivery. The differences in definition lie in the degree of abnormality required to make a diagnosis. Oedema is difficult to quantitate and is, in any case, present in 40% of normal pregnant women (Thomson, Hytten and Billewicz, 1967). The diagnosis of pre-eclampsia is usually made, therefore, on the basis of sustained hypertension and proteinuria developing during the second half of pregnancy (Page, 1972).

An editorial in The Lancet (1975) suggested the use of the term "hypertensive disease of pregnancy" to avoid the "largely semantic debate over whether a patient is "toxaemic" or not. Such a group, which would include patients with pre-existing hypertension and chronic renal disease, would be even more heterogenous than that included under the term "pre-eclampsia".

In the present project pre-eclampsia was defined as the development of a diastolic blood pressure of greater than 90 mm of mercury, on at least two occasions separated by 24 hours, and proteinuria in excess of 0.5 g/24 hours. All the patients were normotensive and did not have detectable proteinuria at their first ante-natal visit (at 10-14 weeks gestation). The signs developed after the 24th week of pregnancy and resolved following delivery (although in some cases this took several months). These patients would all conform to the definition of "severe pre-eclampsia" as outlined in the British Perinatal Mortality Survey (Butler and Bonham, 1963).

Although many biochemical and other abnormalities have been demonstrated in association with pre-eclampsia, it has not

yet been possible to identify the primary factor (or factors) responsible for the syndrome. Definite abnormalities found in association with pre-eclampsia (in addition to those suggestive of intravascular coagulation discussed in Chapter V.2) include disturbances in the renin-angiotensin system, in the immunological response to pregnancy and in carbohydrate and protein metabolism.

In normotensive pregnancy renin values rise to the very high levels usually associated with a hypertensive response (Weir, Brown, Fraser et al., 1973). The reason for this resistance to renin in normal pregnancy is not yet understood. Paradoxically it has been shown that plasma renin, renin substrate, angiotensin II and aldosterone are all decreased in pre-eclampsia falling towards the non-pregnant values (Weir et al., 1973). The suggested explanation was that an unknown pressor agent was suppressing the renin-angiotensin-aldosterone mechanism and resulting in the hypertension found in pre-eclampsia. This work has not been confirmed in a more recent study (Symonds, Broughton Pipkin and Craven, 1975), in which elevated angiotensin II levels were found in pre-eclampsia. In this study a significant positive correlation was established between the diastolic blood pressure at the time of sample collections and the plasma angiotensin II levels. The reasons for the discrepancies between these two studies are not yet apparent.

Evidence in favour of an immunological cause of pre-eclampsia has been supported by the demonstration of immuno-globulins and complement in the placental bed (Kitzmiller and Benirschke, 1973), renal glomeruli (Petrucco, Thomson, Lawrence et al., 1974) and hepatic sinusoids (Arias and Mancilla-Jimenez, 1976). It is not known, however, whether the deposition of immunoglobulins and complement indicates active involvement in

the pathogenesis of pre-eclampsia, because these proteins could have been passively trapped as part of a separate pathological process. A recent study has failed to show evidence of complement activation in the plasma of pre-eclamptic patients (Thomson, Stevenson, Behan et al., 1976) and therefore does not support the idea that circulating immune complexes are involved in the primary aetiology of pre-eclampsia.

Abnormalities in carbohydrate metabolism with low fasting plasma glucose levels and low fasting plasma placental lactogen levels have been found in association with pre-eclampsia (Singh, 1976). Plasma uric acid levels are higher in pre-eclamptic patients than in normal pregnant women, and the rise seems to occur usually about 3 weeks before the onset of proteinuria (Redman, Beilin and Bonnar, 1976). The seromucoid fraction of maternal serum glycoproteins is increased in pre-eclampsia (Good, 1975) and this may reflect "progressive placental deterioration with increasing trophoblastic fragmentation and deportation".

It is probable that all these effects are secondary and are not related to the primary pathology of the pre-eclamptic syndrome. The various theories of the pathogenesis of pre-eclampsia need not be mutually exclusive. Page (1972) with his "vicious circle of pre-eclampsia and eclampsia" linked many of these findings together. It should, however, be stressed that pre-eclampsia is a syndrome which may encompass several primary disease processes. Theobald (1974) warned against the dangers of trying "to pinpoint one lesion as being the cause of, or always being associated with pre-eclampsia".

Nevertheless the concept of an association between low grade disseminated intravascular coagulation and pre-eclampsia is an attractive one, because if intravascular coagulation was impairing blood flow to organs such as the placenta, kidney, liver

and brain, many of the clinical features of pre-eclampsia would be explained. The evidence in favour of such an association has come from several sources including:-

(a) histology of human pre-eclampsia

(b) animal experiments

(c) haematological changes in human pre-eclampsia.

These will be discussed in turn in the next section.

V.2 The Association between Pre-eclampsia and Intravascular Coagulation

This subject has attracted widespread interest over the last few years. A recent editorial in The Lancet (1975) concluded that "We cannot yet demote intravascular coagulation from its pre-eminence as an explanation of toxaemia of pregnancy".

V.2(a) Histology of Human Pre-eclampsia

The original link between eclampsia and microvascular thrombosis was described by Schmorl in 1893. In 1909 Holland stated that "a widespread thrombosis of capillaries is a consistent feature of fatal cases of eclampsia".

The development of safe techniques for obtaining biopsy material and the facilities for good electron microscopy have allowed this work to be extended to the study of pre-eclampsia. Many reports have now been published on renal biopsy histology (including Pollak and Nettles, 1960; Pirani, Pollak, Lannigan et al., 1963; Thomson, Paterson, Smart et al., 1972). There appears to be a glomerular lesion fairly typical of pre-eclampsia, which includes swelling of the endothelial and mesangial cells ("endotheliosis"), platelet aggregates and the deposition of an amorphous fibrinoid material within the cells and beneath the basement membrane. Using immunofluorescence this amorphous fibrinoid material was shown to contain fibrinogen or fibrinogen

derivatives (by among others Vassalli, Morris and McCluskey, 1963; Petrucco et al., 1974).

Deposition of fibrinogen-related protein has also been demonstrated by immunofluorescence around the hepatic sinusoids in liver biopsy specimens from pre-eclamptic patients, but not in those from healthy pregnant controls (Arias and Mancilla-Jimenez, 1976).

Changes have also been found in placental histology suggesting increased fibrin deposition in pre-eclampsia. In normal pregnancy the initimal endothelium of the spiral arteries is largely replaced by cytotrophoblast and the elastic lamina and smooth muscle of the media by an amorphorous matrix containing variable amounts of insoluble fibrin (Brosens, Robertson and Dixon, 1967; Sheppard and Bonnar, 1974a and b).

In pregnancies complicated by maternal hypertension a lesion described as "acute atherosis" appears (Zeek and Assali, 1950; de Wolf, Robertson and Brosens, 1975). The first evidence of arterial damage may be "fibrinoid necrosis" (Dixon and Robertson, 1958) while massive intramural fibrin deposition and luminal thrombosis occur in the later stages of the disease process (de Wolf et al., 1975). Sheppard and Bonnar (1976) describe a similar lesion with marked increase in the fibrin content of the media and occlusive atheromatous lesions of the spiral arteries in pregnancies complicated by placental infarction and foetal growth retardation, regardless of whether the mother is hypertensive or not.

These studies indicate that placental fibrin deposition occurs in normal pregnancy. Local intravascular coagulation within the placenta is increased in pre-eclampsia but is not specific for this condition.

Although no direct histological evidence of intravascular coagulation is available in the lungs, perfusion defects have been

found on lung scans from pre-eclamptic patients (The Birmingham Eclampsia Study Group, 1971). Criticisms of this paper would include the lack of studies in a normal pregnancy control group and the non-specific nature of a perfusion defect in the absence of any information on ventilation, but nevertheless the finding could suggest the occurrence of intravascular coagulation with platelet-fibrin aggregates blocking the pulmonary capillaries in pre-eclampsia.

In summary, histological studies in pre-eclampsia have indicated that fibrinogen-related material (presumably fibrin) is laid down in the renal glomeruli, the hepatic sinusoids and to an increased extent in the maternal placental vessels. There is, in addition, presumptive evidence that a similar process may occur in the pulmonary vasculature. These studies do not, however, indicate at what stage in the pathological process the fibrin is deposited, nor do they indicate the stimulus to intravascular coagulation. In an attempt to answer these questions animal experiments have been undertaken, although it has been difficult to obtain an entirely satisfactory animal model.

V.2(b) Animal Experiments

In 1909 Englemann and Stade injected placental extracts into pregnant rabbits and the animals died with widespread intravascular coagulation, which could be prevented by prior anticoagulation with hirudin. Following a similar series of experiments Schneider, (1947; 1951) suggested that intravascular coagulation was a cause of eclampsia and that thromboplastin release from the placenta might be the precipitating factor, a suggestion also made by Page (1948).

During placental separation or following trauma to the placenta, "white emboli" enter the maternal circulation in

experimental animals and the animals develop histological evidence of intravascular coagulation (Brown and Stalker, 1969). Vassalli, Simon and Rouiller (1963) demonstrated that infusion of thromboplastin in pregnant rabbits produces renal glomerular changes similar to those seen in human pre-eclampsia.

Thromboplastin release from the placenta into the maternal circulation caused by trauma to the placenta or minor episodes of abruptio might therefore give rise to some of the features of pre-eclampsia as well as intravascular coagulation. In this connection it is interesting that trophoblastic cells have been encountered more frequently in the uterine vein blood of pre-eclamptic women compared with normal controls (Jäämeri, Koivuniemi and Carpén, 1965) and that the trophoblast has very high thromboplastic activity (Chargaff, 1945).

Other animal models of pre-eclampsia have been used. Wardle and Wright (1973) carried out suture of the placental bed of pregnant rabbits. The spectrum of clinical changes produced ranged from abruptio placentae to "toxaemia" with mild hypertension and proteinuria. ^{125}I fibrinogen studies demonstrated intravascular coagulation with fibrin deposition in the lung, liver and kidney. The renal histology, however, showed marked tubular degeneration and no proliferative response. It is, therefore, not certain that these animals were suffering from a "pre-eclamptic" type of illness.

Arhelger, Douglas and Langford (1967) induced hypertension and proteinuria in unilaterally nephrectomised pregnant rats by feeding them deoxycorticosterone acetate and a high salt diet. The renal histology resembled that of human pre-eclampsia and in particular the glomeruli were filled with fibrin-like material. McKay, Goldenberg, Kaunitz and colleagues (1967) fed pregnant rats a diet low in tocopherol and containing molecular distillates of oxidised cod liver oil (lipid peroxidases). Disseminated intra-

vascular coagulation was produced, the earliest changes being platelet damage and clumping with adhesion to endothelium in the intestine, spleen and placenta. The placenta was the first organ to be affected. It was suggested that platelets might act as a trigger to a process of disseminated intravascular coagulation, but it could be argued that this animal model was rather different from human pre-eclampsia.

Studies using radioactively labelled platelet aggregates infused into the inferior vena cava of the rabbit have shown that they become lodged within the pulmonary vasculature (Wardle, 1973). This finding would be in keeping with the perfusion defects detected in lung scans on pre-eclamptic patients, (see Chapter V. 2(a)). Thromboplastin, however, does cross the pulmonary circulation in the same experimental model (Wardle, 1973). A similar process may occur in human pre-eclampsia and could result in the development of disseminated intravascular coagulation.

It is debatable how far such animal experiments reflect the pathogenesis of human pre-eclampsia. It does, however, seem possible that thromboplastin release from the placenta (Schneider, 1947) may trigger changes in coagulation factors and platelets in the mother's circulation resulting in low-grade intravascular coagulation. Placental ischaemia or infarction could be responsible for the thromboplastin release, which in its turn would cause further placental ischaemia and infarction, thus setting up a vicious circle.

In this context it may be important that pregnant animals are particularly at risk of developing the Generalised Shwartzman Reaction or GSR. (For a discussion of the GSR see Chapter I. 5(c)). It is known that only one injection of endotoxin is required to produce the GSR in pregnant animals (Apitz, 1935; Beller and Graeff, 1967). Histological studies have drawn attention to the similarity between

human eclampsia and the GSR (McKay, Merrill, Weiner et al., 1953). It would therefore appear that pregnancy itself produces a state similar to that produced by the preparative injection in the GSR. This "hypercoagulable state" (see also Chapter V.2(c)) might include changes in the vascular endothelium as well as in the coagulation factors and the platelets. McKay and colleagues in their original paper (1953) stated, with reference to eclampsia, that "pregnancy prepares the blood vessels" and that "premature separation of the placenta releases "toxin" from the necrotic decidua in the circulation which amounts to the "provoking" dose in the Shwartzman phenomenon". The evidence from histology in human pre-eclampsia and also from the variety of animal experimental models already discussed, suggests that a similar although less dramatic process may occur in pre-eclampsia (Schneider, 1947; Page, 1948). The "toxin" may be thromboplastin, while placental ischaemia or infarction may be responsible for its release rather than actual premature separation of the placenta.

Valuable as such animal studies may be in unravelling the complex nature of pre-eclampsia, there are obvious problems in extrapolating the results that are obtained to the human situation.

V.2(c) Haematological Changes in Human Pre-eclampsia

(i) Microangiopathic haemolytic anaemia

In 1908 Zangemeister noted the presence of haemoglobin in serum in 5 out of 14 cases of eclampsia. The haematological abnormality was investigated further in 3 cases of severe pre-eclampsia (Pritchard, Weisman, Ratnoff et al., 1954). A haemolytic anaemia was found with blood film appearances similar to those later described in microangiopathic haemolytic anaemia (Brain and Hourihane, 1967). This is now known to be

closely related to disseminated intravascular coagulation (Rubenberg, Regoeczi, Bull et al., 1968). Microangiopathic haemolytic anaemia is, however, only present in severe or longstanding cases of pre-eclampsia, and reflects florid disseminated intravascular coagulation. Several studies have described more subtle changes suggestive of intravascular coagulation by measuring coagulation, fibrinolytic and platelet function and these will be described below.

(ii) Coagulation system

During normal pregnancy the levels of many of the blood coagulation factors rise reaching a peak in the third trimester (several reports including Pechet and Alexander, 1961 and Nilsson and Kullander, 1967). Bonnar (1976) summarised the current literature and stated that increases have been found in plasma fibrinogen levels (reaching as high as 400-600 mg/100 ml in late pregnancy and labour) and in Factors VII, VIII, IX and X with diminished concentrations of Factors XI and XII. A slight increase in the ratio of Factor VIII related antigen to Factor VIII activity (Bouma, Sixma, van Mourik et al., 1973; Bonnar, 1976) together with significantly raised levels of "thrombin activated Factor V" (Factor Va) have also been found in normal pregnancy (van Royen and ten Cate, 1976).

Since the circulating blood volume increases in normal pregnancy by 30-50% many of the coagulation factors double in absolute amount compared with the non-pregnant state. These changes may have a physiological importance in assisting the pregnant woman to achieve and maintain haemostasis during and after the third stage of labour. On the other hand, the very changes which help to maintain the integrity of the vascular tree may also predispose the pregnant woman to disseminated intravascular coagulation.

Compared with normal pregnancy concentrations of some

blood coagulation factors rise still further in association with pre-eclampsia. Howie, Prentice and McNicol (1971) noted a further increase in Factor VIII activity levels in a group of pre-eclamptic women compared with a matched pregnant control group. Reid, Frigoletto, Tullis and colleagues (1971) noted a shortened Quick prothrombin time in their group of pre-eclamptic patients suggesting that Factors II, V, VII or X might be elevated. They also noted a strikingly shortened ratio for silicone-to-glass clotting times, which was interpreted as suggesting the presence of Factors XIIa and/or XIa in association with pre-eclampsia. In contrast, Factor V levels were found to be lower in hypertensive compared with normotensive pregnant patients (Davidson and Phillips, 1972). A recent study has shown significantly raised Factor XII levels in pre-eclampsia compared with normal pregnancy, while Factors XI and X were slightly lower in the patient group (Condie, 1976). The ratio of Factor VIII related antigen to Factor VIII activity is increased in women with pre-eclampsia compared with the changes in normal pregnancy (Bonnar, 1976).

Conflicting results have been published comparing the levels of plasma fibrinogen in a group of patients with pre-eclampsia to those from a matched pregnant control group. Usually no statistically significant differences is found in plasma fibrinogen levels (Wardle and Menon, 1969; Bonnar, McNicol and Douglas, 1971; Howie et al., 1971; Kitzmiller, Lang, Yelenosky et al., 1974) but both higher (Dienst, 1905) and lower levels (Birmingham Eclampsia Study Group, 1971; Dube, Bhattacharya and Dube, 1975) have also been reported.

A number of techniques have been used to identify abnormal or activated forms of fibrinogen in pre-eclampsia (see Chapter I. 6(a)).

(i) Cryofibrinogen

Increased cryofibrinogen levels have been found in pre-eclampsia compared with normal pregnancy (McKay and Corey, 1964; Wardle and Menon, 1969; Howie et al., 1971). A similar abnormal form of fibrinogen "heparin precipitable cold fibrinogen" has also been found to be increased in pre-eclampsia (Smith, 1957).

(ii) Protamine Sulphate Tests

Increased numbers of positive protamine sulphate tests have been found in pre-eclampsia compared with the numbers found in normal pregnancy (Hyde, Joyce, Gurewich et al., 1973; Kitzmiller, Lang, Yelenosky et al., 1974; Pinango, Linares, Cova et al., 1976).

(iii) Ethanol Gelation Tests

One study has shown 4 positive ethanol gelation tests out of 6 samples taken from 2 pre-eclamptic patients, but no positive tests were found in samples from 10 healthy pregnant women (Reid et al., 1971).

(iv) Fibrinogen Series

At a more sophisticated level a "fibrinogen series" has been developed which is claimed to distinguish between predominant coagulation, predominant fibrinolysis and coagulation balanced by fibrinolysis (Wood, Burnett, Picken et al., 1974). Immunoreactive fibrinogen, heat precipitable fibrinogen and cryofibrinogen are measured. Immunoreactive fibrinogen measures fibrinogen-related antigen in addition to intact fibrinogen and is therefore usually raised above the heat precipitable fibrinogen level during fibrinolysis, but falls below the heat precipitable fibrinogen level when thrombin action predominates. In several cases of pre-eclampsia changes have been found in this fibrinogen series to suggest that intravascular coagulation (i.e. thrombin action) is associated with deterioration in the pre-eclamptic state, while enhanced fibrinolysis occurs during clinical improvement.

All these tests for abnormal or activated fibrinogen are somewhat unsatisfactory (see discussion Chapter I. 6(a)). Despite this there is clearly some evidence to suggest that increased thrombin-activated fibrinogen (i. e. soluble fibrin monomer) is present in pre-eclampsia compared with normal pregnancy.

It is known that soluble fibrinogen-fibrin complex concentration demonstrated by fibrinogen chromatography does increase in normal pregnancy reaching a peak concentration at term (Hafter, Schneebauer, Tafel et al., 1975) and further increases occur in the puerperium (Graeff, Wiedemann, von Hugo et al., 1976). A marked increase in soluble complex concentration has been well documented in a case of eclampsia (Graeff and von Hugo, 1972) and also in two cases of abruptio placentae (Vermylen, Donati and Verstraete, 1971; Graeff and von Hugo, 1974). Wardle (1973) found "both high and low molecular derivatives of fibrinogen are in the circulation ... in hypertension in pregnancy" using the plasma fibrinogen chromatography technique, but the details of this work have never been published (Wardle, 1976).

In summary, therefore, the increased concentrations of several coagulation factors and changes in fibrinogen suggest that the tendency towards hypercoagulability in normal pregnancy is enhanced in pre-eclampsia.

The coagulation system only reflects one aspect of the mechanisms responsible for haemostasis and the changes in the fibrinolytic system and the formation of fibrinogen-fibrin degradation products will now be discussed.

(iii) Fibrinolytic system

In normal pregnancy there is an increase in the level of

plasma plasminogen (Bonnar, McNicol and Douglas, 1969), which is in step with the rise in plasma fibrinogen (Bonnar, McNicol and Douglas, 1969). Despite this the spontaneous fibrinolytic activity in the blood is diminished (as measured by prolongation of the euglobulin lysis time) in normal pregnancy (Gillman, Naidoo and Hathorn, 1959; Shaper, MacIntosh and Kyobe, 1966; Bonnar, McNicol and Douglas, 1969). The euglobulin lysis time rapidly returns to normal following delivery (Bonnar, McNicol and Douglas, 1970).

The low level of circulating plasminogen activator, suggested by the prolonged euglobulin lysis time, could be due to diminished activator synthesis, to inhibition of the release of activator from the endothelium and also to circulating inhibitors.

It is known that local release of plasminogen activator in response to venous occlusion is progressively diminished during pregnancy and is barely detectable at term (Åstedt, 1972a). Furthermore an impaired fibrinolytic response to physical exercise has been reported in late pregnancy (Woodfield, Cole and Cash, 1968). Venous biopsy specimens show diminished fibrinolytic activity during pregnancy, although the actual level of activity is much higher than that found in the blood (Åstedt, 1972a). This could suggest the presence of an activator-release inhibiting mechanism.

Placental biopsy specimens have also shown lack of fibrinolytic activity in the cytotrophoblastic cells lining the spiral arteries (Sheppard and Bonnar, 1976). This might be due to local production of an activator inhibitor for it is also known that the placenta is rich in inhibitors to urokinase activation of plasminogen (Kawano, Morimoto and Uemura, 1968).

Increased inhibition of urokinase by pregnant serum compared with non-pregnant serum was demonstrated in one study (Kawano et al.,

1968) but not confirmed in further studies using serum (Hedner and Åstedt, 1971) or plasma (Bonnar, McNicol and Douglas, 1969). The differences between batches of urokinase and between urokinase and naturally produced plasminogen activator are clearly important in assessing these results (Chapter I. 3(b)). Although inhibitors of urokinase activation of plasminogen may not rise during pregnancy, it should be noted that antiplasmin activity and α_2 macroglobulin and α_1 antitrypsin concentrations do rise (Hedner and Åstedt, 1971).

The explanation for the inhibition of fibrinolysis in normal pregnancy is therefore far from clear, but it is probable that the placenta is important, because the depressed fibrinolytic activity only returns to normal following placental delivery (Åstedt, 1972b). It may be that inhibitors are produced in the placenta, which enter the maternal blood and in some way prevent the release of plasminogen activator from endothelial cells.

It is probable that fibrinolysis is only partially inhibited because serum fibrinogen-fibrin degradation product (FDP/fdp) levels rise during pregnancy, especially during the third trimester (Woodfield, Cole, Allan et al., 1968). This rise appears to be episodic and may not occur in all women (Hoq, Pepper, Prescott et al., 1975). There is a further increase in serum FDP/fdp during labour and in the puerperium (Bonnar, Davidson, Pidgeon et al., 1969), which presumably reflects lysis of insoluble fibrin formed at the time of placental separation.

The depression of fibrinolytic activity, whatever its cause, will tend to aggravate any tendency towards intravascular coagulation in pregnancy. This may be important in relation to pre-eclampsia.

In pre-eclampsia lower levels of plasma plasminogen are present than in normal pregnancy (Bonnar, McNicol and Douglas, 1971; Howie et al., 1971). There is also an increase in the level of inhibitor to urokinase in plasma from patients with pre-eclampsia

(Bonnar, McNicol and Douglas, 1971; Howie et al., 1971).

Serum FDP/fdp levels are higher in pre-eclamptic women than in normal pregnant controls (Bonnar, Davidson and Pidgeon et al., 1969; Henderson, Pugsley and Thomas, 1970; Howie et al., 1971; Hyde et al., 1973; Dube et al., 1975) although sequential studies suggest that consistently raised levels are not always present in pre-eclampsia (Gordon, Ratky, Baker et al., 1976). Urinary FDP/fdp levels are also higher in pre-eclamptic patients than in normal pregnancy, particularly following delivery (Howie et al., 1971).

These results suggest that although there may be increased inhibition of fibrinolysis in pre-eclampsia, this is not complete and some lysis of insoluble fibrin is occurring. The results are therefore compatible with a degree of intravascular coagulation being present in pre-eclampsia.

(iv) Platelet count and function

Thrombocytopenia was first described in eclampsia by Stahnke in 1922 but it also occurs in association with pre-eclampsia (Bonnar, McNicol and Douglas, 1971; Howie et al., 1971; Birmingham Eclampsia Study Group, 1971; Davidson and Phillips, 1972; Kitzmiller et al., 1974). One recent study has shown a correlation between the reduced maternal platelet count and intra-uterine growth retardation in pre-eclampsia (Trudinger, 1976).

Increased platelet adhesiveness was described by McKay, de Bacalao and Sedlis (1964) in pre-eclampsia compared with normal pregnancy. They claimed that there was a direct relationship between the severity of the pre-eclampsia and the "platelet adhesiveness index". In contrast to this report, however, other studies have suggested that platelets are less reactive in pre-eclampsia as compared with normal pregnancy. Thus there is a reduced response to adenosine diphosphate induced aggregation

in pre-eclampsia (Howie et al., 1971) and platelet adhesion and aggregation following exposure to connective tissue may also be diminished in some cases of pre-eclampsia (Kitzmiller et al., 1974).

In an on-going study Whigham, Howie, Drummond and colleagues (1977) have found that platelets in women with severe pre-eclampsia are generally less reactive (using a variety of aggregating agents) than platelets from normal pregnant women. The diminished maximum rate of aggregation was particularly marked when collagen was used as the aggregating agent. It was also found that platelet serotonin content was significantly reduced in severe pre-eclampsia compared with normal pregnancy. The general loss of platelet reactivity is further emphasised by the observation that platelet factor 3 is reduced in association with pre-eclampsia (Howie et al., 1971).

It is difficult to interpret these findings at present but they are not incompatible with a low grade intravascular coagulation in which platelets are being consumed in the formation of micro-thrombi. In the process some platelets might disaggregate from these deposits and circulate in the blood in a less reactive form.

(v) Summary

Pre-eclampsia is therefore associated with abnormalities in a number of tests of coagulation, fibrinolytic and platelet function, which can be broadly summarised as follows:

(i) Indirect evidence of activation of the coagulation system.

(ii) Depression of fibrinolytic activity

(iii) Production of fibrinogen-fibrin degradation products

(iv) Indirect evidence of activation and consumption of platelets.

Taken together this evidence would favour a degree of intravascular coagulation, although quite profound changes do occur even in normal pregnancy. It would appear that certain trends are established in normal pregnancy, which become accentuated in association with pre-eclampsia.

It seemed that the technique of plasma fibrinogen chromatography was well suited to provide further and rather more direct evidence of increased activation of the coagulation system in pre-eclampsia. Accordingly the study comparing groups of pre-eclamptic, normal pregnant and normal non-pregnant women was undertaken and is described in the next section.

V. 3 Design of Clinical Study

Three groups of subjects were studied (see Table 16). Group I consisted of 10 patients with a clinical diagnosis of pre-eclampsia which was defined as a sustained diastolic blood pressure of greater than 90 mm of mercury and proteinuria in excess of 0. 5 gram/24 hours. The signs developed after the 24th week of pregnancy and resolved following delivery (for discussion of this definition see Chapter V. 1). These women were all ambulant in-patients at either the Royal Maternity Hospital or the Queen Mother's Hospital in Glasgow. Short case summaries will be found in Appendix II.

Group II consisted of 10 healthy pregnant women, who were attending the antenatal clinic at the Royal Maternity Hospital in Glasgow. They were carefully matched for age, parity and length of gestation with those subjects in Group I. It proved impossible to obtain suitable in-patient normal pregnant women, as the reason for admission usually made them unsuitable for inclusion in a "control" group. All the women in Groups I and II were receiving iron and folic acid, but the pre-eclamptic patients received a variety of drugs (e. g. antihypertensive agents, sedatives, diuretics

etc.) not given to the control pregnant women. The drug therapy for each individual pre-eclamptic patient is listed in Appendix II. Every normal pregnant woman recruited into the control group was reviewed following her post-natal visit to ensure that no obstetrical or other complication had occurred during pregnancy or the puerperium. If such a complication (e.g. hypertension) had developed after the time of study this patient was withdrawn and another recruited.

Group III consisted of 10 healthy non-pregnant women, none of whom were taking the oral contraceptive "pill" or any other drugs. They were matched for age with those in Groups I and II. They were all normotensive and no significant proteinuria was found on testing a single urine specimen with Albustix (Ames). All subjects in Groups I, II and III gave informed consent to inclusion in the study which amounted to the taking of 20 ml (Groups I and II) or 30 ml (Group III) of venous blood. (The different volumes were related to the changes in haematocrit in normal pregnancy). The details used in matching the three groups are shown in Table 17. Ten sets of three matched subjects were obtained and these were coded into Sets 1-10 for convenience. Exact matching was impossible but age was matched to within 3 years, length of gestation (calculated from the patient's last menstrual period) to within 2 weeks and parity was matched exactly in every Set except Set 9 in which the patient with pre-eclampsia had suffered one more abortion in the past than the control pregnant woman. The pre-eclamptic patient in Set 8 had a twin pregnancy, while the control pregnant woman had a single pregnancy. The differences between twin and single pregnancies were investigated separately (Chapter VII.2). They were found to be minimal and therefore could not have caused the differences in results found in Set 8 (see results section). Statistical analysis

using the "t" test failed to show any difference between the groups for age and between the pregnant groups for length of gestation. Systolic and diastolic blood pressure measurements for all three groups at the time of blood sampling are shown in Tables 18(a) and 18(b). The quantitative proteinuria results on the day of study for the pre-eclamptic patients in Group I are shown in Table 18(c). All the patients in Group I had some degree of oedema of their face and hands (as well as ankles) and a rough clinical assessment was made on a scale from a trace to +++. It can be seen that there was a wide spectrum of severity within the pre-eclamptic group and this will be discussed further in Chapter VI. 3.

The following packed agarose gel columns were used for the work described in the pre-eclamptic patients (Chapters V and VI):-

Columns B5 to B8 (Batch II)
C5 (Batch II)
C9 (Batch II)

Details regarding these packed gel columns can be found in Appendix I.

V.4 Results

(a) Plasma fibrinogen

The plasma fibrinogen levels (measured by the Ratnoff and Menzie technique) are shown in Table 19. It can be seen that there was a significant rise associated with normal pregnancy (Group II/ Group III). This would have been expected in view of previous published results (see discussion Chapter V. 2(c)). There was no statistically significant difference in plasma fibrinogen levels between the two pregnant groups (Group I/Group II), although the general trend was towards lower levels in association with pre-eclampsia. Although the literature is rather confusing on this

point, this result is in keeping with previous work in which either normal or low plasma fibrinogen levels have been found in association with pre-eclampsia (see discussion Chapter V.2(c)).

(b) Serum FDP/fdp levels

These were measured by two techniques - the tanned red cell haemagglutination inhibition immunoassay (T.R.C.H.I.I.) and the staphylococcal clumping test (SCT). The results are shown in Tables 20 and 21.

Using the T.R.C.H.I.I. it can be seen that there was a small, but statistically significant increase in serum FDP/fdp levels in normal pregnancy, when compared with the normal non-pregnant control group (Group II/Group III). A further small, statistically significant increase in serum FDP/fdp levels was found in association with pre-eclampsia when compared with normal pregnancy (Group I/Group II).

The SCT proved a less sensitive method of measuring serum FDP/fdp, as only half of the pre-eclamptic and none of the control groups showed a positive result. There was, however, a significant rise in association with pre-eclampsia (Group I/Group II). There was also a significant positive correlation for the paired results on the samples from all three groups taken together using the two different techniques (Table 22).

(c) Plasma fibrinogen chromatography

Ten sets of matched results were obtained. Each set contained a sample from a pre-eclamptic patient, a matched pregnant control and a matched non-pregnant control. A set of matched results for FR-antigen curves is shown in Figure 46. The shape of the antigenic curve was consistently abnormal in association with pre-eclampsia, and suggested soluble complex formation. In addition, antigenic material (indicated by the arrow)

seemed to be eluted much earlier (close to the void volume fractions) in pre-eclampsia compared with normal pregnancy. Further examples of elution curves in pre-eclamptic patients can be found in Figures 49-51 (Chapter VI).

The results were examined in a number of different ways in order to obtain the maximum amount of information:-

(i) FR-antigen ratio
(ii) SCT Titre of the V_o fractions
(iii) T.C.P. ratio
(iv) T.C.T. ratio
(v) Areas m, n and p

(i) FR-antigen Ratio (Chapter II.4(k))

This is a measurement of the elution volume at which fibrinogen-fibrin related (FR-) antigen could first be detected. A ratio was used rather than the actual elution volume to minimise the minor differences between the packed gel columns. The results are shown in Table 23. It can be seen that the ratio was consistently lower in association with pre-eclampsia, indicating that the antigenic material was eluted earlier than in normal pregnancy close to the void volume. The difference between the results in pre-eclampsia and normal pregnancy was highly significant. There was also a significant difference between the results from the pregnant and non-pregnant normal control groups.

The lower FR-antigen ratios found in some of these samples could have been associated with a corresponding rise in plasma fibrinogen levels, therefore the paired results were studied using the technique of linear regression analysis (Table 24). The only significant correlation was found when the results for Groups II and III were considered together. This was negative and only reached borderline levels of significance ($2p < 0.05$). It may therefore be

suggested that the higher fibrinogen levels in normal pregnancy resulted in the FR-antigen material being eluted from the packed column at a slightly earlier volume. This clearly did not happen in the samples from the pre-eclamptic patients (Group I) because in these samples antigenic material appeared close to the void volume, regardless of the fibrinogen level.

It was already known that the Mancini technique was not ideal for measuring the amount of antigen in the fractions close to the void volume (see Chapter III. 4 and III. 6), therefore, the SCT titre was determined for these fractions.

(ii) SCT titre of V_o fractions (Chapter II. 4(1))

Several fractions close to the void volume were tested. The highest concentration of fibrinogen-related material measured was used for statistical analysis. This was, in fact, usually one or two fractions later than the crest of the total protein V_o peak as determined by the optical density elution curve. The results are shown in Table 25. The small batch variations in the sensitivity of the staphylococci were not taken into account. It can be seen that normal pregnancy was associated with a positive test in 5 out of 10 cases. The difference between the normal pregnant and the non-pregnant control groups only reached borderline significance ($2p < 0.05$) and this could possibly have been due to a degree of batch variation. The difference between the pre-eclamptic group and normal pregnant control groups was, however, highly significant and it seems improbable that this could be explained in terms of batch variation.

The results for FR-antigen concentration for the same fractions (close to the void volume) measured by the radial immuno-diffusion (Mancini) technique are shown in Table 26. The correlation between the two techniques for measuring FR-antigen was calculated

by linear regression analysis and the results are shown in Table 27. A significant positive correlation was obtained when the data from all three groups was considered together (although as no antigen was recorded using the radial immunodiffusion technique in Groups II and III no correlation could be expected within these groups).

(iii) T.C.P. ratio (Chapter II.4(i))

This is a measurement of the earliest elution volume at which "fibrin" clot or strands could be produced following incubation with thrombin. A ratio was used to try to minimise the variation between different packed gel columns. The results are shown in Table 28. A much lower ratio was consistently seen in association with pre-eclampsia indicating that material which will produce "fibrin" clot or strands was present close to the void volume in these samples. The difference in results between the pre-eclamptic and normal pregnant control groups was again highly significant. The difference in results between the pregnant and non-pregnant control groups also reached statistical significance.

It is possible that a higher fibrinogen level could have resulted in a lower T.C.P. ratio, however, as can be seen from Table 29 this did not seem to be the case. The only significant correlation found using this data was positive (Group I data alone) and this only reached a borderline level of significance ($2 \propto < 0.05$). This presumably reflects the tendency for plasma fibrinogen levels to fall slightly while the T.C.P. ratio is also lower in pre-eclampsia. The two changes might not be directly related.

There was, however, a significant positive correlation between the results of the FR-antigen and T.C.P. ratios when the paired data from all three groups was considered together (Table 30). This might have been expected as both measure the first elution volume at which fibrinogen-related material could be detected.

The results for the FR-antigen ratio, the SCT titre at V_o and the T.C.P. ratio all suggested that fibrinogen-related protein was present in the very early void volume fractions in association with pre-eclampsia. In all three tests there was a statistically significant difference between the results found in pre-eclampsia and normal pregnancy. This material is likely to represent polymer of very high molecular weight (see Chapter III.2).

(iv) T.C.T. ratio (Chapter II.4(i))

This is a measurement of the earliest elution volume at which a T.C.T. of less than 3 minutes was recorded. A ratio was used to minimise minor differences between the packed gel columns. The results are shown in Table 31. It can be seen that there was a trend towards lower values in association with pre-eclampsia, but that this failed to reach statistical significance. The lower values found in normal pregnancy compared with the non-pregnant control group did, however, reach statistical significance ($2p < 0.02$). This ratio, therefore, did not produce as good a separation between the different groups as the T.C.P. ratio did and will therefore not be considered further.

(v) Areas m, n and p. (Chapter III.4(k))

Measurements of these areas are related to changes in the shape of the FR-antigen profile. Area m will largely reflect the formation of dimers and area p the formation of early degradation products.

Area m (Table 32 and Figure 47)

There was a consistent increase in area m in association with pre-eclampsia compared with normal pregnancy and this difference was highly significant ($2p < 0.001$). There was also a significant increase in area m, when the results obtained in normal pregnancy were compared with those from the non-pregnant control group.

The correlation between changes in area m and plasma fibrinogen levels is considered in Table 33. When the results of Groups II and III were considered together a significant positive correlation was found. It may therefore be that the rise in area m levels in normal pregnancy is associated with the higher fibrinogen levels. This is not the case for the pre-eclamptic samples, because when the Group I samples were considered a significant negative correlation was found (which still applied to the result when the Group I and II data were considered together). The negative correlation in the Group I data presumably reflects the tendency for plasma fibrinogen levels to fall slightly and area m levels to rise in pre-eclampsia but these changes may not be directly related.

The correlation between changes in area m and serum FDP/fdp levels is shown in Table 34. There was a significant positive correlation when the data from Groups II and III and Groups I, II and III were considered together but not in any of the groups taken separately. It is most likely that both area m and the serum FDP/fdp levels rise independently in association with pregnancy and pre-eclampsia, but there might be a true association (e.g. serum FDP/fdp might be derived from soluble complexes in area m).

Area p (Table 35 and Figure 48)

Although area p tended to be slightly higher in normal pregnancy than in the non-pregnant control women the trend did not reach statistical significance. Area p also tended to be increased in pre-eclampsia compared with normal pregnancy and this trend reached borderline statistical significance ($2p < 0.05$).

The correlation between area p and plasma fibrinogen levels is considered in Table 36. The only significant correlation was found when the Group II data was considered separately. In this

a negative correlation was found which just reached significance levels ($2 < 0.05$). This was most probably due to chance.

The correlation between area p and serum FDP/fdp levels is considered in Table 37. It can be seen that it was only when the results from Groups I, II and III were considered together that a significant positive correlation was found ($2 \propto < 0.05$). This may simply have been due to chance or both values might rise independently in association with pregnancy and pre-eclampsia. There may, however, be a true association for it would not be unreasonable to imagine that serum FDP/fdp levels are derived from the early clottable degradation products found in area p.

There was no significant correlation between values for area m and area p, except when the data from Groups I, II and III were considered together in which case a positive correlation of borderline significance ($2 \propto < 0.05$) was found (Table 38). It is most likely that both values increase independently in pregnancy and pre-eclampsia, but there could be a degree of true association (e.g. area p could be derived from area m).

Area n (Table 39)

The changes in area n obviously mirrored the changes in areas m and p. The pre-eclamptic group had much lower values than the normal pregnant group ($2p < 0.001$). Area n was also significantly lower in association with normal pregnancy compared with the non-pregnant control group ($2p < 0.005$).

There was no significant correlation between area n and plasma fibrinogen levels (Table 40), while the correlation between area n and serum FDP/fdp levels only reached significance when the data from all three groups was considered together (Table 41). In this case a significant negative correlation was found ($2 \propto < 0.01$). It is probable that the two values are not directly related, but change independently in pregnancy and pre-eclampsia.

(vi) Summary

This carefully matched study using plasma fibrinogen chromatography demonstrated several differences between a group of pre-eclamptic patients and a normal pregnant control group. There were also small differences between the normal pregnant and the normal non-pregnant groups. These results are summarised in Table 42, together with the plasma fibrinogen and serum FDP/fdp levels. For many of these results there appears to be a spectrum of abnormality extending from the non-pregnant control group, through normal pregnancy, into the pre-eclamptic group.

Thus the FR-antigen ratio, the SCT titre at V_o and the T.C.P. ratio all suggest that a polymer containing fibrinogen related protein is present in samples from patients with pre-eclampsia. The SCT titre suggests that lower concentrations of this polymer may also be present in normal pregnancy (at least in some cases). The area m results suggest that a dimer containing FR-antigen is present in association with pre-eclampsia in higher concentrations than in normal pregnancy, although the concentration appears to rise even in association with normal pregnancy.

It has to be accepted that there may be an appreciable error in some of the results, particularly in the calculation of areas m, n and p. A small error in estimating the FR-antigen concentration in any fraction of the main antigenic peak will produce an appreciably larger change in the area m or p concentrations. This must be

remembered in assessing the results. There may also have been a degree of subjective bias in the calculation of these results, as it was not possible to carry out the study "blind". Ideally the blood samples should have been collected and coded by someone outwith the laboratory and the code not broken until the end of the study; but only one person was involved in organising the study, collecting the blood samples, performing the gel filtration and laboratory work and in calculating the results.

Sequential studies in several pre-eclamptic patients will be considered in the next chapter (VI. 2). An attempt will also be made to determine whether there is any correlation between the laboratory results and the patient's clinical condition (Chapter VI. 3). The significance of the results in pre-eclampsia and normal pregnancy in relation to intravascular coagulation will be discussed in detail in the final chapter (Chapter IX).

CHAPTER VI

SOLUBLE FIBRINOGEN-FIBRIN COMPLEXES IN PRE-ECLAMPSIA - SEQUENTIAL STUDIES

VI. 1 Introduction

The study described in Chapter V demonstrated significant differences between samples from patients with pre-eclampsia and normal pregnant women using the technique of plasma fibrinogen chromatography. In the present chapter sequential data from six of the pre-eclamptic women will be described and discussed in detail (Chapter VI. 2). This study followed the evolution of the plasma fibrinogen chromatography changes during the ante-natal period and related them to changes in the clinical picture. The study was continued into the phase of clinical recovery following delivery. The final samples were taken at the post-natal visit (approximately six weeks after delivery) at which time almost complete clinical recovery had occurred.

For the purpose of these studies area m and SCT titre at V_o were considered to represent soluble complexes and as such to reflect coagulation activity (see Chapter III. 4 and 6). Fibrinogen-related material eluting close to the void volume and identified by the SCT must be incorporated into a polymer of high molecular weight (see Chapter III. 2). The fibrinogen-related material identified in area m has a lower molecular weight and must be incorporated into an oligomer (probably a dimer). In contrast

the serum FDP/fdp and area p results were considered to reflect fibrinogenolytic/fibrinolytic activity (see Chapter III. 5 and 6).

These results, together with the plasma fibrinogen levels, were considered in relation to the diastolic blood pressure and quantitative proteinuria results obtained on the day of study.

These results can be evaluated in different ways. In the first part of the chapter the results will be described and discussed in detail from the point of view of the individual patient. The results can, however, also be studied in different groups, using statistical analysis to see whether there is any relationship between the laboratory findings and the clinical picture and outcome of pregnancy. These studies will be described and discussed in the second part of the chapter (VI. 3). The results were grouped in the following ways for these statistical analyses:-

(i) All the results from samples taken ante-natally from the pre-eclamptic patients were used.

(ii) Only the results from samples from pre-eclamptic patients taken closest to delivery or intrauterine death were used. (Samples taken while the patient was in labour were not included). This group was selected as probably representing the samples at the time the patient was most critically ill ante-natally.

(iii) The calculations performed using groups (i) and (ii) were repeated including the results from the normal pregnant women studied in Group II (Chapter V).

The features of pre-eclampsia used in this analysis were:-

(a) A "clinical index" based on the diastolic blood pressure and quantitative proteinuria results on the day of study (Howie, Purdie, Begg et al., 1976).

(b) Foetal survival.

(c) Percentile birth weight.
(Lubchenco, Hansman, Dressler et al., 1963).

Finally, the results used for Group I in Chapter V were compared with those currently available from 35 samples taken from 15 pre-eclamptic patients, in order to see if there were any differences between the smaller, selected group and this larger group (Chapter VI.4).

VI.2 Sequential Studies on Six Pre-Eclamptic Patients

The sequential fibrinogen chromatography results for three pre-eclamptic women (Patients 3, 7 and 9) are shown in Figures 49, 50 and 51. It can be seen that many of these samples appear qualitatively abnormal, although it is difficult to follow the changes in any but a very general way. It is simpler to use the semi-quantitative measurements of areas m and p and the SCT titre at V_o in this respect. Therefore in this section, in which the sequential data from six pre-eclamptic women will be discussed in detail, these semi-quantitative measurements will be used.

In each case a short clinical summary will be followed by a discussion of the changes in plasma fibrinogen levels, soluble complexes (area m representing oligomers and SCT titre at V_o representing polymers) and fibrinogen/fibrin degradation products (area p and serum FDP/fdp levels).

(i) Patient 3 (M.H.) Figures 49 and 52.

This elderly primigravida developed pre-eclampsia at 26-27 weeks gestation. Over the next two weeks she developed gross

proteinuria and sustained severe hypertension. Elective Caesarean section was performed at 29 weeks. The baby was born alive but developed severe Respiratory Distress Syndrome (as a complication of extreme prematurity) and died 31 hours after birth. The patient recovered gradually following delivery, although she was still mildly hypertensive at her post-natal visit. She was normotensive when reviewed by the renal physicians and the diagnosis of pre-eclampsia was accepted (see summary Appendix II).

It can be seen that as the patient's clinical condition deteriorated the plasma fibrinogen level fell to below normal levels. As this happened both area m and the SCT titre at V_o rose, while area p and the serum FDP/fdp levels fell. Before delivery, however, both area p and the serum FDP/fdp levels had risen above the normal range. Following delivery the plasma fibrinogen level rose while area m and SCT titre at V_o started to fall. The area p and serum FDP/fdp levels peaked following delivery and then fell. At the post-natal visit the laboratory results were within the range found in the normal non-pregnant control group (Group III, Chapter V) apart from slightly raised area p and serum FDP/fdp levels.

Comment

This patient was an example of steadily deteriorating severe pre-eclampsia. The increasing proteinuria was mirrored by the pattern of increasing coagulation (as measured by area m and SCT titre at V_o), while fibrinolysis (area p and serum FDP/fdp levels), remained either low or only slightly increased. After delivery, there was a surge of fibrinolytic activity and a decrease in coagulation activity.

(ii) Patient 5 (J.T.) Figure 53

This patient developed hypertension and proteinuria at 24-25 weeks gestation. Her condition gradually deteriorated with intra-

uterine death occurring at about 28 weeks. She was delivered at 29 weeks and gradually recovered. She was still slightly hypertensive at her post-natal visit but was normotensive when reviewed by the renal physicians. They concluded that the diagnosis of pre-eclampsia had been correct (see summary Appendix II).

Plasma fibrinogen levels were low ante-natally but not below the normal range. Area m levels were consistently above the normal range, but fell slightly following intrauterine death. (No SCT titre at V_o values are available on this patient). The area p and serum FDP/fdp levels were raised above normal but both fell around the time of intrauterine death.

Following delivery the plasma fibrinogen level rose, but area m did not, while both area p and the serum FDP/fdp levels peaked sharply. At the post-natal visit the plasma fibrinogen, serum FDP/fdp and plasma fibrinogen chromatography results were within the range found in the non-pregnant control group, apart from a slightly raised area m level.

Comment

In this patient, as in the previous one, the developing pattern of pre-eclampsia was associated with increasing coagulation activity (area m) and diminishing fibrinolytic activity (area p and serum FDP/fdp levels). It was interesting to note that coagulant activity fell after foetal death but that the post-natal surge of fibrinolytic activity was not evident until after placental delivery. This would be in keeping with the hypothesis that a placental factor is responsible for inhibition of fibrinolytic activity (see Chapter V.2(c)).

(iii) Patient 7 (M.M.) Figures 50 and 54

This patient was in some ways one of the least severely affected of those studied sequentially. She developed hypertension and

proteinuria at 35 weeks gestation, but her hypertension was only moderately severe and the proteinuria only gradually increased. The baby was slightly premature and dysmature at birth. The patient made a good recovery following delivery. Her blood pressure had returned to normal and proteinuria disappeared by the time of her post-natal visit.

The plasma fibrinogen levels were normal ante-natally. Both area m and the SCT titre at V_0 were raised above normal, although in the case of area m the level was only slightly above the normal range. The serum FDP/fdp levels were normal but area p rose towards the time of delivery. Following delivery the plasma fibrinogen level rose slightly. Area m also rose slightly, but the SCT titre at V_0 fell. The serum FDP/fdp and area p levels peaked following delivery. The laboratory findings at the post-natal visit were within the normal range found in normal non-pregnant women, except for a slightly raised serum FDP/fdp level.

Comment

This patient again followed the pattern noted in the two previous cases. Deteriorating clinical pre-eclampsia was associated with increased coagulant activity (area m and SCT at V_0) and low or only slightly increased fibrinolytic activity (area p and serum FDP/fdp). A surge of fibrinolytic activity was again noted after delivery with a return to fairly normal results six weeks after delivery.

(iv) Patient 9 (A.McN.) Figures 51 and 55

This patient is interesting in that she developed hypertension 2-3 weeks before she developed proteinuria (i.e. pre-eclampsia according to the definition used for this thesis). She was a multi-parous patient with a past obstetric history of two ante-partum haemorrhages due to abruptio placentae. At no time, however, had she been noted to be hypertensive before the pregnancy under

study. She was delivered by elective Caesarean section at 35 weeks gestation. The baby was thought to be both premature and dysmature at birth, but thereafter progressed well. The patient continued to have mild, fluctuating hypertension for some months following delivery. This was controlled with a low dose of propranolol. It is therefore possible that there was a degree of underlying essential hypertension in this case, which had not become manifest until the pregnancy under study (see summary Appendix II).

Over the period of study (from 30-35 weeks gestation) the plasma fibrinogen level progressively fell, reaching below normal shortly before delivery. Area m was consistently raised throughout the ante-natal period and tended to rise, while the SCT titre at V_o was raised above normal in 3 out of the 6 ante-natal samples. The serum FDP/fdp levels were raised slightly above normal in the earlier samples but fell during the period of clinical deterioration. Area p rose to above the normal range in the earlier samples but fell to within the normal range in the samples closest to delivery. Immediately following delivery the plasma fibrinogen level remained low and area m and SCT titre at V_o remained high, while the area p and serum FDP/fdp levels peaked. All the laboratory measurements had returned to within the normal non-pregnant range by the time of the post-natal visit, apart from a raised serum FDP/fdp level.

Comment

This patient was most interesting in that she was studied sequentially before the onset of proteinuria. In the early results when she had either mild pre-eclampsia or essential hypertension, there was evidence of only slightly increased coagulant activity (area m). In addition there was some evidence of increased fibrinolytic activity (serum FDP/fdp and area p). As the proteinuria developed and deteriorated, both factors of coagulant activity showed high levels while fibrinolytic activity diminished before

delivery (area p and serum FDP/fdp). After delivery, coagulant activity fell and fibrinolytic activity peaked as in the previous patients.

(v) Patient 10 (D.F.) Figure 56

This patient is interesting in that she had gross proteinuria from 28 weeks gestation until delivery at 32 weeks. Her hypertension fluctuated but never reached high levels. The baby grew well and, although slightly premature at birth, progressed satisfactorily thereafter. Marked proteinuria occurred post-partum. Significant proteinuria was still present at the post-natal visit. This disappeared by 3 months post-partum. Full renal investigation was carried out and the diagnosis of pre-eclampsia was accepted by the renal physicians (see summary, Appendix II).

The plasma fibrinogen levels were consistently low in this patient and fell below the normal range from 29 weeks onwards. The area m levels were always above normal but were only slightly raised between 29 and 31 weeks. The SCT titre at V_o was raised above the normal range in 3 out of the 5 ante-natal samples. The serum FDP/fdp levels tended to rise towards delivery, while area p levels were high in the three samples taken closest to delivery. Following delivery there was a rise in the plasma fibrinogen level, while area m and the SCT titre at V_o remained high, only gradually returning to the normal values found at the post-natal visit. The serum FDP/fdp level peaked following delivery while area p remained high. These measurements also gradually returned to within the normal non-pregnant range by the post-natal visit.

Comment

This patient was unusual in that she had high levels of proteinuria for several weeks and despite this, did not suffer eclampsia or foetal loss. Her fibrinogen chromatography changes also differed

from the previous cases. Coagulant activity (area m and SCT at V_o) was only slightly increased, while fibrinolytic activity (area p and serum FDP/fdp) appeared to increase in the later ante-natal samples. This pattern of "compensatory" fibrinolytic activity may have been a factor in enabling the pregnancy to continue despite the adverse clinical features. After delivery, the fibrinolytic surge seen in the previous cases was reflected by the serum FDP/fdp levels, but not by area p.

(vi) Patient 1 (M.C.) Figure 57

This patient is considered last because very little ante-natal data is available on her. She is worth discussing, however, because she was the only patient in the group to develop eclampsia. She developed hypertension and gross proteinuria at 25-26 weeks gestation and this necessitated delivery within a few days. She was induced by prostaglandin infusion and developed eclampsia during labour. The baby was stillborn. The patient gradually recovered post-partum, although she was still mildly hypertensive at her post-natal visit, only becoming normotensive 3 months post-partum. The renal physicians agreed that the diagnosis of pre-eclampsia with intra-partum eclampsia was correct (see summary, Appendix II).

Only two samples were studied on this patient ante-natally. (The second sample was taken at the time of induction). In both samples the plasma fibrinogen levels were normal. Area m was markedly raised on both occasions, but the SCT titre at V_o was normal in the first sample. Area p was normal in the first sample, but had fallen to below the normal range in the second sample although the serum FDP/fdp level was slightly raised in both samples. In the sample taken 2 days after delivery there was virtually no change in plasma fibrinogen or area m levels, although the SCT titre at V_o had fallen slightly. Similarly the area p and serum FDP/fdp levels remained unchanged. It may be that in this patient fibrinolytic

activity was slower to return to normal because of the severity of her illness. It is recognised that this may happen in patients with severe pre-eclampsia/eclampsia (Bonnar, McNicol and Douglas, 1971). Apart from a raised serum FDP/fdp level all the results had returned to within the normal non-pregnant range by the time of the post-natal visit.

Comment

This patient had a very short but acute clinical course. Coagulant activity was increased in both ante-natal samples, as measured by area m, and appeared to be increasing, as measured by SCT titre at V_o. Fibrinolytic activity, although slight increased as measured by serum FDP/fdp levels, was low and indeed falling as measured by area p. The post-natal increase in fibrinolytic activity observed in the other patients was not seen. This may have been due to an unusually marked degree of inhibition of fibrinolysis and might have been observed had a sample been taken slightly later in the puerperium.

It had been noted in passing that some of the laboratory tests were still slightly abnormal at the time of the post-natal visit, when they were compared with the range of values found in the normal non-pregnant control women in Group III (Chapter V). None of these differences, however, reached statistical significance when the results in the two groups were compared (Table 43).

Comment on the Sequential Studies

The following observations can be made:-

(a) Plasma fibrinogen levels

These tended to fall as the patient became clinically less well (see Patients 3, 9 and 10). The reason for this is uncertain. Possible explanations might be diminished hepatic synthesis of fibrinogen, increased "consumption" of fibrinogen in forming fibrin or possibly

increased renal loss. None of the patients had particularly high fibrinogen levels at any point ante-natally. The level of plasma fibrinogen rose following delivery (see Patients 3, 5, 7, 9 and 10) and always returned to lower values (similar to the non-pregnant state) at the post-natal visit.

(b) Serum FDP/fdp levels

These tended to be slightly raised ante-natally although they fluctuated considerably. A fall in the serum FDP/fdp levels to "normal" accompanied clinical deterioration in three cases (Patients 3, 5 and 9). The serum FDP/fdp level was never raised above "normal" in Patient 7. After delivery the level of serum FDP/fdp tended to rise (see Patients 3, 5, 7, 9 and 10) but had fallen again by post-natal visit. The values in Patient 1 were constant throughout the study.

(c) SCT titre at V_o (polymer)

This tended to be raised in the ante-natal samples although some fluctuation occurred. The highest values were not necessarily associated with the most seriously ill patients. A rising value was associated with clinical deterioration in three cases (Patients 1, 3 and 5). The values tended to fall post-natally (Patients 1, 3, 7 and 9). Patient 10 was the only case to have a recordable SCT titre at V_o in the post-natal visit sample but she was also the only patient with significant proteinuria at the post-natal visit.

(d) Area m (dimer)

Raised levels were found in all the ante-natal samples. A rising area m level appeared to accompany clinical deterioration in Patients 3, 5 and 9 and possibly also Patient 7. In Patient 1 (who became eclamptic) high levels of area m were found. Area m fell following delivery in Patients 3, 9 and 10. Low values were found

at the post-natal visit.

(e) Area p (early degradation products)

Considerable fluctuation was noted in area p values. Area p tended to fall during phases of clinical deterioration in some cases (Patients 1, 3, 5 and 9). Rising area p values accompanied periods of diminishing proteinuria in two cases (Patients 3 and 10). The sequential changes in area p and serum FDP/fdp values were broadly similar in four cases ante-natally (Patients 3, 5, 9 and 10). Area p tended to increase post-partum (Patients 3, 5, 7 and 9) but thereafter fell to low levels by the post-natal visit.

In summary it might be suggested that worsening of the clinical picture in pre-eclampsia is associated with:-

(a) a falling plasma fibrinogen level

(b) a falling serum FDP/fdp level

(c) a rising area m

(d) a falling area p

Because of the small numbers, further work would be required to establish whether these observations are in fact reproducible, but the present study does suggest that a pattern of increased coagulation and diminished fibrinolytic activity is associated with deteriorating pre-eclampsia.

Serial studies in normal pregnant women have not as yet been performed; but serial studies in two patients considered to be "at risk" of developing pre-eclampsia, but who, in fact, had uncomplicated pregnancies can be found in Appendix III.

VI. 3 Relationship of the Clinical Picture and the Outcome of Pregnancy to the Plasma Fibrinogen Chromatography and Other Laboratory Results

In order to determine whether any such relationship could be proven statistically, the results were examined in terms of the clinical index at the time of blood sampling, foetal outcome and percentile birth

weight. These will be discussed in turn.

(a) Clinical Index

The "clinical index" (Howie et al., 1976) is based on the diastolic blood pressure and quantitative proteinuria results and is calculated according to the formula:-

$$\text{Clinical index} = 0.542\,X_1 + 12.917 \log_e (1 + X_2) - 13.374$$

where X_1 = increase in diastolic blood pressure (mmHg) compared with reading early in pregnancy

X_2 = proteinuria (g/24 hours)

(The authors arranged this equation so that all the clinically normal patients in their study had negative indices, while positive indices were only found in the pre-eclamptic patients).

The clinical index was calculated for the day (or days) when blood samples were studied from all the pre-eclamptic and normal pregnant women included in Groups I and II, Chapter V (Table 44). As would be expected all the normal pregnant women had negative indices, but negative indices were also found in results from pre-eclamptic Patient 9 in the early samples (1-3) before the onset of proteinuria and in pre-eclamptic Patient 4 (sample 2) after intra-uterine death had occurred and the proteinuria disappeared.

The clinical indices were compared with measurements of:-

(i) area m

(ii) SCT titre at V_o

(iii) area p

(iv) serum FDP/fdp

There was no significant correlation using linear regression analysis between the paired clinical index and chromatography findings when all the ante-natal results were examined (Table 45a). No significant

correlation emerged when the results from the sample closest to delivery (or intrauterine death) were examined (Table 45b).

When the results from 4 patients in whom the necessary information was available for 3 or more ante-natal samples was examined (Table 45c) some significant correlations did emerge. These results confirmed statistically the impressions already gained in these patients (Chapter VI. 2). These relationships became more obvious when a single clinical index incorporating both blood pressure and proteinuria results was used (see also Figures 58a-d).

It is also worth noting that although virtually no significant correlation existed when the pre-eclamptic data was considered alone, if the results from the 10 normal pregnant women (Group II, Chapter V) were also included significant positive correlations emerged using all four laboratory results (Tables 45d and e). This was particularly true when the last samples taken before delivery in intrauterine death in the pre-eclamptic patients were considered together with the normal pregnant results (Table 45e). Presumably at this point in time the patients with pre-eclampsia were most severely affected.

In view of the differences between pre-eclampsia and normal pregnancy already demonstrated in Chapter V. 4 these results are perhaps not surprising, but it is most interesting that if the clinical index is used to follow a spectrum of clinical severity there appears to be a related spectrum of severity in the laboratory results. It is important to stress, however, that within the pre-eclamptic group of results virtually no correlations were apparent except in some individual patients.

(b) Foetal Survival

The ante-natal results were considered in terms of foetal survival. They were divided into two groups - "live babies" and "perinatal deaths", a perinatal death being defined as a still-birth or death within the first week of life (Donald, 1969a). As can be

seen from Table 46a Patients 1, 3, 5, 6 and 8 all suffered "perinatal deaths" according to this definition. The remaining pre-eclamptic patients and the normal pregnant women (Table 46b) all had "live babies".

As shown in Table 47a when all the ante-natal pre-eclamptic results were examined there was no significant difference in area m, SCT titre at V_o, area p and the serum FDP/fdp levels. In addition when only the results from the last sample taken before delivery (or intrauterine death) were considered no significant difference emerged (Table 47b). This was perhaps not surprising as foetal survival will be affected by so many factors (e. g. the time interval between diagnosis and delivery, see Table 46a).

Not surprisingly the inclusion of the results from the normal pregnant women produced better separation between the two groups, particularly when the results taken closest to delivery or intrauterine death were used (Tables 47c and d). It is worth noting that area m was significantly higher in the pre-eclamptic patients, who later suffered perinatal deaths, but that there was no difference in the area p results between the two groups. This might suggest that coagulation activity was particularly marked in those patients who later suffered perinatal deaths.

(c) Percentile Birth Weight

The percentile birth weight of the babies born to the pre-eclamptic and normal pregnant women (Tables 46a and b) was calculated in terms of sex and gestational age using the Lubchenco charts (Lubchenco et al., 1963; see also Chapter VII. 3). The results from the ante-natal period were divided into two groups:- those in which the percentile birth weight of the baby was below the tenth percentile (i. e. those from pre-eclamptic Patients 4, 7, 8 and 10) and those in which it was above the tenth percentile (i. e. those from pre-eclamptic Patients 1, 2, 3, 6 and 9) and those from all of the normal pregnant women.

As can be seen from Table 48a there was no significant difference between the results from the two groups when all the results from the pre-eclamptic patients were considered. No significant difference emerged when only the results from the last sample taken before delivery (or intrauterine death) were considered (Table 48b). Again it is probably not surprising that no correlation was observed as several factors are likely to be involved in leading to intrauterine growth retardation in these patients (e. g. duration of pre-eclamptic illness which varied considerably - see Table 46a.

When the results from the normal pregnant women were included statistically significant differences were only found with the area p results, which were higher in the pregnancies resulting in babies below the 10th percentile in birth weight (Tables 48c and d). While this could have occurred by chance ($2p < 0.02$ in both analyses) it is an interesting observation, which might indicate increased breakdown of insoluble fibrin in these patients. (Intrauterine growth retardation in non-hypertensive pregnancies will be considered in Chapter VII. 3).

VI. 4 Total Pre-eclamptic Data

At the time of preparing this chapter a further 5 patients with pre-eclampsia have been studied. The results are not presented in detail as they are similar to those already described. Therefore the results from 35 samples from 15 pre-eclamptic women are now available together with the results from 14 normal pregnant women (to be described in Chapter VII. 2).

When the results of these larger but unmatched groups are compared (Table 49) the differences are similar to those found in Groups I and II in Chapter V (see Table 42). It is also worth noting that there were in fact no significant differences between the small selected group of 10 results (Group I, Chapter V) and the total group of 35 results (Table 50).

VI. 5 Summary

When the results in Chapters V and VI are considered as a

whole there is good evidence that:-

(i) a small, but definite increase in soluble complex concentration occurs in association with normal pregnancy compared with the non-pregnant state

(ii) a marked increase in soluble complex concentration occurs in association with pre-eclampsia compared with normal pregnancy.

These results would suggest that in normal pregnancy there is either increased production of soluble complexes or diminished clearance of these complexes or possibly both. This tendency (or tendencies) would seem to be increased in samples from women who have developed pre-eclampsia.

The actual soluble complex concentration in the pre-eclamptic patients did not correlate with the clinical severity of the condition as assessed by the "clinical index" of Howie and colleagues (1976). If, however, there is considered to be a spectrum of clinical severity extending from normal pregnancy to severe pre-eclampsia (Howie et al., 1976) a good positive correlation can be demonstrated between the chromatography findings and the clinical index. This was particularly true of the soluble complex measurements (area m and SCT titre at V_o).

The soluble complex levels did not help to distinguish which of the pre-eclamptic pregnancies would result in perinatal deaths or babies with evidence of intrauterine growth retardation. This was not surprising because the foetal outcome will depend on many factors including the gestational age at the time of onset of the pre-eclamptic symptoms, the management and the duration of the illness.

The results from Chapters V and VI, together with the results obtained in the obstetrical conditions studied in Chapter VII will be discussed further in Chapter IX, in which conclusions from the work described in the entire thesis will be considered.

CHAPTER VII

SOLUBLE FIBRINOGEN-FIBRIN COMPLEXES IN OBSTETRICAL CONDITIONS CLINICALLY RELATED TO PRE-ECLAMPSIA

VII. 1 Introduction

The results described in Chapters V and VI showed a small, but significant increase in soluble fibrinogen-fibrin complex concentration in plasma samples from healthy pregnant women and in addition a further marked increase in samples from women with pre-eclampsia. In order to assess these results more fully, further studies were performed in obstetrical patients with:-

(1) apparently uncomplicated single and twin pregnancies (Chapter VII. 2)

(2) pregnancies complicated by intrauterine growth retardation (Chapter VII. 3)

(3) essential hypertension diagnosed before the start of pregnancy (Chapter VII. 4)

(4) chronic renal disease diagnosed before the start of pregnancy (Chapter VII. 5)

These groups will be discussed in turn. The relevant background literature and the design of each study will be described first followed by the results that were obtained.

The following packed agarose columns were used for the work described in this chapter:-

Columns B5 (Batch II)
B5 to B10 (Batch II)
C5 to C10 (Batch II).

Details regarding these packed columns can be found in Appendix I.

VII. 2 Apparently Uncomplicated Single and Twin Pregnancies

To investigate further the increase in soluble complex concentration in normal pregnancy 21 apparently normal pregnant women were studied (6 with twin pregnancies) and the results assessed in terms of the development of complications (e. g. hypertension), differences between single and twin pregnancies and the gestational age at the time of study. The results in the twin compared with the single pregnancies were of particular interest, because it is thought that there is a higher incidence of pre-eclampsia in twin pregnancies (Donald, 1969b).

To be included in these groups all the women had to be normotensive and lack proteinuria. There was nothing in their past medical or obstetrical history to suggest that any of these women would develop hypertension or pre-eclampsia during the pregnancy under study.

The clinical details for the single and twin pregnancy groups are shown in Tables 51 and 52. The gestational age ranged from 24 to 39 weeks. Patients 15, 20 and 21 developed hypertension during the antenatal period and so are placed separately at the foot of the tables. Patient 15 developed hypertension (B. P. 150/105) at 36 weeks gestation, but this settled after admission to hospital and bed rest (B. P. 130/80). She never developed proteinuria but had "significant" facial and digital oedema. Patient 20 developed hypertension (B. P. 135/90) at 36 weeks gestation and later developed proteinuria and eclampsia post-partum. Patient 21 developed hypertension (B. P. 140/90) at 37 weeks gestation but never developed proteinuria. Therefore none of these patients would have been included under the definition of "pre-eclampsia" used in this thesis. Patients 15 and 21 might be considered to have had a

less severe form of the syndrome, while Patient 20 had post-partum eclampsia. Patients 1-14 and 16-19 had uncomplicated pregnancies. (Several of these patients were included in Group II, Chapter V).

The results of this study are shown in Tables 53 and 54. The patients who developed hypertension were not included in the statistical analysis of the results, but it should be noted that none of the three patients had very abnormal results. This would not encourage the idea that this technique would be able to predict which patients would develop hypertension later in pregnancy, but a much larger study would be required to confirm this.

When the results in the 14 single and 4 twin pregnancies in which no complications developed were compared, areas m and p and the SCT titre at V_o tended to be slightly higher in the twins group, while area n and the FR-antigen and T. C. P. ratio tended to be slightly lower. Only the differences in the T. C. P. ratio, however, reached statistical significance (Table 55). With larger numbers in the twins group the levels of significance might have improved, particularly for the area m results which only just failed to reach significance.

It has, however, to be concluded from this study with small numbers of patients that there were only minor differences between the two groups. Slightly higher values for areas m and p and SCT titre at V_o and lower values of area n, FR-antigen ratio and T. C. P. ratio would, nevertheless, have to be accepted as "normal" in twin pregnancies.

There was no significant correlation between the gestational age (x) and area m (y). The equation calculated by linear regression analysis was $y = 6.340 - 0.060\,x$ using the data from the 14 uncomplicated single pregnancies ($\gamma = -0.266$, $2\alpha > 0.1$). If the

data from the 4 uncomplicated twin pregnancies was also included the equation became $y = 7.522 - 0.088x$ ($r = -0.320$, $2\alpha > 0.1$). A gradual increase in soluble complex concentration (using a measurement similar to area m) to a maximum of $4.9 \pm 1.3\%$ at term has been reported (Hafter, Schneebauer, Tafel et al., 1975). It may be that in the present series insufficient numbers of patients have been examined, especially in early pregnancy, to show a significant positive correlation. It might also be necessary to examine the same patient serially through pregnancy in order to demonstrate a correlation.

From this study it can be concluded that:-

(i) the soluble complex concentration was not raised in patients, who subsequently developed hypertension, if they were clinically normal at the time of study

(ii) the trend towards increased soluble complex concentration demonstrated in normal single pregnancies was slightly increased in normal twin pregnancies

(iii) for this small series there was no correlation between soluble complex concentration and gestational age.

VII. 3 Intrauterine Growth Retardation

Intrauterine growth retardation (dysmaturity, placental insufficiency, "small-for-dates" or "light-for-dates") is an important obstetrical complication, which may be associated with local intravascular coagulation within the placental circulation (see below).

There is a complicated inter-relationship between pre-eclampsia, hypertension in pregnancy and intrauterine growth retardation. Pre-eclampsia may be accompanied by intra-uterine growth retardation (Donald, 1969c) and indeed several of the babies born to the mothers studied in Group I (Chapter V)

were suffering from intrauterine growth retardation at birth (see Chapter VI. 3).

Increased fibrin deposition is a feature of placental histology in intrauterine growth retardation cases (when compared with placental histology from uncomplicated pregnancies) and this seems to be independent of whether the mother is hypertensive or not (Bonnar, Redman and Sheppard, 1975; Sheppard and Bonnar, 1976).

A variety of coagulation, fibrinolytic and platelet function tests have been used to study blood samples from patients with intrauterine growth retardation. One report has shown increased levels of plasma Factor V and fibrinogen in patients with intrauterine growth retardation compared with normal pregnant women (Elder and Myatt, 1976). In contrast another report has shown no difference in plasma Factors V and VIII or fibrinogen in patients with intrauterine growth retardation compared with normal pregnant women (Howie, Prentice and McNicol, 1971).

There is little evidence of altered systemic fibrinolysis in patients with intrauterine growth retardation. Howie and colleagues (1971) found normal euglobulin lysis times, normal urokinase sensitivity tests, normal plasma plasminogen levels and normal antiplasmin levels in a group of these patients. Conflicting results have been published regarding serum FDP/fdp levels. These have been found to be within normal limits (Howie et al., 1971), raised in a small percentage of patients (Gordon, Ratky, Sola et al., 1975) and lower than normal (Elder and Myatt, 1976) in different series. Placental extracts, however, from pregnancies complicated by intrauterine growth retardation exhibit greater inhibition of urokinase induced fibrinolysis using the fibrin plate technique, than placental extracts from normal pregnancies (Elder and Myatt, 1976).

Platelet counts are normal in intrauterine growth retardation patients (Howie et al., 1971; Elder and Myatt, 1976). Platelet factor 3, platelet adhesiveness and adenosine diphosphate induced aggregation are all normal in these patients, however, platelet disaggregation appears to be reduced (Howie et al., 1971).

When all this evidence is considered together it would not favour the occurrence of disseminated intravascular coagulation in these patients. An increased degree of local intravascular coagulation within the placenta might, however, be present in these patients, although this does not seem to produce marked changes in the peripheral maternal blood. The technique of plasma fibrinogen chromatography seemed particularly suitable to study these patients. In addition, it seemed important to determine any differences between the intrauterine growth retardation and pre-eclamptic patients. It was difficult to know to what extent the findings in the pre-eclamptic patients simply reflected local intravascular coagulation within the placenta.

A group of 10 patients in whom the diagnosis of intrauterine growth retardation was suspected on clinical grounds were therefore studied. After delivery this total group was divided into a sub-group in whom the diagnosis of intrauterine growth retardation was confirmed (5) and a sub-group in whom it was not (5). The diagnosis was confirmed if the baby's birth weight was below the 10th percentile for the appropriate sex and gestational age using the Lubchenco charts (Lubchenco, Hansman, Dressler et al., 1963). The clinical details are given in Table 56. All the patients were normotensive and none had significant proteinuria at the time of study and none of the patients subsequently developed pre-eclampsia. Patient 9 did, however, develop mild fluctuating hypertension (B.P. 140/90) at 37 weeks gestation. In 5 patients

(1, 2, 5, 8 and 9) the diagnosis of intrauterine growth retardation was confirmed at birth. In one case (Patient 2) the baby's birth weight was greater than 2.5 Kg (i.e. not "low birth weight" by some definitions, Hutchison, 1969). Nevertheless the baby's birth weight was below the 10th percentile on the appropriate Lubchenco chart and therefore the baby was suffering from intrauterine growth retardation according to definition used for this present study.

The results of plasma fibrinogen and serum FDP/fdp estimations for the total group (10 patients) are shown in Table 57. These are split into the diagnosis confirmed (5 patients) and not confirmed (5 patients) sub-groups in Table 58. The plasma fibrinogen chromatography results for the total group are shown in Table 59. These are split into the two sub-groups in Table 60.

Statistical analysis on the data from the two sub-groups is shown in Table 61. The gestational age did not differ significantly between the two sub-groups. The plasma fibrinogen level appeared to be significantly higher in the sub-group in which the diagnosis was confirmed, and while this may simply be due to chance ($2p < 0.02$) similar results have been published by Elder and Myatt (1976). There was no significant difference in the serum FDP/fdp levels. This is in keeping with the results from a much larger series using a radioimmunoassay (Gordon et al., 1975) although lower than normal serum FDP/fdp levels in these mothers have also been reported (Elder and Myatt, 1976). Area m was significantly higher in the sub-group in which the diagnosis was confirmed ($2p < 0.01$) while area p was also significantly increased in this sub-group ($2p < 0.05$). There was no significant difference in the SCT titre at V_o or in the FR-antigen or T.C.P. ratios.

Significant differences in areas m, n and p are also found when the data from the 5 patients in whom the diagnosis was confirmed was compared with that from the 14 normal pregnancies described earlier in this chapter (Table 62). There were no significant differences when the data from the 5 patients in whom the diagnosis was not confirmed were compared with that from the 14 normal pregnancies (Table 63).

It would therefore seem that increased soluble complex concentrations, as measured by area m (i.e. oligomers, probably mainly dimers) and early fibrinogen/fibrin degradation products, as measured by area p, are present in plasma samples from patients in whom the diagnosis of intrauterine growth retardation is subsequently confirmed on grounds of birth weight. The numbers are, however, very small. Subjective bias was unlikely as the results were calculated before the diagnosis was confirmed or refuted. The results would suggest increased formation or diminished clearance of soluble complexes (or both) in intrauterine growth retardation patients compared with normal pregnant patients. In addition there may either be an increase in fibrinogenolysis/fibrinolysis in the intrauterine growth retardation patients or diminished clearance of the early degradation products (or both).

The results in the 5 patients with confirmed intrauterine growth retardation were compared with those from the 10 samples from 10 pre-eclamptic patients (Group I, Chapter V) and the results of this statistical analysis are shown in Table 64. There was no difference in the plasma fibrinogen levels between the two groups. There were, however, higher values for area m and SCT titre at V_o and lower values for the FR-antigen and T.C.P. ratios in the pre-eclamptic patients. These findings would suggest higher levels of soluble complexes (both oligomers and polymers) in samples from

the pre-eclamptic patients. There was no significant difference, however, in the area p results, although the serum FDP/fdp levels were significantly higher in the pre-eclamptic patients. These findings could suggest comparable or only slightly increased fibrinogenolysis/fibrinolysis in the pre-eclamptic patients. Clearly the results in the intrauterine growth retardation patients show some overlap with the results in pre-eclamptic patients, but the marked abnormalities found in many of the pre-eclamptic patients are not seen in the intrauterine growth retardation patients.

Since the number of confirmed intrauterine growth retardation patients was so small, care must be taken not to make unwarranted conclusions from these results. It has, however, been shown that there are significant differences in the plasma fibrinogen chromatography results, particularly with respect to the soluble complexes measured in area m between:-

(i) Patients with suspected intrauterine growth retardation in whom the diagnosis is confirmed and those in whom it is not.

(ii) Patients with confirmed intrauterine growth retardation and a group of normal pregnant subjects.

(iii) Patients with confirmed intrauterine growth retardation and patients with pre-eclampsia.

In contrast there was no significant difference between the patients with suspected intrauterine growth retardation in whom the diagnosis was not confirmed and a group of normal pregnant subjects.

These results would be in keeping with the histological evidence that increased intravascular coagulation occurs within the placenta in pregnancies complicated by intrauterine growth retardation (Bonnar, Redman and Sheppard, 1975). The higher soluble complex concentration in association with pre-eclampsia might

suggest that in pre-eclampsia the intravascular coagulation occurs not only within the placental circulation, but is more widespread throughout the maternal circulation.

VII.4 Essential Hypertension in Pregnancy

It was decided to study a group of patients in whom essential hypertension had been diagnosed before the start of pregnancy, because it was important to establish whether or not the abnormalities found in the pre-eclamptic patients reflected changes invariably present in hypertensive, pregnant women. A previous study had failed to show any difference in coagulation, fibrinolytic or platelet tests between pregnant women with essential hypertension and normal pregnant subjects (Howie et al., 1971). It was necessary, however, to see whether there was any difference in plasma fibrinogen chromatography results between these groups.

To qualify for inclusion in this group the pregnant woman had to have been fully investigated for hypertension before the start of pregnancy and all causes of secondary hypertension excluded (Table 65). Patient 5 had a minor degree of renal artery stenosis, which was not thought to be of any clinical significance. Each patient had definite hypertension on the day of study, but none of the patients subsequently developed worsening hypertension or proteinuria suggesting the onset of superimposed pre-eclampsia. As it proved rather difficult to find patients who fulfilled all these criteria only 5 patients are considered in this section.

The clinical details are shown in Table 65. The plasma fibrinogen chromatography, plasma fibrinogen and serum FDP/fdp results are shown in Table 66. The statistical analysis comparing the results in essential hypertension with those in normal pregnancy are shown in Table 67. It can be seen that there is some difference in gestational age between the two groups (lower in the essential

hypertensive group 2p $\langle 0.05$). It proved so difficult to find suitable patients, that this had to be accepted. Apart from this and the obvious higher blood pressure readings in the essential hypertension group, no other statistically significant difference was found.

When the results in the essential hypertensive group were compared with those found in the 10 samples from the 10 pre-eclamptic patients (Group I, Chapter V) there was no difference in either systolic or diastolic blood pressure readings between the two groups, nor was there any difference in plasma fibrinogen levels (Table 68). Area m and SCT titre at V_o, however, were both significantly higher, while the FR-antigen and T.C.P. ratios were significantly lower in the pre-eclamptic group. The soluble complex levels (both oligomers and polymers) were therefore higher in the pre-eclamptic patients. Area p levels were not significantly different between the groups, although the serum FDP/fdp levels were significantly higher in the pre-eclamptic patients. It would therefore seem possible that fibrinogenolysis/fibrinolysis was comparable or only slightly increased in the pre-eclamptic patients.

From these results it can be concluded that:-

(i) there was no increased concentration of soluble complexes in pregnancies in which the mother suffered from essential hypertension, compared with pregnancies in which the mother was normotensive

(ii) there was a definite increase in soluble complex concentration in pregnancies complicated by pre-eclampsia, compared with pregnancies in which the mother suffered from essential hypertension with comparable systolic and diastolic blood pressure levels.

VII. 5 Chronic Renal Disease in Pregnancy

Finally a group of pregnant patients, in whom chronic renal disease had been documented before the start of pregnancy were studied. It is recognised that many cases diagnosed as "pre-eclampsia" during pregnancy will demonstrate significant renal pathology post-natally. Bedford and Taylor (1972) found an incidence of 38% renal pathology (demonstrated by intravenous pyelography carried out post-natally) in 100 women "with moderate, severe or recurrent pre-eclampsia". 8 out of the 10 patients described in Group I (Chapter V) were referred for renal investigation (including intravenous pyelography) post-natally. In all 8 cases no renal abnormality was found. It was therefore concluded that in at least 8 out of the 10 patients in Group I no significant renal disease was present, but it was important to establish the chromatography findings in patients with a combination of chronic renal disease and pregnancy.

It is difficult to conduct a study in chronic renal disease patients during pregnancy because, since hypertension and proteinuria are often present throughout pregnancy, it is virtually impossible to decide whether pre-eclampsia has developed in addition to the background renal disease. The aim of the present study was therefore to see whether the combination of chronic renal disease and pregnancy inevitably produced abnormalities in the plasma fibrinogen chromatography results.

To qualify for inclusion in this group the woman had to have been fully investigated before the start of pregnancy and a definite diagnosis of chronic renal disease made. As each of the 5 patients studied had either hypertension, proteinuria or both throughout most of the pregnancy it was not possible to make or exclude a clinical diagnosis of superimposed pre-eclampsia.

The clinical details are shown in Table 69. 4 out of the 5 patients were known to have chronic pyelonephritis while the remaining patient had systemic lupus erythematosus with renal involvement. The plasma fibrinogen chromatography, plasma fibrinogen and serum FDP/fdp results are shown in Table 70. The results are compared with the group of 14 normal single pregnancies in Table 71. Only the blood pressure, serum FDP/fdp and FR-antigen and T.C.P. ratio results differed significantly.

The results in the chronic renal disease patients were then compared with those from the 10 pre-eclamptic patients in Group I, Chapter V (Table 72). The blood pressure readings were comparable in the two groups, as were the plasma fibrinogen levels. Area m and the SCT titre at V_o were significantly higher, while the FR-antigen and T.C.P. ratios were significantly lower in the pre-eclamptic patients. These findings suggest higher soluble complex levels (both oligomers and polymers) in the pre-eclamptic patients. The area p and serum FDP/fdp results were similar in the two groups suggesting comparable fibrinogenolysis/fibrinolysis.

This study suggests that the combination of chronic renal disease and pregnancy does not <u>necessarily</u> produce marked abnormalities in the plasma fibrinogen chromatography results. The FR-antigen and T.C.P. ratios did tend to be slightly lower in the chronic renal disease patients compared with normal pregnant women, suggesting increased polymer in the renal disease patients, but this was the only significant difference. The results in the chronic renal disease patients were markedly different from those found in the pre-eclamptic group, suggesting a much higher soluble complex concentration in the pre-eclamptic patients. One additional interesting observation was the raised serum FDP/fdp levels found in the chronic renal disease patients compared with normal pregnancy. The levels were similar to those found in pre-

eclampsia. The raised levels in the chronic renal disease patients may reflect the diminished clearance of the low molecular weight fragments (D/d and E/e) in patients with loss of functioning renal tissue (Atsushi, Rutherford, Wochner et al., 1976) rather than increased production.

It would, however, be incorrect to conclude that increased soluble complex levels (as measured by area m) cannot be found in pregnant women with chronic renal disease. Patient 5 was followed serially, the results selected for statistical analysis were from the first blood sample taken. In Figure 59 the results of area m and p measurements are shown together with the clinical index on the day of study (see Chapter VI. 3 for formula). It can be seen that area m was markedly increased in the three samples taken closest to delivery. The rise in area m did accompany a phase of clinical deterioration, but since both hypertension and proteinuria were present throughout pregnancy, it is impossible to know whether this indicated the development of superimposed pre-eclampsia. In addition area p levels were slightly raised above normal pregnant values in 4 out of the 5 ante-natal samples. The birth weight of the baby was above the 10th percentile and he was in good condition at birth. It is therefore unlikely that the baby was seriously affected by the pre-eclamptic process if present.

From the results in this series of chronic renal disease patients it can be concluded that:-

(i) the plasma fibrinogen chromatography results may be within the normal limits for pregnancy in pregnant women with definite chronic renal disease, apart from a possible increase in polymer levels (FR-antigen and T.C.P. ratios). In particular the quantitative measurements (area m and SCT titre at V_o) were normal in the chronic renal disease patients studied.

(ii) Serum FDP/fdp levels tend to be increased in pregnant chronic renal disease patients compared with normal pregnancy.

VII. 6 Summary

The studies described in this chapter have shown that there may be a trend towards slightly higher soluble complex concentration in twin as compared with single pregnancies. It was not possible, however, to demonstrate a correlation between soluble complex concentration and gestational age.

Intrauterine growth retardation was accompanied by a significant increase in soluble complex concentration, when compared with that occurring in cases where the diagnosis was suspected, but not confirmed and also when compared with normal pregnancy. The increases found in intrauterine growth retardation were not as marked, as those already demonstrated in pre-eclampsia. There would therefore seem to be a difference between pre-eclampsia and intrauterine growth retardation. This may result from the intravascular coagulation being limited to the placental vasculature in the intrauterine growth retardation patients and being more widespread throughout the maternal circulation in pre-eclampsia.

The combination of essential hypertension and pregnancy did not appear to be associated with a significant increase in soluble complex concentration. It is therefore unlikely that the increased soluble complex concentration found in the pre-eclamptic patients was simply secondary to the hypertension. Finally, it was shown that the combination of chronic renal disease and pregnancy did not necessarily lead to the increases in soluble complex levels found in the pre-eclamptic patients.

CHAPTER VIII

SOLUBLE FIBRINOGEN-FIBRIN COMPLEXES IN ASSOCIATION WITH OESTROGEN-CONTAINING ORAL CONTRACEPTIVE DRUGS

VIII. 1 Introduction

Women receiving oestrogen-containing oral contraceptive drugs suffer from an increased incidence of thrombo-embolic episodes (Royal College of General Practitioners, 1967; Inman and Vessey, 1968; Vessey and Doll, 1968; Collaborative Group for the Study of Stroke in Young Women, 1973 etc.). Such women, therefore, might be regarded as suffering from a "hypercoagulable" state, at least in the epidemiological sense.

In his recent review article on the subject of oestrogens and blood coagulation, von Kaulla (1976) pointed out that the acceleration of blood clotting by oestrogens was well known before the time that oral contraceptive drugs were introduced. He quotes the observation by Bablik (1935) that "the investigations of various authors indicate that clotting is strongly promoted by follicular hormone".

The studies described in this chapter used the technique of plasma fibrinogen chromatography to investigate this "hypercoagulable" state, because of the possible similarity to the "hypercoagulable" state occurring in normal pregnancy (Chapter VIII. 2). In addition, a group of women diagnosed as suffering from "pill-related hypertension" were studied, because of the possible similarity of this condition to "pregnancy-related hypertension" or pre-eclampsia (Chapter VIII. 3).

A short account of the relevant literature on each subject will be discussed first, followed by the design of the study and the results that were obtained.

VIII. 2 Healthy (Normotensive) Women Taking Oestrogen-Containing Oral Contraceptive Drugs

There are several reasons for suggesting that women taking such drugs suffer from a "hypercoagulable" state. The epidemiological evidence already mentioned has shown an increased incidence of venous thrombo-embolism and cerebral thrombosis or ischaemia in these women. These women may also suffer from a "hypercoagulable" stage in the haematological sense (many papers including Howie, Mallinson, Prentice et al., 1970).

The alterations in the components of the coagulation and fibrinolytic systems together with changes in platelet function have been summarised by von Kaulla (1976):-

(1) Coagulation system:

Increases in plasma fibrinogen, prothrombin and Factors V, VII, VIII, IX and X have been demonstrated.

(2) Fibrinolytic system:

Increases in plasma plasminogen, antiplasmin and decreased plasminogen activator in the venous wall have been found.

(3) Platelet function:

Increased platelet aggregability has also been demonstrated. In addition to these changes von Kaulla mentions diminished antithrombin III activity and increased "soluble fibrin".

Abnormal or activated forms of fibrinogen have been detected in these women as cryofibrinogen (Pindyck, Lichtman and Kohl, 1970), as plasma soluble fibrinogen-fibrin complexes (Alkjaersig, Fletcher and Burstein, 1970; Asbeck, Bebber and van de Loo, 1974; Alkjaersig, Fletcher and Burstein, 1975) and as soluble

fibrin (Pilgeram, Ellison and von dem Bussche, 1974).

There is conflicting evidence regarding the production of soluble complexes and soluble fibrin by incubation of fibrinogen with oestrogens in vitro. The original work suggested that this did occur (Pilgeram and von dem Bussche, 1974; Pilgeram and von dem Bussche, 1975) but these claims have not been supported by other studies (Gaulton and Doolittle, 1975; Stemberger, Hafter, Graeff et al., 1976).

The present in vivo study was undertaken with two main aims. Firstly, to try to confirm the results of previous studies and secondly, to compare the results with those found in normal pregnancy. Such a comparison is clearly of interest in helping to unravel the problem of relating the plasma fibrinogen chromatography results to the different forms of intravascular coagulation.

Study and Results

Ten healthy, normotensive women were studied. They all had taken a form of oestrogen-containing oral contraceptive drugs for a minimum of 3 months. The actual preparation varied but each contained 50 micrograms of either ethinyl-oestradiol or mestranol. As the numbers were small no attempt was made to compare different preparations. Each woman was taking the drug on the day of study. The packed gel columns used for this study were:-

Columns C5 and C6 (Batch II)
B5 and B7 (Batch II).

(Details regarding these packed columns can be found in Appendix I).

The results obtained are shown in Tables 73 and 74. They were first compared with the results from a group of normotensive, non-pregnant women who were not taking oestrogen-containing drugs (i.e. Group III in Chapter V). The results of the statistical analysis

are shown in Table 75. It can be seen that only one result (area m) differed significantly between the two groups. This would suggest increased soluble complex concentration of the oligomer type. If the upper limit of normal for area m in the non-pregnant women not taking oestrogen-containing drugs is taken as 3.5% (mean + 2 S.D.) 8 out of the 10 women taking such preparations had values for area m above this. These results are broadly speaking in agreement with those of Alkjaersig and colleagues (1975) and Asbeck and colleagues (1974) although the method of calculation of the soluble complex concentration is different. In particular, the results resemble those of Asbeck and colleagues (1974), who claim to have demonstrated the appearance of a dimer in plasma samples from women taking oestrogen-containing oral contraceptive therapy by comparing results before and during treatment.

The results in the women taking the oestrogen-containing drugs were then compared with those found in normal single pregnancies (i.e. the 14 patients in Chapter VII.2). The results of the statistical analysis are shown in Table 76. The plasma fibrinogen levels were significantly higher in the pregnant women compared with those taking oestrogen-containing drugs. The SCT titre at V_o was significantly higher in the pregnant patients and the FR-antigen and T.C.P. ratios significantly lower. These results suggest that there is a higher concentration of polymer in the pregnant patients, than in those taking the oestrogen-containing drugs. There was, however, no difference between the two groups in measurements of area m and this would suggest that the concentration of dimer is similar. Although the pregnant patients tended to have higher levels of area p (early degradation product) this failed to reach statistical significance, although the serum FDP/fdp levels were significantly

higher in the pregnant group.

The results from this study can be summarised:-

(i) There are increased soluble complex levels (oligomers, probably dimers) in the plasma samples from women taking oestrogen-containing oral contraceptive drugs, compared with a control group of women not taking these drugs.

(ii) Although there appears to be no significant difference between the soluble complex concentrations of the oligomer/dimer type between samples from healthy women taking oestrogen-containing drugs and normal pregnant women, there is an increase in polymer concentration in pregnancy.

(iii) Plasma fibrinogen and serum FDP/fdp levels were both significantly higher in normal pregnancy than in women taking oestrogen-containing drugs but there was no difference in plasma fibrinogen and serum FDP/fdp levels between women taking oestrogen-containing oral contraceptive therapy and those who did not.

VIII.3 Hypertensive Women Taking Oestrogen-Containing Oral Contraceptive Therapy

One complication of long-term oral contraceptive therapy is an increase in the blood pressure. In a controlled prospective study significant increases in both mean systolic and diastolic blood pressure were found after 4 years treatment, the blood pressure returning to pre-treatment levels within three months of discontinuing therapy (Weir, Briggs, Mack et al., 1974). A small percentage of women (5-7%) actually become hypertensive (B.P. $>140/90$) while taking these drugs (Spellacy and Birk, 1972). It has been suggested that a history of pre-eclampsia may be a predisposing factor in such cases (Spellacy and Birk, 1972). It is known that this "pill-related hypertension" may be reversible on

discontinuing treatment (Weir, Tree and McElwee, 1973).

The concept of a definite diagnostic syndrome of "pill-related hypertension" has been strengthened by the demonstration of abnormalities of the peripheral renal vessels in these patients by angiography and histology (Boyd, Burden and Aber, 1975). The vessels may show irregular narrowing, beading and increased tortuosity but no evidence of microthrombi. Microthrombi were only found in patients, who had remained hypertensive after pregnancies complicated by pre-eclampsia and were taking oestrogen-containing oral contraceptive drugs or in patients developing acute oliguric renal failure while taking these drugs.

Study and Results

Five patients in whom the diagnosis of "pill-related hypertension" had been made were examined. They had all been taking oestrogen-containing oral contraceptive therapy for several years (range $2\frac{1}{2}$ - 9 years). In each case the woman had been known to be normotensive before starting therapy and systemic hypertension had developed while taking therapy. Each patient had been referred by the Family Planning Association to Dr. R. Weir (Gartnavel General Hospital, Glasgow), who made the diagnosis on the basis of:-

(i) sustained hypertension developing while taking the drug

(ii) no other obvious cause of secondary hypertension.

The possibility that essential hypertension had developed quite fortuitously while the patient was on therapy clearly existed.

All five patients had significant hypertension on the day of study and 4 out of the 5 had taken oestrogen within the previous 24 hours. The remaining patient had taken the last tablet of a three week course, two days before the time of blood sampling. The

other clinical details are shown in Table 77.

All 5 patients discontinued therapy shortly after they had been studied. On review three months later 3 out of the 5 patients showed a definite fall in blood pressure (Patients 3, 4 and 5). One patient showed worsening of hypertension and was started on antihypertensive therapy (Patient 1), while the remaining patient (2) showed a fluctuating pattern of blood pressure readings, similar to those found before stopping therapy. Three out of the five patients may therefore have had "reversible" hypertension.

The following packed agarose columns were used for this study:-

Column B10 (II)
Column C10 (II)
Column C12 (II)
Column C13 (II)

(Details regarding these packed columns can be found in Appendix I).

The results obtained in this group of patients are shown in Tables 77 and 78. The statistical analysis comparing the results with those obtained in the normotensive women taking oestrogen-containing oral contraceptive drugs is shown in Table 79. The hypertensive group were significantly older and this presumably reflects the increased incidence of hypertension with increasing age. The hypertensive women also had significantly higher plasma fibrinogen and serum FDP/fdp levels. The only significant differences in the chromatography results were higher SCT titres at V_o and lower FR-antigen ratios in the hypertensive group. These findings suggest increased polymer, but not increased dimer in the hypertensive patients.

The findings in this study can be summarised:-

(i) Soluble complex concentration as measured by area m (i.e. oligomer or dimer) does not differ significantly between a group of hypertensive and a group of normotensive women taking

oestrogen-containing oral contraceptive drugs.

(ii) Increased levels of polymer as measured by SCT titre at V_o and the FR-antigen ratio may, however, be present in the hypertensive women, but the relative importance of polymer as compared to oligomer (dimer) is not yet understood.

(iii) Plasma fibrinogen and serum FDP/fdp levels were both higher in the hypertensive patients.

VIII.4 Summary

The two studies described in this chapter have shown a significant increase in soluble complex of oligomer (dimer) type in plasma samples taken from women receiving oestrogen-containing oral contraceptive therapy. It is impossible to say whether this is due to pure "hypercoagulability" or whether some degree of insoluble fibrin deposition has occurred.

This tendency would seem to be increased, at least in the formation of polymers, in samples from women who have become hypertensive while taking these drugs, but again the relationship of this observation to insoluble fibrin deposition requires further clarification.

CHAPTER IX

CONCLUSION

IX. 1 Introduction

In this thesis the biochemistry of fibrinogen has been discussed in detail with particular reference to the formation of soluble fibrinogen-fibrin complexes (Chapter I). To allow identification of these soluble complexes the technique of plasma fibrinogen chromatography was set up (as described in Chapter II) and the methodology checked in a series of *in vitro* experiments (Chapter III) and in studies of the *in vivo* acute defibrination state induced by intravenous ancrod infusion (Chapter IV).

Samples from normal pregnant women and patients with pre-eclampsia were then studied (Chapters V and VI) and the results compared with those found in several obstetrical conditions related to pre-eclampsia (Chapter VII) and with those found in normotensive and hypertensive women taking oestrogen-containing oral contraceptive therapy (Chapter VIII).

Discussion of the work in this thesis can be considered under two main headings, the first dealing with an assessment of the technique itself and the second with the significance of the results as evidence of intravascular coagulation.

IX. 2 An Assessment of the Technique of Plasma Fibrinogen Chromatography

The following questions have to be answered:-

(a) What conclusions can be drawn from the various measurements obtained using the plasma fibrinogen chromatography technique?

How do these measurements relate to each other and to the plasma fibrinogen and serum FDP/fdp results?

(b) What practical problems are associated with the plasma fibrinogen chromatography technique?

(c) What modifications could be introduced to improve the technique?

(d) Is the plasma fibrinogen chromatography technique ever likely to be useful in the clinical diagnosis and management of patients or does it solely have a role at a research level?

These questions will be discussed in turn beginning with a summary of the results.

IX.2(a) Summary of the Results in this Thesis

There are two main types of abnormality found using plasma fibrinogen chromatography - the first indicating the presence of soluble fibrinogen-fibrin complexes and the second indicating the presence of early fibrinogen-fibrin degradation products:-

(1) The presence of increased soluble complex levels in a patient group is suggested, when compared with an appropriate control group, there are:-

(i) increased SCT titres at V_o

(ii) lower FR-antigen and T.C.P. ratios

(iii) increased amounts of FR-antigen in area .

Area m probably identifies oligomers (mainly dimers), while the SCT titre at V_o identifies polymers of high molecular weight. The ratios give an indication of the molecular weight of fibrinogen related material first eluted from the packed gel column.

(2) The presence of increased early fibrinogen-fibrin degradation product concentration in a patient group is suggested by finding increased amounts of FR-antigen in area p when the results are compared with those from an appropriate control group.

A summary of the results (mean $\pm$ 1 S.D.) is shown in Table 80. The results from a group of patients with established local venous thrombosis (Appendix IV) are also included for comparison. The scatter of results for area m, the SCT titre at V_o and area p are shown in Figures 60, 61 and 62 respectively.

(i) Plasma fibrinogen levels (Table 80)

Plasma fibrinogen levels rose in association with normal pregnancy. Slightly lower than normal values were found in the total pre-eclamptic group, but the individual results were not helpful in distinguishing pre-eclamptic patients.

(ii) Area m (Table 80 and Figure 60)

Although this was at best only a semi-quantitative method of measuring the soluble complex concentration in a sample, it proved useful in distinguishing between the different clinical groups studied. Increased levels of area m were found in association with normal pregnancy and oestrogen-containing oral contraceptive therapy, when compared with appropriate control subjects. In the obstetrical patients a further increase was found in association with intrauterine growth retardation and pre-eclampsia, but not in samples from patients with essential hypertension. Increased levels were found after 6 hours of intravenous ancrod infusion.

Area m was inevitably affected by quite small errors in the radial immunodiffusion (Mancini) technique, particularly in the dimer molecular weight range. Such errors were liable to lead to an over-estimation of the amount of antigen in area m. In addition, as shown in Chapter III, the radial immunodiffusion assay was not a good method

of measuring the amount of polymer present, which was likely to have been underestimated. It is possible that these two sources of error compensated for each other to some extent, but it is still likely that area m was overestimated in some cases.

Area m was less likely to have been affected by changes in the separation characteristics of the agarose gel medium than the ratio tests. It was also less likely to have been affected by changes in the sensitivity of the assay than the FR-antigen ratio or the SCT titre. Overall it proved the best test for distinguishing between the different groups.

(iii) SCT titre at V_o (Table 80 and Figure 61)

There was considerable overlap in the results obtained in the different groups. Particularly high titres were found in samples from patients with pre-eclampsia and after 6 hours of intravenous ancrod therapy. No positive tests were recorded in samples from a group of 10 healthy control women but the other groups showed considerable overlap. One problem was almost certainly variation in the sensitivity of different batches of staphylococcal clumping factor. Incorporation of a standard fibrinogen solution into the test-system would have helped avoid this problem, but would also have limited the number of other tests which could have been performed on each batch. The test was also affected by the inevitable inaccuracy in performing a doubling dilution technique and difficulty in determining the end-point. The SCT titre at V_o was used as a test for soluble complexes of high molecular weight (i.e. polymers). It was better than the ratios in this respect, because it achieved a degree of quantitation.

(iv) FR-antigen and T.C.P. ratios (Table 80)

Despite a degree of overlap these tests proved useful in distinguishing between the different groups. A low ratio identified

the presence of polymer, but gave no indication as to amount. The ratios tended to fall in association with normal pregnancy but not with oestrogen-containing oral contraceptive therapy. Low ratios were found in association with pre-eclampsia and after 6 hours intravenous ancrod therapy. The ratio tests were inevitably affected by the particular batch of gel medium.

(v) Area p (Table 80 and Figure 62)

This was the only test used to identify early fibrinogen-fibrin degradation products and there was considerable overlap in the range of values between the groups. Higher values were found in some patients with intrauterine growth retardation and pre-eclampsia compared with pregnant control subjects, while very high values were found after 6 hours intravenous ancrod infusion.

Most of the difficulties associated with the measurement of area m also applied to area p. In particular overestimation of area p was likely to be caused by the "trailing effect" found with several technical faults (e.g. non-uniform cross-sectional bed resistance). Such faults could usually be detected by examination of the O.D. traces and the sample discarded, but minor degrees probably escaped detection.

(vi) Serum FDP/fdp levels (Table 80 and Figure 63)

Serum FDP/fdp levels rose slightly in association with normal pregnancy. They tended to increase further in association with pre-eclampsia, but individual results were not particularly helpful in distinguishing the pre-eclamptic patients.

The question as to how these individual measurements relate to each other has already been considered (Chapter V.4). A much larger number of samples were included in the linear regression analysis results shown in Table 81. Although there

was a wide scatter in the results, several significant correlations were found.

The different methods of detecting and measuring soluble complexes showed significant degrees of association, as might have been expected (e.g. SCT titre at V_o and FR-antigen ratio). These results would suggest that oligomers and polymers tend to be increased in the same conditions.

There was, however, no significant correlation between area m or area p and the plasma fibrinogen levels. It is therefore unlikely that changes in plasma fibrinogen levels affected the results (except perhaps in normal pregnancy, see Chapter V.4).

The serum FDP/fdp and area p results gave a correlation coefficient of 0.198, which reached a borderline level of significance ($2 \alpha < 0.05$) because of the large numbers involved, but the degree of correlation was obviously not close. Although both assays measure degradation products the serum FDP/fdp assay will only measure non-clottable degradation products, while most of the material in area p is probably clottable. There was no significant correlation between the serum FDP/fdp and area m results.

It should be noted also that there was a significant degree of correlation between area m and area p measurements. This could simply reflect a tendency for both to be increased in the same samples, but might also indicate that the one is derived in some way from the other.

Despite the broad correlation between the measurements used to assess the chromatography results, the separation between the four main groups studied (i.e. the non-pregnant control group, Group III, Chapter V.4; the pregnant control group, Chapter VII.2; the total pre-eclamptic group, Chapter VI.4 and the normotensive women taking oestrogen-containing oral contraceptive therapy or the "pill", Chapter VIII.2) could be improved by considering more

than one measurement. This was particularly true when measurements of area m (oligomer) and the FR-antigen ratio (polymer) were considered for the same sample (Figure 64a and b). It can be seen that there is a progression towards increasing abnormality starting with the samples from the normal non-pregnant women and extending through those from women taking oestrogen-containing oral contraceptive drugs and normal pregnant women, to those from pre-eclamptic patients. There clearly might be some advantage in using two of the results from a single sample in this way.

It might also be possible to determine whether the predominant abnormality in a plasma sample was one of increased coagulation, increased fibrinolysis/fibrinogenolysis or a balanced increase in coagulation and fibrinolysis/fibrinogenolysis by using the measurements of area m and area p to reflect coagulation and fibrinolysis/fibrinogenolysis respectively. In Figure 65 the graph is divided in half by the line indicating balanced coagulation and fibrinolysis/fibrinogenolysis (area m = area p). When an individual sample is plotted on this graph if it lies above this dividing line the predominant abnormality is coagulation. Similarly, if the point lies below the dividing line the predominant abnormality is fibrinolysis/fibrinogenolysis. It is also obvious that the further a point lies from zero, the greater is the degree of abnormality.

As would be expected when the four main groups are examined in this way (Figure 66 a-b) the pre-eclamptic patients show the most marked degree of abnormality. It is interesting that 26 out of the 35 samples from the pre-eclamptic patients, while only 5 out of the 14 samples from the pregnant controls and 3 out of the 10 samples from the non-pregnant controls lie above the dividing line. These observations stress the fact that the

predominant abnormality in pre-eclampsia appears to be increased coagulation rather than fibrinolysis/fibrinogenolysis, while in samples from control subjects in which only minor abnormalities are present, fibrinolysis/fibrinogenolysis is slightly more marked than coagulation. In the samples from women taking oestrogen-containing oral contraceptive therapy 7 out of the 10 samples lie above the line, suggesting rather more marked coagulation than fibrinolysis/fibrinogenolysis in these subjects.

IX. 2(b) Problems with the Plasma Fibrinogen Chromatography Technique

Some of these have been discussed at various stages throughout this thesis. It has to be stressed that the technique is laborious and time consuming. Only one sample can conveniently be handled each day when only one set of fractionation equipment is available. If blood samples are analysed immediately after collection, inevitably much evening and weekend work is required. This is especially true when studying samples from acutely ill patients.

The agarose gel columns are prone to develop minor and major faults some of which (e.g. clogging of the bed support) may not be readily recognised until the filtration experiment is complete. Meticulous attention to detail is required at every stage, when omitted this usually leads to the sample having to be discarded. On average 2 out of every 10 samples studied for the projects described in this thesis had to be discarded, because of problems with separation at the gel filtration stage or with the radial immunodiffusion assay of the eluant fractions. It is possible that some samples were included in this thesis in which minor technical faults were not detected.

IX. 2(c) Modifications which could be Introduced to Improve the Plasma Fibrinogen Chromatography Technique

The technique as described in Chapter II could probably be

simplified in several ways without too much loss of information. The β -alanine precipitation step could be omitted and this would also have the advantage of reducing the volume of blood required for the technique (only 2 ml of plasma would be needed instead of 9 ml). Time would be saved (approximately 1 hour) and the sample could be applied to the agarose column within 30 minutes of venesection in most cases.

The disadvantage would be the presence of higher concentrations of the other plasma proteins. Optical density traces would therefore be of even less value and polyacrylamide gel electrophoresis without further purification would be impossible.

In the analysis of the eluant fractions the radial immunodiffusion (Mancini) assay might be sufficient on its own. Areas m and p and the FR-antigen ratio could all be calculated. Although the SCT titre at V_o, T.C.P. ratio and abnormalities in the T.C.T. curve add some further useful information, they could probably be omitted.

An alternative way of obtaining results more rapidly has been described by Graeff and colleagues (Hafter, Schneebauer, Tafel et al., 1975). The β -alanine precipitation step is still included, but the analysis of the eluant fractions is limited to optical density measurements at 325 and 280 nm. The first measurement is subtracted from the second to obtain an estimate of the amount of FR-antigen present. Graeff and colleagues claim to find this method quite satisfactory.

IX. 2(d) Clinical Applications of the Plasma Fibrinogen Chromatography Technique

The time delay in obtaining results with this technique (about 3 days) would seem to seriously handicap its use in the diagnosis and management of thromboembolic disease and disseminated intravascular

coagulation, where rapid laboratory tests are essential. Fletcher and colleagues have introduced several modifications to the technique (e. g. small packed gel columns, automated FR-antigen analysis and a computerised calculation of results) which in their hands appear to produce accurate results rapidly (Alkjaersig, Roy and Fletcher, 1973). These changes would require a major financial outlay, which would have to be justified by the quality and value of the results obtained.

Quite apart from the time delay, the difficulty in interpreting the results in terms of the type of intravascular coagulation (see later, Chapter IX. 3) would seem to limit the clinical usefulness of the technique, but it is possible that it has a role at a research level. Fletcher and Alkjaersig (1973) suggest that the technique might be particularly useful in determining "in vivo drug efficacy", but while this is possible there remain problems over the interpretation of the results. Diminishing soluble fibrinogen-fibrin complex levels could indicate their removal from the circulation by the reticulo-endothelium system or some other mechanism without insoluble fibrin deposition, but another explanation would be the precipitation of the soluble complexes as insoluble fibrin thrombi or micro-thrombi. The first would seem desirable, while the second would not.

The time involved in analysing a single sample is clearly a drawback to building up a large series of samples on any one group of patients. The technique would therefore seem to be of limited use in clinically orientated projects. It might seem more sensible to put the time and effort involved in the plasma fibrinogen chromatography technique into a more specific test for soluble fibrin. This would be particularly true if this test could be performed more rapidly. Affinity chromatography, although still a somewhat

laborious technique, does hold promise. Other newer tests (e.g. using erythrocytes coated with soluble fibrin monomer described by Largo, Heller and Straub, 1976) would seem worth investigating further.

IX.3 The Significance of Abnormal Plasma Fibrinogen Chromatography Results

What do these results (particularly the increased soluble complex concentration) mean regarding the different forms of intravascular coagulation (see Preface)? It was confirmed in Chapter III that soluble complexes could be produced in vitro by:-

(i) the incubation of fibrinogen with low concentrations of a coagulant enzyme (i.e. thrombin or ancrod)

(ii) the incubation of early fibrinogen degradation products with a coagulant enzyme

(iii) the lysis of insoluble fibrin.

It is probable, though not certain, that similar mechanisms operate in vivo. The formation of soluble complexes should therefore reflect the action of thrombin on fibrinogen with the formation of fibrin monomer. Soluble complexes might also be produced by F XIIIa crosslinking fibrinogen, but this would probably also imply a degree of activation of the coagulation pathway (see Chapter I. 5(b)). Soluble complexes in plasma samples should therefore indicate activation of the final stage of the coagulation pathway and to this extent intravascular coagulation.

The fact that soluble complexes are present in samples analysed by agarose gel filtration at room temperature does not necessarily mean that they are present in the blood circulating in vivo at 37°C. This question has already been discussed in Chapter IV. 5. It is possible that soluble complexes demonstrated at room temperature composed of units with degraded α chain are not polymerised at 37°C (Edgar, McKillop, Howie et al., 1976),

although work currently in progress suggests that the situation in relation to complex formation at different temperatures may be even more complicated than this.

It must be stressed, however, that these observations do not alter the basic premise that soluble complexes provide biochemical markers of intravascular coagulation. For the reasons discussed in detail in Chapter I. 5 and 6 it still seems highly probable that they do.

What conclusions can be made from the results described in Chapters IV-VIII? The results themselves have already been summarised in Table 80 and Figures 60-63. How do they relate to the different forms of intravascular coagulation (i. e. hypercoagulability, local thrombosis, local and disseminated intravascular coagulation, see Preface)? The main conditions studied in this thesis will be considered separately:-

(i) Normal Non-Pregnant Women (Group III, Chapter V. 4)

In this group there is very little evidence of intravascular coagulation. The soluble complex and degradation product concentrations in areas m and p are likely to have been overestimated by about 2 percent, because of the "tails" in the Gaussian type elution profile found with a pure protein are not included in calculating area n. Therefore the amount of soluble complex or degradation product in area m and area p is even less than is suggested by the actual values. A minor degree of fibrinogenolysis/fibrinolysis is suggested by the low levels of serum FDP/fdp and the tendency for area p values to exceed area m values.

(ii) Normotensive Women taking Oestrogen-Containing Oral Contraceptive Therapy (Chapter VIII. 2)

In this group there is some evidence of increased coagulation as evidenced by increased oligomer/dimer concentration (area m

results), but there is no evidence of increased fibrinogenolysis/fibrinolysis compared with a non-pregnant control group. These findings may reflect pure hypercoagulability, although oestrogens might be able to alter fibrinogen metabolism producing soluble complexes without activation of the coagulation pathway. There is, however, little experimental evidence to support this second suggestion.

(iii) Normal Pregnant Women (Chapters V. 4 and VII. 2)

In this group there is evidence to suggest increased coagulation (area m and SCT titre at V_o results) and possibly increased fibrinolysis (serum FDP/fdp results) compared with the non-pregnant control group. These results could suggest pure hypercoagulability but might also reflect a degree of local intravascular coagulation within the maternal side of the placental vasculature.

(iv) Intrauterine Growth Retardation Patients (Chapter VII. 3)

In this group of patients there was evidence of increased coagulation (area m results) and increased fibrinogenolysis/fibrinolysis (area p results) compared with the normal pregnant women. These findings could reflect increased local intravascular coagulation within the placenta in the intrauterine growth retardation patients.

(v) Essential Hypertension in Pregnancy (Chapter VII. 4)

No differences were found between this group of patients and the normal pregnant control group. There would therefore seem to be no difference in the extent of intravascular coagulation between the two groups (i. e. hypercoagulability and/or local intravascular coagulation in the placenta).

(vi) Pre-eclampsia (Chapters V and VI)

In these patients there is evidence to suggest increased

coagulation (area m and SCT titre at V_o results) and increased fibrinogenolysis/fibrinolysis (area p and serum FDP/fdp results) compared with the normal pregnant control group. The results are more markedly abnormal than those found in the other obstetrical groups and could reflect widespread low-grade disseminated intravascular coagulation.

(vii) Intravenous Ancrod Infusion (Chapter IV)

In this clinical situation, in which a controlled dose of a coagulant enzyme is infused into the circulation, there is evidence of both increased coagulation (area m and SCT titre at V_o results) and increased fibrinogenolysis/fibrinolysis (area p and serum FDP/fdp results). The results in pre-eclampsia and ancrod therapy differ most markedly in the considerable evidence for fibrinogenolysis/fibrinolysis found in the ancrod patients. It may be that the difference between the two conditions lies in the capacity of the fibrinolytic system to rapidly lyse micro-clots in the ancrod patients, a capacity which is inhibited (or partially inhibited) in patients with pre-eclampsia. As it is possible that insoluble fibrin micro-clots may not form during ancrod therapy, it is impossible to determine whether ancrod infusion is an example of gross hypercoagulability or widespread disseminated intravascular coagulation. It is certain, however, that ancrod infusion is a good example of acute defibrination.

(viii) Local Thrombosis

This condition has not been studied in detail in this thesis, but abnormalities have been detected in plasma samples from several patients with venographic evidence of local thrombosis (see Table 80 and Appendix IV). Although definite and highly significant abnormalities are present in plasma samples from these patients, the markedly abnormal soluble complex results (area m and SCT titre at V_o) seen in the ancrod and pre-eclamptic

patients were not found in the local thrombosis patients.

While it is, of course, not completely possible to exclude the presence of local thrombosis in the obstetrical and other groups studied in Chapters V-VIII, the markedly abnormal results found in pre-eclampsia are most unlikely to have been associated simply with local thrombosis. It is also not possible to be certain that the local thrombosis patients were not in addition suffering from some other form of intravascular coagulation (e.g. 2 of the 6 patients studied had malignant disease with secondary spread and might have had a degree of unsuspected low-grade disseminated intravascular coagulation).

While it is possible to interpret the results in this thesis as examples of hypercoagulability, local thrombosis, local and disseminated intravascular coagulation, all that has really been shown is a difference in the degree of abnormality and it is only possible to speculate as to how such abnormalities relate to underlying types of intravascular coagulation (Figure 67). It must be appreciated that although soluble fibrinogen-fibrin complexes probably represent reaction products of the limited proteolysis of fibrinogen by thrombin, their presence does not indicate the site or sites at which this reaction occurs, whether insoluble fibrin has been deposited and if it has been, whether it is in the form of a single thrombus or multiple microthrombi.

IX.4 Final Comments and Aspects for Further Investigation

Pre-eclampsia obviously involves the complicated interaction of many factors (Chapter V.1 and 2). Page (1972) has produced the "vicious circle" theory of pre-eclampsia and eclampsia which has been slightly modified to include the conditions studied in this thesis in Figure 68. There are clearly several points of entry into this circle and although highly speculative it does explain many

of the clinical and biochemical features of pre-eclampsia including the presence of soluble fibrinogen-fibrin complexes.

As a working hypothesis the occurrence of low-grade disseminated intravascular coagulation in pre-eclampsia is attractive. Whether involved in the primary aetiology of the syndrome or occurring as a secondary complication to an unknown primary pathology, disseminated intravascular coagulation explains many of the signs and symptoms found in pre-eclamptic patients.

The confusion over terminology in the field of intravascular coagulation has been discussed elsewhere (see Preface). It was suggested that there is a need for a term, such as "haematological disseminated intravascular coagulation", to apply to circumstances in which the presumptive diagnosis of disseminated intravascular coagulation is based simply on the results of blood tests and other investigations, without histological evidence of insoluble fibrin deposition in several organs. Where such evidence is available another term, such as "histological disseminated intravascular coagulation" could be used.

The evidence currently available in the literature (Chapter V. 2) and that produced in the original work in this thesis strongly supports an association between "haematological disseminated intravascular coagulation" and pre-eclampsia. There is, of course, also evidence of insoluble fibrin deposition within the kidney, liver and placenta of patients with severe pre-eclampsia. Thus histological disseminated intravascular coagulation may also be present in this syndrome.

If disseminated intravascular coagulation is present in these patients, there is the possibility that it might prove amenable to some form of treatment. It is possible that although conventional anticoagulant and anti-platelet therapy (heparin and dipyridamole) may not be helpful in patients with established severe pre-eclampsia

(Howie, Prentice and Forbes, 1975), such therapy might be more useful if started earlier in the clinical course of the syndrome. This might be particularly true if therapy were started "prophylactically" in "high-risk" patients (see Appendix III). While it might well be worth attempting such a study, there also remains the possibility that newly developed drugs might prove more useful in this clinical situation and should therefore be evaluated. The plasma fibrinogen chromatography technique might have a role to play in the laboratory assessment of such studies.

Although it is probable that in due course the plasma fibrinogen chromatography technique will be replaced by more specific, rapid techniques for identifying and measuring soluble fibrin, it has played a useful research role in contributing to the study of intravascular coagulation.

To those sceptical about the technique it should be pointed out that when performed carefully, abnormal results are only found in patients in whom there is some other good reason to suspect a degree of intravascular coagulation. There is also at present no other single technique which will demonstrate the presence of soluble complexes and uncomplexed early fibrinogen-fibrin degradation products and distinguish between the two.

Intravascular coagulation in a variety of disease processes remains an important cause of morbidity and mortality in many branches of medicine. The study of soluble fibrinogen-fibrin intermediates (whether as soluble complexes or as soluble fibrin) may well have a role to play in the improved clinical diagnosis and management of the different varieties of intravascular coagulation. The need is for a rapid, specific and sensitive test for the detection of these intermediates.

Appendix I

Variations in the V_e of Fibrinogen in the Column Systems used throughout this Thesis

It proved impossible to obtain exact identity of the elution profiles of purified fibrinogen or normal plasma using different packed gel column systems and indeed some variation occurred even using the same column system on different occasions. A short-hand method of identifying each packed gel column was devised:-

(1) Each empty column apparatus was identified using a capital letter (e.g. Column A, B, C, etc.).

(2) Each packed gel column was identified using an Arabic numeral following the letter for the particular column apparatus (e.g. Column A1, B2, etc.).

Column A had an internal diameter of 2.5 cms while Columns B, C and D each had an internal diameter of 2.6 cms. The packed height proved impossible to reproduce exactly. Every attempt was made to ensure a packed height of 34-35 cms but it was found that some variation in height occurred even in the same packed gel column run under apparently standardised conditions. Biogel A5m is provided as swollen particles and therefore a dry weight cannot be obtained to help in standardisation.

(3) Each batch of gel was recorded as a Roman numeral (e.g. I, II etc.) and placed in brackets after the Column letter and number (e.g. Column A1 (I), Column C5 (II) etc.). It became obvious that the batch of gel was very important in determining the elution characteristics of fibrinogen and other proteins. Several jars of gel from the same batch were therefore purchased

at the same time to ensure continuity. The best separation was always obtained with previously unused gel. After being used for several packed gel column systems the number of fractured and fine gel spheres greatly increased. (This could be checked microscopically). There was a corresponding decrease in the quality of the separation obtained and so that particular quantity of gel was discarded.

The V_o and V_e of fibrinogen for the packed gel columns used in this thesis is shown in Table 82 (a-c). Any obviously abnormal elution profiles were excluded from these calculations as it proved very difficult to calculate the V_e of fibrinogen if a pronounced shoulder was present on the ascending limb of the peak. The V_e of fibrinogen remained reasonably constant for each packed gel column system.

In order to properly standardise each column system several samples of purified fibrinogen or plasma from a normal individual should have been analysed before attempting to analyse patient samples. Ideally analysis of each patient sample should be followed by analysis of a sample from an appropriate control subject. This proved impracticable in the present project because of the difficulty in obtaining patient samples at regular time intervals. Only four or five samples could be analysed in a normal working week and in addition all the samples were analysed without storage on the day of collection. Long periods of time passed in which few or no patient samples were available, particularly in the pre-eclampsia study. Therefore when patient samples were available as many as possible were studied and the remaining time was used to analyse samples from normal controls.

Appendix II

Case Summaries

A. Pre-eclamptic patients

No: 1 (M.C.)

Age: 16 years — **Occupation:** Hairdresser

Past medical history: No serious illness

Past obstetric history: Para $0 + {}^{0}$

Pregnancy studied: 1975

	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24 hrs)
First visit to G.P.:	12	120/75	None
First hospital visit:	20	130/90	None
Progress: Admitted -	25-26	140/90	10.5
	26-27	160/110	12.5

Drug therapy: Oral iron, folic acid and vitamins. Sodium amylobarbitone 200 mg orally b.d. Sodium amylobarbitone 250 mg intra-muscularly as required.

The patient's condition rapidly deteriorated making termination of pregnancy necessary.

Delivery: 26-27 weeks gestation.

Induction: Prostaglandin infusion. Eclamptic fit in labour.

Means of delivery: Assisted breech delivery.

Baby: Stillborn female

Birth weight: 1.11 Kg.

Placenta: 0.25 Kg.

Post mortem on baby:	Marked external bruising of buttocks and lower limbs.
CNS:	Bilateral tentorial tear. Small subdural and subarachnoid haemorrhages.
RS:	Subpleural haemorrhages.
GIT:	Hepatic tear, subcapsular haemorrhages.
Summary:	(i) Intra-partum asphyxia (following maternal eclamptic fit). (ii) Extreme prematurity. (It was difficult to determine how many of the changes had occurred after foetal death).
Placental pathology:	Two large areas of infarction (one old and one recent) occupying more than half of the placenta. Large area of recent retro-placental clot and haemorrhage. Histology showed extensive necrosis of the decidua capsularis and decidua basalis with extensive fibrin deposition in the maternal vessels and perivillous fibrin.
Summary:	Placental infarction. Abruptio placentae. Compatible with pre-eclampsia.
Puerperium:	Remained very ill for several days with gradual improvement in B.P. and lessening of proteinuria.
Post-natal visit:	Well although B.P. still 130/100 mmHg (125/90 mmHg) and a trace of proteinuria at 6 week visit.
Renal Unit follow-up:	Fully investigated. IVP and all other renal investigations normal. B.P. gradually settled (normotensive by 3 months post-

partum) and proteinuria disappeared. Diagnosis of pre-eclampsia with eclamptic seizure during labour would seem to have been correct.

No: 2 (E.S.)

Age: 18 years — Occupation: Student

Past medical history: No serious illnesses

Past obstetric history: Para $0 + {}^{0}$

Pregnancy studied: 1974

	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24 hrs)
First visit to G.P.:	12	110/70	None
First hospital visit:	26	130/90	None
Progress: Admitted -	34	160/110	5.6
	35	160/110	6.4

Drug therapy: Oral iron, folic acid and vitamins. Sodium amylobarbitone 200 mg orally nocte.

The patient's condition deteriorated making delivery necessary.

Delivery: 35 weeks gestation

Induction: Forewater amniotomy and Syntocinon infusion

Means of delivery: Kielland's forceps

Baby: Live female

Birth weight: 3.07 Kg.

Placenta: 0.83 Kg.

Baby's condition at birth and progress: Baby in satisfactory condition at birth. Subsequently progressed well apart from bilateral conjunctivitis.

Summary: Baby was well grown for gestation (P75-90) and in good condition.

Placental pathology: Gross inspection showed 4 small infarcted areas. Detailed histology not performed.

Comment: Compatible with pre-eclampsia.

Puerperium: B.P. rose following delivery to 178/130 mmHg. Treated with Heminevrin and diazoxide. Thereafter good recovery.

Post-natal visit: Well. B.P. 140/90 mmHg (130/85 mmHg). No proteinuria.

Renal Unit follow-up: Not referred but diagnosis of pre-eclampsia would seem likely to have been correct.

No: 3 (M.H.)

Age: 35 years

Occupation: Medical Secretary

Past medical history: Recurrent rectal bleeding and abdominal pain for several years. Left-sided hemicolectomy 3 years ago. Diagnosis uncertain (? purgative colitis, ? ulcerative colitis).

Past obstetric history: Para $0 + {}^{0}$

Pregnancy studied: 1975

	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24 hrs)
First visit to G.P.:	10	130/80	None
First hospital visit:	11	140/80	None
Progress: Admitted - B.P. settled, allowed home	23	150/90	None
Readmitted:	27	120/90	0.47

Drug therapy: Oral iron and vitamins. Sodium amylobarbitone 200 mg orally t.i.d. Sodium amylobarbitone 250 mg intra-muscularly as required. Salazopyrin 1 g orally mane. Betnovate rectal cream as required. Predsol suppositories - 1 suppository nocte. Dulcolax suppositories as required.

Delivery:	29 weeks
Means of delivery:	Elective Caesarean section
Baby:	Live female
Birth weight:	1.00 Kg.
Placenta:	0.25 Kg.
Baby's condition at birth:	Premature baby. Despite intensive care died of Respiratory Distress Syndrome at 31 hours.
Post mortem on baby:	Main findings -
RS:	Hyaline membrane formation and resorption atelectasis. Focal intrapulmonary haemorrhages and primary atelectasis. No bacterial infection noted.
GIT:	Subcapsular haemorrhages.
Summary:	(i) Severe respiratory distress syndrome (ii) Prematurity.
Placental pathology:	Cord was very oedematous but gross inspection of placenta revealed no marked abnormality. Histology showed that the villi were small and spindly for this degree of prematurity and that there were an excessive number of syncytial knots.
Comment:	"Ageing" phenomena are compatible with pre-eclampsia/placental insufficiency.
Puerperium:	Gradual improvement, on discharge B.P. 140/90 mmHg.
Post-natal visit:	Well although still slightly hypertensive. B.P. 145/95 mmHg (140/90 mmHg).
Renal Unit follow-up:	Fully investigated. B.P. 130/85 mmHg. IVP and all other renal investigations were normal. Diagnosis of pre-eclampsia seemed fairly certain.

No: 4 (S. S.)

Age: 23 years

Occupation: Clerkess

Past medical history: Nocturnal enuresis. Obesity.

Past obstetric history: Para $1 + 0$

(1) 1970 Pre-eclampsia with an intrauterine death at 28 weeks. Normotensive 3 months after delivery.

Pregnancy studied: 1974

	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24 hrs)
First visit to G.P.:	12	130/80	None
First hospital visit:	17	150/90	None
	22	145/90	None
	32	145/90	None
	35	150/90	1.9
	36	150/95	3.1

Drug therapy: Oral iron, folic acid and vitamins. Sodium amylobarbitone 200 mg orally b.d. Sodium amylobarbitone 250 mg intra-muscularly as required.

The patient's condition gradually deteriorated. It was decided to deliver by Caesarean section at 36-37 weeks.

Delivery: 37 weeks gestation.

Means of delivery: Elective Caesarean section.

Baby: Live dysmature and premature male.

Birth weight: 2.25 Kg.

Placenta: 0.60 Kg.

Baby's condition at birth and progress: Baby was mildly asphyxiated at birth. Despite the development of hypoglycaemic attacks and Respiratory Distress Syndrome he subsequently started to gain weight and was discharged home in good condition.

Placental pathology: Several small areas of infarction. No histology available.

Puerperium: Uneventful recovery.

Post-natal visit: Well. (B.P. 148/80 mmHg).

Renal Unit follow-up: Fully investigated. IVP and all other renal investigations were normal. Diagnosis of pre-eclampsia seemed fairly certain.

No: 5 (J.T.)

Age: 29 years Occupation: Clerkess

Past medical history: No serious illnesses

Past obstetric history: Para $0 + {}^{1}$

(1) 1974 Spontaneous abortion at 10 weeks

Pregnancy studied: 1974

	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24hrs)
First visit to G.P.:	13	130/80	None
First hospital visit:	18	120/90	None
Progress: Admitted -	24+	145/95	+++
	26	150/100	3.1
	28	150/110	6.7

Drug therapy: Oral iron, folic acid and vitamins. Sodium amylobarbitone 200 mg orally nocte. Methyl dopa 250 mg orally q.i.d. Sodium amylobarbitone 250 mg intra-muscularly as required. The patient's condition showed progressive deterioration. Intrauterine death occurred 28 weeks.

Delivery: 29 weeks gestation.

Induction: Prostaglandin infusion - intrauterine death.

Means of delivery: Spontaneous vertex delivery

Baby: Stillborn female.

Birth weight:	0.81 Kg.
Placenta:	0.17 Kg.
Post mortem on baby:	Degree of maceration suggested death some time before delivery. (Foetal heart last heard about 1 week before induction). No gross congenital abnormality.
Summary:	Intrauterine death about 1 week before delivery.
Placental pathology:	Macerated with old blood clot and several areas of old infarction. The usual coarse pattern of alternating pink and white areas associated with an intrauterine death. Histology showed patchy, squamous change with small villi and small fresh and old haemorrhages in the decidua capsularis. Maternal vessels showed fibrinoid in walls.
Puerperium:	Remained slightly hypertensive (B.P. 140/95 mmHg) with some proteinuria until discharge.
Post-natal visit:	B.P. 130/90 mmHg (130/85 mmHg). Trace of protein.
Renal Unit follow-up:	Fully investigated. (B.P. 125/80 mmHg). IVP and all other renal investigations were normal. Diagnosis of pre-eclampsia seemed fairly certain. (Further pregnancy in 1975 in which she did not develop pre-eclampsia).

No: 6 (M.McL).

Age: 21 years	Occupation: Machinist
Past medical history:	Obesity
Past obstetric history:	Para $1 + {}^{0}$
(1) 1972	? urinary tract infection

Pregnancy studied: 1974

	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24 hrs)
First visit to G.P.:	24	120/70	-
First hospital visit:	28	130/70	None
Progress: Admitted -	29+	130/80	+++
	30	130/90	5.0
	30+	125/85	+

Drug therapy: Oral iron, folic acid and vitamins.

The patient's condition rapidly deteriorated. It is possible that intrauterine death occurred shortly after admission (29-30 weeks).

Delivery: 30 weeks gestation.

Induction: Prostaglandin infusion.

Means of delivery: Breech extraction.

Baby: Stillborn male.

Birth weight: 0.950 Kg.

Placenta: 0.220 Kg.

Post mortem on baby: Degree of maceration and autolysis suggested death some time before delivery. (Foetal heart last heard about 3 days before delivery). No gross congenital abnormality.

Comment: Intrauterine death about 3 days before delivery.

Placental pathology: Multiple retroplacental haematomata related to areas of red and white infarction. 60% of the placenta showed pathological change. Histology showed small villi with excessive perivillous fibrin and syncytial knots. Maternal vessels showed excessive fibrinoid in walls with frequent complete occlusion. Foetal arteries showed endarteritis.

Puerperium: Good recovery.

Post-natal visit: Well. (B.P. 120/75 mmHg and no proteinuria).

Renal Unit follow-up: Fully investigated. IVP and all other renal investigations were normal. Diagnosis of pre-eclampsia seemed fairly certain. (Further pregnancy in 1976 in which she did not develop pre-eclampsia).

No: 7 (M.M.)

Age: 27 years Occupation: Housewife

Past medical history: No serious illnesses

Past obstetric history: Para $0 + {}^{0}$

Pregnancy studied: 1975

	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24 hrs)
First visit to G.P.:	-	-	-
First hospital visit:	9	110/60	None
Progress: Admitted -	35+	130/104	1.8
	36	130/95	3.1
	36+	160/96	2.2

Drug therapy: Oral iron, folic acid and vitamins. Sodium amylobarbitone 200 mg orally b.d.
The patient's condition gradually deteriorated and she was induced at 37 weeks gestation.

Delivery: 37 weeks gestation

Induction: Forewater amniotomy and Syntocinon infusion.

Means of delivery: Kielland's forceps; manual removal of placenta.

Baby: Live female.

Birth weight: 2.17 Kg.

Placental weight:	0.48 Kg.
Baby's condition at birth and progress:	Baby was mildly asphyxiated at birth. She was thought to be both dysmature and premature (assessed as 36 weeks). She did not, however, develop any serious complications and developed well.
Placental pathology:	There were several areas of old infarction and blood clot. Histology showed perivillous fibrin and syncytial knots in excess. Foetal vessels showed endarteritis. Maternal vessels showed excessive fibrinoid deposition and one large maternal vessel was completely occluded.
Summary:	Definite placental insufficiency, compatible with pre-eclampsia.
Puerperium:	Good recovery.
Post-natal visit:	Well. B.P. 110/64 mmHg. No proteinuria.
Renal Unit follow-up:	Not referred but diagnosis of pre-eclampsia seemed fairly certain despite this. (Subsequent pregnancy in 1976 in which the patient did not develop pre-eclampsia).

No: 8 (M.D.)

Age: 23 years — Occupation: Secretary

Past medical history: No serious illnesses

Past obstetric history: Para $0 + {}^{0}$

Pregnancy studied: 1975

TWINS	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24 hrs)
First visit to G.P.:	10	120/75	None
First hospital visit:	22+	130/90	None
Progress: Admitted -	34	165/90	3.15

Drug therapy:	Oral iron, folic acid and vitamins. Sodium amylobarbitone 200 mg orally nocte. Sodium amylobarbitone 250 mg intra-muscularly as required. Methyldopa 250 mg orally t. i. d. Rapid deterioration with falling urinary oestriol levels. One week after admission only one foetal heart heard and so patient induced.
Delivery:	34 weeks gestation
Induction:	Forewater amniotomy and Syntocinon infusion
Means of delivery:	(1) Assisted breech delivery (2) Breech extraction
Babies:	(1) Stillborn male (2) Stillborn male
Birth weights:	(1) 1. 24 Kg. (2) 1. 00 Kg.
Placenta:	0. 35 Kg.
Post mortem on babies:	Exact sequence of events difficult to determine. Clinically one foetal heart was thought to disappear some hours before induction. Second foetal heart disappeared during labour. Twin (1): Signs of intra-partum asphyxia. No gross congenital abnormality. Twin (2): Macerated infant. Foetal vascular thrombosis. No gross congenital abnormality.
Summary:	Twin (1) died of intra-partum asphyxia. Twin (2) intrauterine death some time before induction.
Placental pathology:	Diamniotic, monochorionic placenta. No abnormal circulation demonstrated. Maternal vessels showed marked "atherosis" with fibrinoid in vessel wall and fibrin in lumen

of veins and sinusoids. No obvious area of infarction. Almost all of large vein on foetal surface (Twin 2) was occluded with antemortem thrombus. Histology compatible with pre-eclampsia.

Puerperium: Gradually settled.

Post-natal visit: Well. B.P. 140/100 mmHg (120/85 mmHg).

Renal Unit follow-up: Not followed up but IVP was normal. (B.P. 120/80 mmHg). Diagnosis of pre-eclampsia seemed fairly certain.

No. 9 (A.McN)

Age: 24 years Occupation: Housewife

Past medical history: No serious illnesses

Past obstetric history: Para 2 + 2

(1) 1969 Ante-partum haemorrhage 28 weeks anencephalic.

(2) 1970 Ante-partum haemorrhage 32 weeks. Live female.

(3) 1971 Spontaneous abortion 6 weeks.

(4) 1972 Spontaneous abortion 12 weeks.

Never hypertensive.

Pregnancy studied: 1975

	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24 hrs)
First visit to G.P.:	10	125/80	None
First hospital visit:	16	145/85	None
Progress:	30	120/90	None
	32	146/110	0.75
	33	160/110	0.62
	34	158/110	1.7

Drug therapy:	Oral iron, folic acid and vitamins. Sodium amylobarbitone 200 mg orally nocte. Sodium amylobarbitone 250 mg intra-muscularly as required. Methyldopa 250 mg orally t. i. d. Gradual deterioration in condition with development of proteinuria.
Delivery:	35 weeks gestation.
Means of delivery:	Elective Caesarean section
Baby:	Live female
Birth weight:	2.16 Kg.
Placenta:	0.44 Kg.
Baby's condition at birth and progress:	Baby was mildly asphyxiated at birth. She was thought to be both premature and dysmature. No serious complications but required several weeks in hospital. Thereafter good progress.
Placental pathology:	No details available.
Puerperium:	Hypertension and proteinuria gradually settled.
Post-natal visit:	Well although still hypertensive - B.P. 160/100 mmHg (145/90 mmHg).
Renal Unit follow-up:	Still slightly hypertensive at first visit 130/100 mmHg. IVP and other renal investigations were all normal although B.P. remained slightly raised until treated with a low dose of propranolol. It is possible that there may be underlying essential hypertension, but the diagnosis of pre-eclampsia can probably be accepted.

No: 10 (D.F.)

Age: 22 years Occupation: Shop assistant and housewife.

Past medical history: No serious illnesses.

Past obstetric history: Para 0 + 0

Pregnancy studied: 1974

	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24 hrs)
First visit to G.P.:	12	110/70	None
First hospital visit:	18	110/70	None
Progress:	28	136/100	11.5
	30	140/100	7.0
	32	120/100	6.0

Drug therapy: Oral iron, folic acid and vitamins. Sodium amylobarbitone 200 mg orally t.i.d. Methyldopa 250 mg orally t.i.d., later increased to 500 mg orally t.i.d. (at 30 weeks).
Despite marked proteinuria the patient remained surprisingly well. It was possible to continue pregnancy until 32 weeks.

Delivery: 32 weeks gestation.

Means of delivery: Elective Caesarean section.

Baby: Live male.

Birth weight: 1.60 Kg.

Placenta: 0.33 Kg.

Baby's condition at birth and progress: Premature baby in good condition. Thereafter made good progress.

Placental pathology: Fibrinoid in maternal vessel walls. Spindly villi and excessive numbers of syncytial knots.

Summary: Compatible with placental insufficiency and pre-eclampsia.

Puerperium: 3 days post-partum proteinuria --) 13.1 g/24 hrs but thereafter settled. When discharged home B.P. 130/85 mmHg and a trace of proteinuria.

Post-natal visit: Well. (B.P. 120/85 mmHg and a trace of proteinuria).

Renal Unit follow-up: Fully investigated. B.P. 110/75 mmHg. Quantitative proteinuria = 0.65 g/24 hrs. Gradually disappeared over next 3 months. IVP and other renal investigations all normal. Diagnosis of pre-eclampsia seemed fairly certain.

B. High risk of pre-eclampsia
Anti-thrombotic treatment

No: 11 (R.S.)

Age: 31 years Occupation: Housewife

Past medical history: No serious illnesses

Past obstetric history: Para $2 + {}^{0}$

(1) 1970 Severe pre-eclampsia at 25 weeks, required termination of pregnancy at 28 weeks. B.P. fell to normal 2 months after delivery.

(2) 1972 Severe pre-eclampsia at 25 weeks resulting in an intrauterine death at 28 weeks. B.P. fell to normal 2 months after delivery.
Investigated at Western Infirmary B.P. Unit:- renal function and IVP normal.

Pregnancy studied: 1974

	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24 hrs)
First visit to G.P.:	8	130/80	None
First hospital visit:	11	125/80	None
	Threatened abortion		

	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24 hrs)
Progress:	20	110/60	None
	24	115/60	None
	28	115/60	None
	32	120/60	None
	34	120/65	None
	36	120/80	None
	38	130/85	None

Well throughout pregnancy. Steady growth of baby. Normal oestriols.

Therapy: Oral iron, folic acid and vitamins.
Heparin 5,000 units subcutaneously b.d. (5 out of 7 days/week).
Aspirin 600 mg orally t.i.d.
Dipyridamole 100 mg orally t.i.d. The anti-thrombotic drugs were discontinued a few days before delivery.

Delivery: 38 weeks.

Means of delivery: Elective Caesarean section

Baby: Live male.

Birth weight: 2.58 Kg (P10-25)

Placenta: 0.520 Kg (no details about pathology)

Baby's condition at birth and progress: Good condition at birth, remained well with satisfactory weight gain.

Puerperium: Good recovery.

Post-natal visit: Well. (B.P. 120/70 mmHg).

No: 12 (J.W.)

Age: 29 years Occupation: Housewife

Past medical history: No serious illnesses

Past obstetric history: Para 2 + ^{0}

(1) 1974 (January) Severe pre-eclampsia at 30 weeks resulting in an intrauterine death at 32 weeks.

(2) 1974 (November) Severe pre-eclampsia at 30 weeks, clinically "small-for-dates" resulting in an intrauterine death at 30 weeks.

Pregnancy studied: 1975

	Gestation (weeks)	B.P. (mmHg)	Proteinuria (g/24 hrs)
First visit to G.P.:	8	115/75	None
First hospital visit:	15	120/75	None
Diagnosed as twin pregnancy.			
Progress: Admitted -	24	120/70	None
	30	115/70	None
	32	120/70	None
	34	120/80	None
	36	120/80	None

Threatened premature labour at 30 weeks.

Babies appeared to grow well.

Drug therapy: Oral iron and folic acid. Dipyridamole 100 mg orally q.i.d. Aspirin 650 mg orally q.i.d. started at 29 weeks and continued until 36 weeks (discontinued temporarily at 30 weeks when threatened premature labour. This was treated with IV salbutamol).

Delivery: 36 weeks

Means of delivery: Elective Caesarean section

Babies: (1) Live female

(2) Live female

Birth weights:	(1) 2.22 Kg. (P 10-25)
	(2) 2.31 Kg. (P 10-25)
Placenta:	1.05 Kg.
	No details about pathology.
Babies condition at birth and progress:	Twin (1): Premature baby. Required resuscitation after delivery. Later developed mild jaundice. 3 weeks after birth started to gain weight and progress well.
	Twin (2): Premature baby. In better condition than twin at birth. Also developed mild jaundice but otherwise progressed well.
Puerperium:	Good recovery. B.P. 125/80 mmHg.
Post-natal visit:	Well. (No B.P. recorded in notes).

Appendix III

Two Pregnant Patients Considered at "High-Risk" of Developing Pre-Eclampsia

These patients are presented as examples of serial studies performed during the ante-natal period on patients who did not develop pre-eclampsia. In view of their past obstetric history it might have been expected that each of these patients would develop hypertensive complications during the pregnancy under study. Both received a form of anti-thrombotic therapy, but it would clearly be impossible to relate the good clinical outcome in each case to this treatment. (Detailed case summaries on each patient can be found in Appendix II).

(i) Patient 11 (R.S.) Figure 69.

This patient had had two previous pregnancies both of which had been complicated by fulminating pre-eclampsia. In both cases the baby had died. She was in hospital for most of this, her third, pregnancy. She was first studied at 20 weeks gestation and thereafter followed serially throughout pregnancy. She received low-dose subcutaneous heparin, Aspirin and dipyridamole as anti-thrombotic therapy in the dosage shown in the summary (Appendix II).

She did not develop any of the clinical features of pre-eclampsia. She was delivered by elective Caesarean section at 38 weeks. The baby was in good condition at birth and progressed satisfactorily thereafter. The patient's post-partum progress was uncomplicated.

The plasma fibrinogen levels were normal (the "low" value at 20 weeks gestation is only low compared with the range in normal third trimester pregnancies shown in the chart and the "high" value just before delivery is not unreasonable for a patient close to term). Area m was consistently within the normal range. The SCT titre at V_o only rose to above the normal range in the sample taken just before Caesarean section, but titres as high as this have been found in individual normal pregnancies (see Table 53). These results would not suggest increased coagulation activity in this patient. Area p levels were raised in 3 out of the 5 ante-natal samples. This could suggest increased fibrinogenolysis/fibrinolysis or diminished clearance of early degradation products (or both). The serum FDP/fdp levels were, however, only raised in the sample taken just before delivery and values as high as this have been found in normal pregnancy (see Table 53).

The laboratory results were therefore largely within the "normal" range for later pregnancy (24-39 weeks) as based on the results in 14 normal pregnant women (Table 53) and as such were compatible with the clinical picture.

(ii) Patient 12 (J.W.) Figure 70.

This was a similar case, the patient having had two previous pregnancies with fulminating pre-eclampsia and intrauterine death. She had the additional complication on this occasion of a twin pregnancy. She was first studied at 28 weeks. She received Aspirin and dipyridamole from about 29 weeks until delivery at 37 weeks in the dosage shown in the summary (Appendix II). This therapy was discontinued briefly at 31 weeks when she threatened to go into premature labour. She was delivered by elective Caesarean section at 36 weeks. Both babies were premature and both developed mild neonatal jaundice. Apart from this, their

condition shortly after birth and subsequent progress was satisfactory.

The plasma fibrinogen levels were normal throughout the period of study. Area m and the SCT titre at V_o were only raised following the episode of threatened premature labour. Possibly both the laboratory and clinical picture at that stage reflected a small abruptio placenta, but unfortunately the placenta was not examined for this after delivery. Area p was slightly raised in 4 out of 7 ante-natal samples studied. Although the true significance of this is uncertain, it is worth noting that slightly raised levels of area p were also found in the previous patient (Patient 11). It may be that increased fibrinogenolysis/fibrinolysis was present with or without diminished clearance of early degradation products in both patients and this might have contributed to the good clinical outcome. The serum FDP/fdp levels were, however, not increased and so the raised area p levels may not be of any real significance.

Comment on the "High-Risk" Patients

It would be impossible to assess the effect of the anti-thrombotic therapy on the clinical course of these patients. It seems most likely that neither were destined to develop pre-eclampsia in the pregnancy studied, but clearly this question cannot at present be answered. These sequential studies do, however, demonstrate that essentially normal soluble complex levels were present in two patients who did not develop pre-eclampsia. The only abnormal area m result occurred in Patient 12 following an episode of premature labour and it is possible that a small abruptio placenta was responsible for both the clinical and laboratory picture.

APPENDIX IV

LOCAL VENOUS THROMBOSIS

Six patients with local venous thrombosis were studied. The clinical diagnosis was confirmed in each case by venography. The clinical details are shown in Table 83. Two patients (Nos. 1 and 5) had metastatic carcinoma. None were hypertensive. The results obtained on samples from these patients are shown in Table 84. A wide range of results were obtained, but this is not surprising in view of the wide variation in clinical picture.

Compared with the results from the normal healthy control subjects (Group III, Chapter V) the results from the deep vein thrombosis patients showed several significant differences (Table 85) although it must be stressed that these groups were not matched (e.g. for age and sex).

These results would suggest that there may be:-

(i) an increase in soluble complex concentration - both oligomer/dimer (area m) and polymer (SCT titre at V_o, FR-antigen and T.C.P. ratios), in plasma samples from local venous thrombosis patients

(ii) an increased concentration of early clottable degradation product (area p) and non-clottable degradation product (serum FDP/fdp) concentration in samples from local venous thrombosis patients.

REFERENCES

The Harvard system is used in this thesis, although occasionally for clarity more than one authors' name is quoted on the second and subsequent reference to the same paper. The references are listed according to the General Regulations on Theses for Higher Degrees, University of Glasgow. In listing references alphabetically according to the initial letter of the surname of the first author, prefixes (e.g. von) are not used (e.g. papers by von Hugo et al. are listed under H). Papers by authors with surnames beginning with Mc or Mac are listed after those beginning with M. The convention of initial capitals for the first word only in the title of a paper, but throughout the title of a journal or book is adhered to, even for titles not in English.

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McKillop C.A. M.D. Thesis 1977

THROMBOSIS RESEARCH
Printed in the United States

Vol. 7, pp. 361-372, 1975
Pergamon Press, Inc.

IN VIVO PRODUCTION OF SOLUBLE COMPLEXES CONTAINING FIBRINOGEN-FIBRIN RELATED ANTIGEN DURING ANCROD THERAPY

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(Received 21.4.1975; in revised form 2.7.1975.
Accepted by Editor B. Blombäck)

ABSTRACT Seven patients were studied during defibrination produced by ancrod infusion. In every case soluble complexes composed of material antigenically related to fibrinogen were demonstrated, using agarose gel filtration, after six hours ancrod infusion. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate showed that the soluble complexes were largely composed of units with a molecular weight less than fibrinogen similar to a minimally degraded product, early Fragment X. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate and mercaptoethanol showed that the component units of the soluble complexes were markedly deficient in intact Aα chain when compared with the uncomplexed material. It is suggested that preferential digestion of fibrin may occur while it is still in the soluble form.

INTRODUCTION

Soluble complexes of material antigenically related to fibrinogen have been demonstrated in the circulation in a number of pathological conditions associated with "hypercoagulability" or thrombosis (1). In vitro studies have shown that different forms of soluble complexes can be produced (2,3,4,5). Little is known, however, concerning the structure, formation and breakdown of soluble complexes in vivo.

Soluble complexes can be demonstrated experimentally

following thrombin infusion in rabbits (4). The therapeutic use of thrombin-like snake venoms as defibrinating agents could, therefore, provide a useful model of soluble complex production in man. Soluble fibrin, demonstrated by the ethanol gelation test and N-terminal amino acid analysis, has been found in the plasma of patients treated with Defibrase, the defibrinating agent from the venom of Bothrops atrox (6). Soluble complexes, demonstrated by agarose gel filtration, have also been found in the plasma of these patients (7). Ancrod is another defibrinating enzyme derived from the venom of the Malayan pit viper (Agkistrodon rhodostoma). In vitro ancrod resembles thrombin in removing fibrinopeptide A from fibrinogen but differs in failing to remove fibrinopeptide B (8). We extend here our preliminary observations (9) on a group of seven patients treated therapeutically with ancrod. Soluble complexes were isolated from plasma in concentrations suitable for the study of the molecular weight and the chains of their component units.

PATIENTS, MATERIALS AND METHODS

Seven patients were studied. Four suffered from deep venous thrombosis and three from severe peripheral arterial disease. There were five males and two females with an age range of 22 to 72 years. Neither female was taking oral contraceptives. Ancrod (Arvin, Berk Pharmaceuticals Limited, Shalford, Sussex, England) was administered as an intravenous infusion in a dose of 2 units/Kg body weight/12 hours. No anticoagulant or fibrinolytic agent was given during the period of study. Blood samples were collected before treatment and after 6 and 24 hours of ancrod infusion. Control samples were obtained from healthy volunteers with a comparable age range.

Collection of Samples: Blood was collected by careful venepuncture with minimal venous occlusion. Each sample was divided into aliquots for preparation of plasma, serum and observation of clot quality.

Plasma: Whole blood was collected in anticoagulant in the proportion of 9 parts blood to 1 part anticoagulant mixture (pH 7.5). This contained (before addition of blood) trisodium citrate 0.13M; N tris (hydroxymethyl) methyl 2-aminoethane sulphonic acid 0.06M; epsilon aminocaproic acid (E.A.C.A.)0.02M and aprotinin (Trasylol, Bayer Pharmaceuticals Limited, Haywards Heath, Sussex, England) 10^6 K.I.U./l. 0.2 ml. ancrod antiserum (snake venom antiserum Agkistrodon rhodostoma, The Lister Institute, Elstree, England) was added to each millilitre of anticoagulant to neutralise any ancrod in the blood sample. The blood was collected in plastic syringes and containers and handled with siliconised glassware. Plasma was prepared by centrifugation at 3,000 g to 20 minutes.

Serum: Whole blood was collected in containers provided for use

with 'Wellcome F.D.P. kits' (Wellcome Research Laboratories, Beckenham, England, HA 14). These contain thrombin and a plasmin inhibitor. Ancrod antiserum (0.05 ml) was added. Blood was allowed to clot at room temperature for several hours and serum separated.

Clot Quality Observation Test: 1 ml of whole blood was incubated in a glass test tube (75 x 10 mm) containing 0.05 ml ancrod antiserum at 37°C for 24 hours. The resultant clot was graded 1 to 5, a normal clot being graded as 1 and no clot as 5. The grades 2, 3, 4 represented mild, moderate and severe defects(10).

Plasma Fibrinogen was measured by the method of Ratnoff and Menzie (11).

Staphylococcal Clumping Test Titre (S.C.T. Titre) was performed by the method of Hawiger et al. (12). Staphylococcal clumping factor was obtained from Sigma London Chemical Company Limited, Kingston-upon-Thames, Surrey, England (850-10).

Serum Fibrinogen-Fibrin Degradation Products (serum F.D.P.) estimation was performed using the Tanned Red Cell Haemagglutination Inhibition Immunoassay (13) using pre-packed kits (Wellcome Research Laboratories, HA 14).

β-alanine precipitate of fresh plasma was prepared by the method of Graeff et al. (14) and gel filtration performed immediately.

6% Agarose Gel Filtration: An adaptation of the method of Fletcher et al. (1) was employed using Biogel A-5m mesh size 200-400 (Bio-rad Laboratories Limited, Bromley, Kent, England, 1510750) with columns of packed height of 35 cms and diameter 2.6 cm. Void volume as measured by Blue Dextran 2,000 (Pharmacia G.B. Limited, 75, Uxbridge Road, London,) was 60 ml. Elution buffer (pH 7.6) contained Tris (hydroxymethyl) methylamine 0.05M; 0.1M HCl 384 ml/l; NaCl 0.115M; trisodium citrate 0.012M; E.A.C.A. 0.05M; aprotinin (Trasylol) 10^5 K.I.U./l. The flow rate of 26 ml/hour was controlled by a constant infusion pump and 2.6 ml fractions of eluant were collected.

Fibrinogen-Fibrin related Antigen (FR-antigen) was estimated in the eluant fractions using a radial immunodiffusion technique (15). Agar, 3% W/V ('Difco' Bacto-Agar, Difco Laboratories, Detroit, Michigan, U.S.A. 0.141-02) was dissolved in barbital-saline buffer (pH 7.2) which contained sodium barbitone 0.02M; sodium chloride 0.29M; 0.1M HCl 161 ml/l; ethylenediaminetetracetic acid disodium salt 0.01M; trisodium citrate 0.012M; E.A.C.A. 0.01M; sodium azide 0.003M. Fibrinogen antiserum (Behringwerke - Hoechst Pharmaceuticals, Hounslow, Middlesex, England, ORCH 05) was used at a 1:120 dilution in 0.9% W/V saline. 6 ml. of dissolved agar was mixed with 6 ml diluted antiserum at 56°C and then poured into a plastic petri dish

(8.5 cm in diameter) and allowed to gel. Samples of 5 µl were applied to this gel and diffusion continued for 48 hours at room temperature in a moist atmosphere. Clear rings were obtained by staining with 1% W/V tannic acid for 3 minutes. Plates were calibrated by use of appropriate dilutions of Protein Standard Plasma (Behringwerke - Hoechst Pharmaceuticals OTF1 03). Where necessary samples were diluted with column elution buffer before assay.

Thrombin Clotting Time (T.C.T.) was performed according to the method described by McNicol et al. (16) and Thrombin Clottable Protein (T.C.P.) was demonstrated using the same system except that it was incubated without mixing at 37°C and examined for clot formation after 1 and 24 hours.

Polyacrylamide Gel Electrophoresis in the presence of sodium dodecyl sulphate (S.D.S.) was performed according to the method of Weber and Osborn (17). Fibrinogen (Kabi, Grade L, AB Kabi, Stockholm, Sweden) was used as a standard. Fragments X, Y and D were prepared by streptokinase-induced lysis of the Kabi fibrinogen (18). Densitometric scans were obtained by scanning the gels at 600 nm using a Gilford spectrophotometer.

RESULTS

Clot Quality Observation Test: In all of the patients the pre-treatment samples produced Grade 1 clot. After 6 hours ancrod infusion the clot remained Grade 1 or 2. After 24 hours infusion the blood was completely incoagulable (Grade 5).

Plasma Fibrinogen, Serum F.D.P. and Serum S.C.T. Titre Levels are shown in Fig. 1. In every case the plasma fibrinogen fell

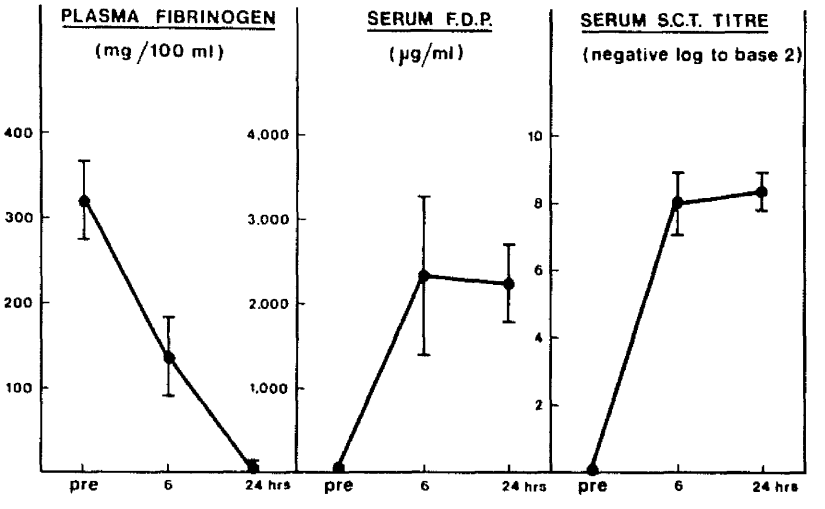

FIG. 1

The mean and standard error of the mean of plasma fibrinogen, serum F.D.P. and serum S.C.T. titre estimations on samples obtained from 7 patients pre-treatment (pre) and after 6 and 24 hours ancrod infusion.

significantly after 6 hours ancrod infusion and after 24 hours had reached negligible levels. There was a corresponding sharp rise in both the serum F.D.P. and serum S.C.T. titre levels after 6 hours infusion and this was maintained after 24 hours.

6% Agarose Gel Filtration: The FR-antigen from normal control plasmas always eluted from the column as a symmetrical curve (main peak) with a peak elution volume of 90 ml. A similarly shaped curve was obtained from the thrombin clotting times. The results of a representative control sample are shown in Figure 2.

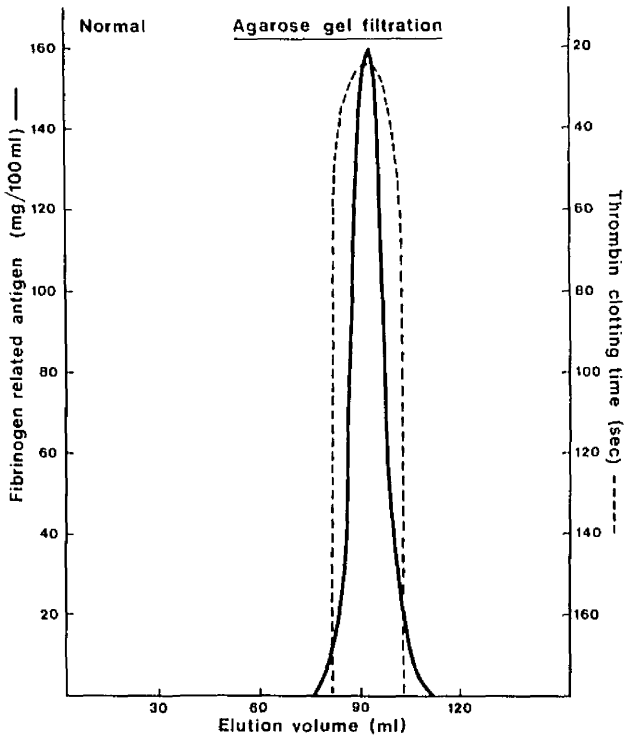

FIG. 2

6% agarose gel filtration of a beta-alanine precipitate of plasma from a normal control subject. FR-antigen measured by radial immunodiffusion techniques and T.C.T. estimations are shown in terms of elution volume.

The elution pattern of FR-antigen for samples from two representative patients are shown in Figure 3(a) and (b). The corresponding T.C.T. curves and range of thrombin clottable protein (T.C.P.) performed on the same eluant fractions from samples from one patient (J.McK.) are shown in Figure 4. In the pre-treatment samples the FR-antigen and T.C.T. curves, together with the range of thrombin clottable protein corresponded closely to results from normal control subjects. In 5 out of the 7 cases small shoulders were present on both sides of the FR-antigen main peak, as in the cases shown. These probably represent low concentrations of soluble complexes and degradation products, compatible with the diagnosis of thrombotic disease.

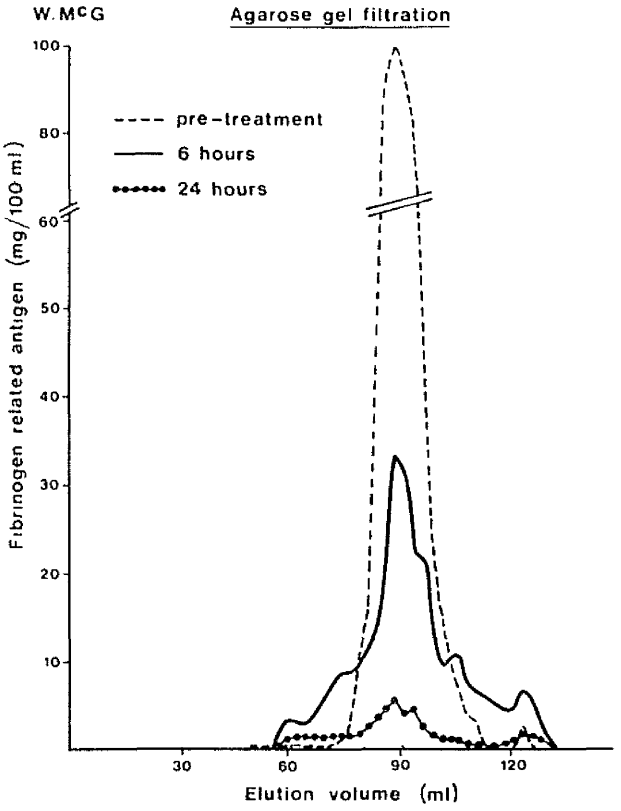

FIG. 3(a)

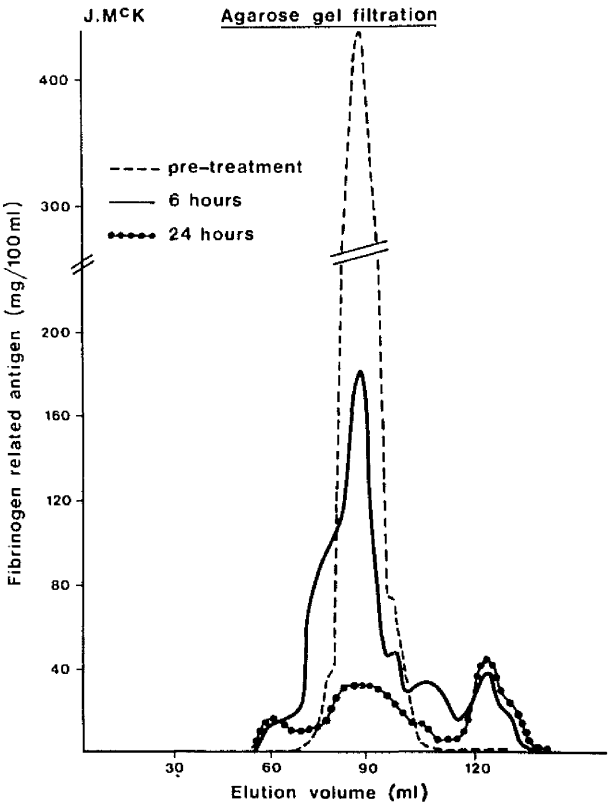

FIG. 3(b)

Elution of FR-antigen following 6% agarose gel filtration of a beta-alanine precipitate of plasma obtained from two representative patients (W.McG. and J.McK.) pre-treatment and after 6 and 24 hours ancrod infusion.

After 6 hours of ancrod infusion definite shoulders were noted on both sides of the FR-antigen main peak indicating the formation of increased quantities of soluble complexes and degradation products. The void volume soluble complexes clotted much more rapidly than an equivalent concentration of fibrinogen and appeared unstable in that they precipitated within a few hours of cooling to 4°C and also on standing at room temperature for 24-48 hours. The intermediate zone soluble complexes did not clot more readily than fibrinogen and in some cases the T.C.T. exceeded 3 minutes. Incubation with thrombin for one hour resulted in clot formation in those cases. These complexes were more stable and did not tend to precipitate on cooling to 4°C or on standing at room temperature for 48 hours.

After 24 hours of ancrod infusion only small quantities of FR-Antigen were eluted but followed the pattern of the 6 hour sample. No recordable T.C.T. was demonstrated in any patient. Small amounts of T.C.P. appeared at the position of the main peak after 1 hour incubation. In some samples T.C.P. was also present after 24 hours incubation.

In every case a high S.C.T. titre was recorded in the void

volume fraction of samples obtained from patients receiving treatment, e.g., in patient W.McG. the pre-treatment titre was zero, rising to 8 at 6 hours and remaining 7 at 24 hours.

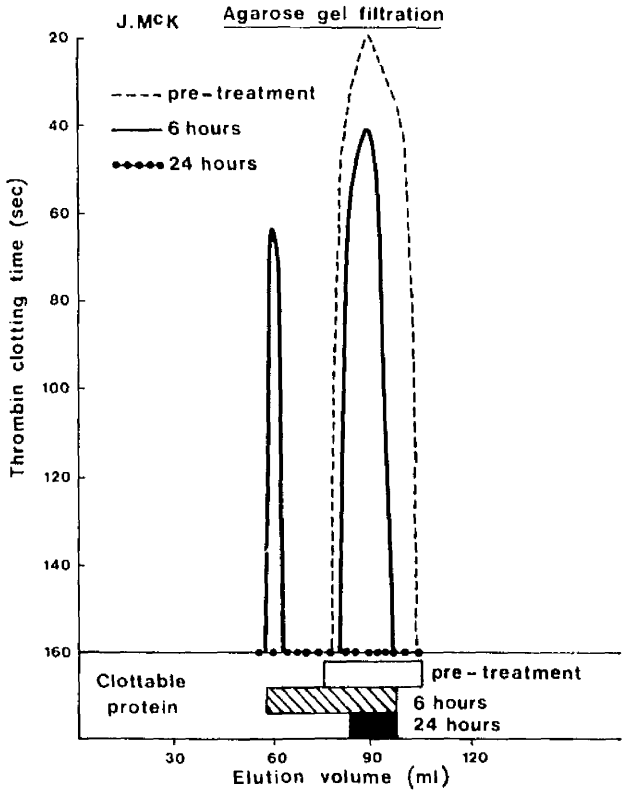

FIG. 4

The upper section shows the T.C.T. curves obtained from the eluant fractions following 6% agarose gel filtration of a beta-alanine precipitate of plasma obtained from patient J.McK. pre-treatment and after 6 and 24 hours ancrod infusion. (The corresponding FR-antigen curves are shown in Fig. 3b). The lower section shows changes in the range of thrombin clottable protein (after 1 hour's incubation) measured on the same eluant fractions.

Polyacrylamide Gel Electrophoresis (P.A.G.E.): The component units of the soluble complexes found in six hour samples at elution volumes 60 ml (void volume) and 80 ml (intermediate zone) were studied and compared with the uncomplexed material eluting at 90 ml (main peak), the position of standard fibrinogen.

P.A.G.E. in presence of S.D.S. alone: This technique allows approximate estimation of the molecular weight (M.W.) of the component units of the soluble complexes and uncomplexed material. Some variation was noted between patients and so the results of two patients illustrating those differences are shown in Fig. 5(a). Patient W.McG. is more typical of the group as a whole than patient J.McK. The component units consisted largely of material with a molecular weight less than fibrinogen similar

to a minimally degraded product early Fragment X. In addition small quantities of protein similar in M.W. to Fragment X, Y and D were found in some cases (particularly J.McK.). The main peak seems to consist of material ranging in M.W. from fibrinogen to Fragment X. Degradation products Y and D were present in some cases.

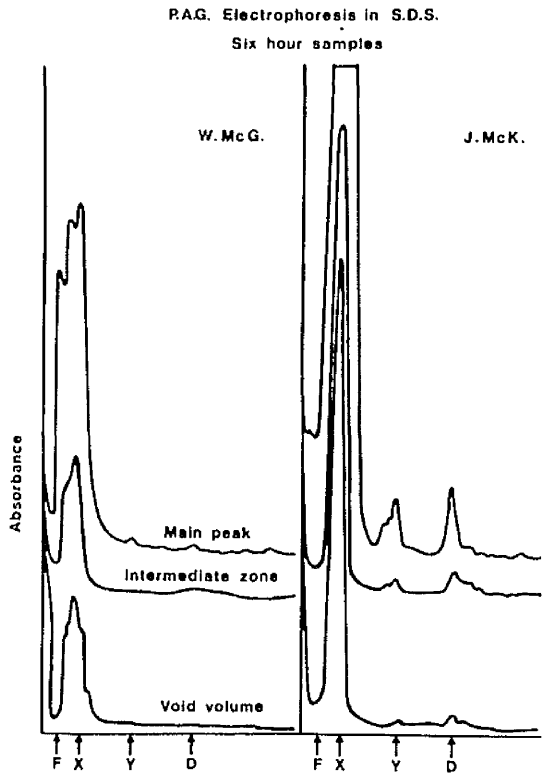

FIG. 5(a)

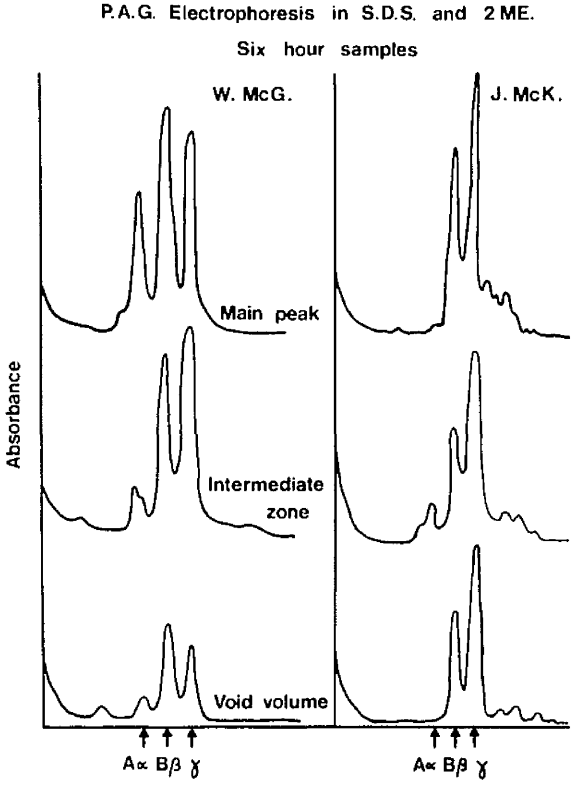

FIG. 5(b)

Densitometric scans of polyacrylamide gels obtained by electrophoresis in (a) sodium dodecyl sulphate (S.D.S.) and (b) sodium dodecyl sulphate and mercaptoethanol (S.D.S. and 2 ME) of selected fractions from agarose gel filtration of the 6 hour samples shown in Fig. 3. The migration position of standard fibrinogen (F), its degradation products X, Y and D and its chain Aα, Bβ and γ are indicated.

P.A.G.E. in presence of S.D.S. and Mercaptoethanol: This allows estimation of the M.W. of the chains within the component units of the soluble complexes and the uncomplexed material. The results for the same two patients are shown in Figure 5(b). In all 7 patients the component units of the soluble complexes were deficient in intact Aα chain. The Aα chain loss varied in degree and was complete in the void volume complexes in 2 patients (see J.McK.). In 6 patients Aα chain loss in the uncomplexed main peak material was minimal or absent (see W.McG). In only one patient (J.McK.) was total loss of Aα chain found.

DISCUSSION

Soluble complexes have been demonstrated in the plasma of patients suffering from a variety of conditions (1, 19, 14, 20, 7). In this present study the formation of a range of soluble complexes has been demonstrated during defibrination by ancrod infusion. It is unlikely that these soluble complexes were produced by the continued action of enzymes contained in the blood sample, as inhibitory concentrations of ancrod antiserum, E.A.C.A. and aprotinin were added with the anticoagulant used in preparing plasma. E.A.C.A. and aprotinin were also present in the column elution buffer.

Lack of intact Aα chain in the component units of the soluble complexes suggests that proteolytic digestion has occurred. High concentrations of ancrod progressively attack the Aα chain of fibrinogen *in vitro* (21,22). A Cohn Fraction I preparation of plasma from patients already defibrinated by ancrod and receiving continued treatment has been shown to be deficient in intact Aα chain (23). While this could be due to digestion of the Aα chain by ancrod itself, the concentration of ancrod likely to be present in the plasma therapeutically is less than that required for Aα chain digestion *in vitro* (22). If ancrod were responsible for the Aα chain digestion two Aα chain fragments (M.W. 39,000 and 31,000) should have been present (22). These were sought but not found in the present study. The rapid fall in plasma plasminogen levels which occurs during initial ancrod infusion (24) suggests that plasmin could be responsible for the Aα chain digestion of the soluble complexes.

The observation, that the Aα chain loss was more marked in the soluble complexes than in the uncomplexed material, in 6 out of 7 cases, suggests that preferential proteolysis of the soluble complexes may have occurred. It has been claimed that soluble fibrin monomer is digested rapidly and selectively by plasmin *in vitro* in preference to fibrinogen (25). This preferential digestion may be limited to the early stages of the reaction (25). If this occurred *in vivo* sufficiently rapidly to prevent the formation of insoluble fibrin, soluble complexes deficient in intact Aα chain could be formed. There would, therefore, be no need to postulate the existence of particulate fibrin 'micro-clots' (24) during ancrod treatment and the benign nature of the acute defibrination state produced could be explained.

Soluble complexes deficient in intact Aα chain could also be produced by the polymerisation of early degradation products of fibrinogen or fibrin (4) or by the lysis of insoluble fibrin (5). *In vitro* studies have shown that soluble complexes deficient in intact Aα chain can be produced in plasma and purified fibrinogen by the sequential action of ancrod and plasmin regardless of which enzyme acts first (unpublished observation). It

is, therefore, possible that the soluble complexes are produced *in vivo* by more than one route. Other enzymes may be involved.

The equilibrium between soluble complex formation and breakdown will depend not only on the rate of ancrod infusion, but also on the rate at which the individual is able to generate proteolytic enzymes and synthesise fibrinogen in response to the challenge. Shifts in this equilibrium might be responsible for the variation in the degree of Aα chain digestion in our patients. It is possible that digestion of soluble fibrin monomer is so rapid that soluble complexes with intact Aα chain never form within the circulation. In such patients (e.g., J.McK.) soluble complexes could be produced by polymerisation of early X fragments lacking fibrinopeptide A.

The suggestion that F.D.P. in ancrod treated patients may be derived from soluble rather than insoluble fibrin has already been made on the basis of studies of the cross-linkage of the F.D.P. (26). The preferential digestion of soluble fibrin could represent an important pathway for the production of F.D.P. *in vivo*.

ACKNOWLEDGEMENTS

We are grateful to Mr. D.W. Short for permission to study 3 patients with severe peripheral arterial disease. We thank the Editor of Nature and Macmillan Journals Ltd. for permission to include Figures 3(a) and 5(b) already published (9). Arvin was supplied as a gift from Berk Pharmaceuticals Ltd., Shalford, Sussex, England. C.R.M. Prentice is a Wellcome Research Fellow.

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McKILLOP C.A. PH. D. THESIS 1977.

Scottish Medical Journal

Reprinted from 1975 20: 139

Soluble fibrin complex production and proteolysis during ancrod therapy

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SCOTTISH SOCIETY FOR EXPERIMENTAL MEDICINE—SELECTED ABSTRACT

SOLUBLE FIBRIN COMPLEX PRODUCTION AND PROTEOLYSIS DURING ANCROD THERAPY

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A soluble fibrin complex is a polymer composed of units antigenically related to fibrinogen. Such complexes have been identified in the plasma of patients suffering from 'hypercoagulable' states and thrombosis (Fletcher *et al.*, 1970). Their presence is thought to indicate activation of the clotting mechanisms with conversion of prothrombin to thrombin within the circulation.

A thrombin-like snake venom, ancrod (Arvin, Twyford Laboratories, High Wycombe), is used therapeutically as a defibrinating agent. *In vitro* ancrod clots fibrinogen by the removal of fibrinopeptide A. The fibrin monomer so produced is thought to polymerise to form, first, a soluble fibrin polymer and then insoluble fibrin. *In vivo* ancrod produces defibrination by the conversion of fibrinogen to fibrin 'micro-clots', which are then removed by a combination of fibrinolysis and the activity of the reticuloendothelial system (Pitney *et al.*, 1969).

A group of 7 patients were studied during the initial stages of defibrination by ancrod with a view to identifying the soluble complexes formed and isolating them in sufficient concentration to allow study of their chain structure. Plasma samples were obtained before treatment and after 6 and 24 hours ancrod infusion at a rate of 2 *u*/Kg./12 hours. Agarose gel filtration was used to separate the soluble complexes from the uncomplexed fibrinogen related material on the basis of their greater molecular size. The concentration of fibrinogen related antigen (FR-antigen) in protein containing elution fractions was measured using a radial immunodiffusion technique. The results from a typical patient are shown in Figure 1.

The FR-antigen from normal control plasmas always eluted on the columns as a symmetrical curve with a peak at an elution volume of 90 ml. In the pre-treatment sample of the representative patient shown in Figure 1, there was a small shoulder on both the ascending limb of the curve, representing soluble complex formation, and on the descending limb indicating the presence of degradation products of fibrinogen or fibrin. Six hours after starting treatment increased quantities of both soluble complexes and degradation products were present. After 24 hours treatment very little FR-antigen remained in the plasma but traces of soluble complexes were still found.

The technique of polyacylamide gel electrophoresis in sodium dodecyl sulphate (S.D.S.) and mercaptoethanol was used to study the chain structure of the fibrinogen related molecules within the soluble complexes. The results of the same patient are shown in Figure 2. Samples were studied from the void volume fraction (60 ml. elution volume) from an intermediate zone fraction (80 ml. elution volume), and were compared with the uncomplexed material present in the main peak fraction (90 ml. elution volume).

Before treatment the main peak fraction showed the three bands of normal fibrinogen corresponding to its alpha, beta and gamma chains. No FR-antigen was present in the void volume fraction. The intermediate zone soluble complexes showed a marked lack of intact alpha-chain, when compared with the main peak fraction. After 6 hours ancrod treatment the soluble complexes in both the void volume and intermediate zone fractions showed a similar lack of intact alpha-chain. The main peak fraction showed the bands of normal fibrinogen with minimal loss of intact alpha-chain.

The relative lack of intact alpha-chain in the soluble complexes suggests that they have undergone preferential digestion by a proteolytic enzyme, such as plasmin. Plasmin initially attacks the alpha-chain of fibrinogen producing a degradation product known as early Fragment X. The soluble complexes consisted of similar units.

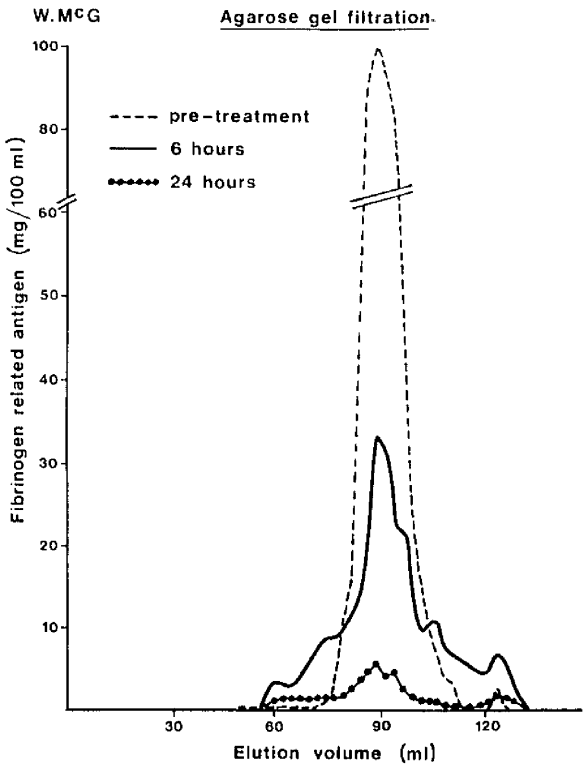

Fig. 1. Agarose gel filtration of fibrinogen related antigen derived from plasma of a patient undergoing ancrod treatment. Samples obtained pre-treatment and after 6 and 24 hours treatment with ancrod.

Soluble fibrin monomer may be attacked selectively by plasmin in preference to fibrinogen *in vitro* (Konttinen *et al.*, 1973). If this occurs *in vivo* at a sufficiently rapid rate the formation of insoluble fibrin 'micro-clots' could be prevented. Instead soluble complexes deficient in intact alpha-chain could be produced. Further digestion of these complexes by plasmin would result in the production of fibrin degradation products (F.D.P.).

The F.D.P. in ancrod-treated patients may, therefore, be derived from soluble rather than insoluble fibrin. As insoluble 'micro-clots' would not form within the circulation, the benign nature of the acute defibrination state produced by ancrod would be explained. It is possible that the preferential digestion of soluble fibrin by plasmin represents an important pathway in the production of F.D.P. *in vivo*.

C.R.M.P. is in receipt of a Wellcome Research Fellowship.

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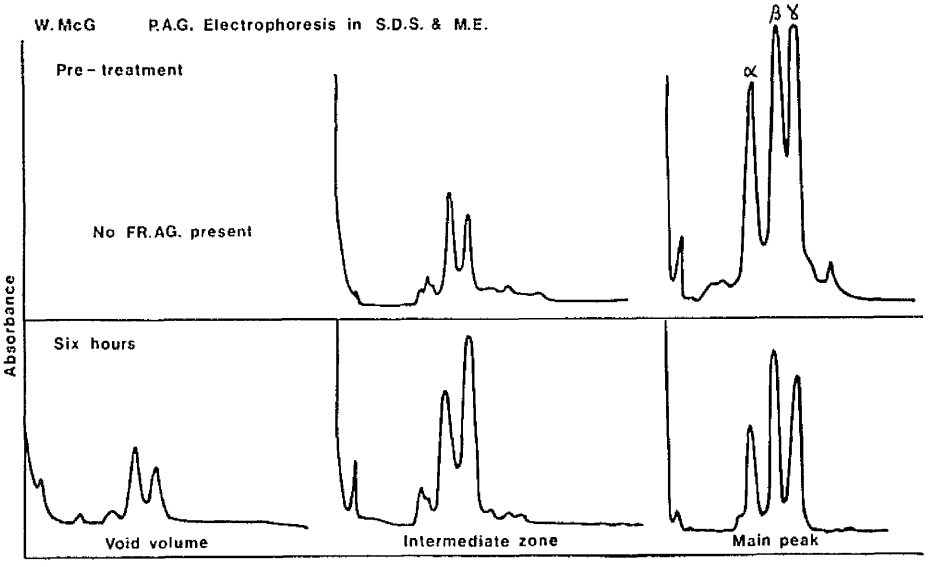

Fig. 2. Densitometric scans of polyacylamide gel electrophoresis in sodium dodecyl sulphate and mercaptoethanol of selected fractions (void volume, intermediate zone and main peak) from the pre-treatment and 6 hour samples in Figure 1.

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(Reprinted from Nature, Vol. 255, No. 5510, pp. 638–640, June 19, 1975)

Possible pathway for formation of fibrin degradation products during ancrod therapy

SOLUBLE complexes composed of protein antigenically related to fibrinogen have been found in the plasma of patients suffering from thrombosis or a hypercoagulable state[1]. These complexes are important as they could provide a sensitive method for the laboratory diagnosis of such conditions at a very early stage in their development[1]. Little is known concerning the structure, formation and breakdown of soluble complexes *in vivo*.

Soluble complexes can be produced by an intravenous infusion of thrombin in rabbits[2]. Although such experiments are not possible in man, the use of thrombin-like snake venoms as defibrinating agents could provide a valuable alternative. Soluble complexes have been identified in the plasma of patients given defibrinating doses of the partially purified venom of *Bothrops moojeni* (Defibrase)[3].

We have studied seven patients receiving treatment with ancrod, a defibrinating agent derived from the venom of *Agkistrodon rhodostoma*. *In vitro* ancrod resembles thrombin in removing fibrinopeptide A from fibrinogen but differs in failing to remove fibrinopeptide B (ref. 4). *In vivo* ancrod infusion is thought to produce rapid defibrination by the formation of insoluble fibrin microclots throughout the circulation, which are removed by fibrinolysis and reticuloendothelial cell activity[5].

Ancrod (Arvin, Berk, Shalford, Surrey, UK) was administered intravenously in a dose of 2 U per kg body weight per 12 h. Blood samples were taken before starting treatment and after 6 and 24 h therapy. Plasma was obtained using trisodium citrate (final concentration 0.013 M) as anticoagulant. Epsilon aminocaproic acid (EACA), aprotinin and ancrod antiserum were added to prevent any *in vitro* proteolysis. Samples were tested immediately without storage, as freezing and thawing may cause the formation of soluble complexes[6].

Soluble complexes were separated from uncomplexed fibrinogen-related protein by 6% agarose gel filtration (Biogel A-5m, BioRad Laboratories, 1510750) of a β alanine precipitate of plasma[1,7]. EACA and aprotinin were added to the elution buffer. Protein-containing eluant fractions were analysed for fibrinogen–fibrin related antigen (FR-antigen) by a radial immunodiffusion technique[8] using fibrinogen antiserum (Behringwerke–Hoechst, ORCH 05). There may be changes in the antigenic structure of the fibrinogen-related molecules when incorporated into soluble complexes and their diffusion properties may also be altered. This method is, therefore, only semiquantitative but changes in the distribution of FR-antigen were clearly shown and the results correlated well with those obtained by continuous absorbance monitoring at 280 nm.

FR-antigen from the plasma of normal control subjects eluted from the column as a symmetrical curve with a peak at 90 ml. The results from a representative patient are shown in Fig. 1. Before starting treatment there is an essentially normal curve but after 6 h of ancrod infusion a range of soluble complexes were present extending from the void volume (60 ml) to the position of standard fibrinogen. Degradation products of lower molecular size were also present.

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate and mercaptoethanol[9] was performed to study the chain structure of the component units of the soluble complexes. Samples were taken from the complexes present at 60 and 80 ml and compared with the uncomplexed material at 90 ml. Where electrophoresis could not be performed immediately, samples were stored at −20 °C and tested within 24–48 h. The results for two patients are shown in Fig. 2.

Fibrinogen is composed of three pairs of polypeptide chains (Aα, Bβ, γ). In all seven patients there was marked loss of intact Aα chain in the soluble complex material. In six out of seven patients there was only minimal loss of intact Aα chain in the main peak fraction (see W.McG.). The remaining patient (J.McK.) showed virtual absence of intact Aα chain.

Proteolytic enzymes initially attack the Aα chain of fibrinogen. The greater degree of Aα chain loss in the soluble complexes in six out of seven patients suggests that

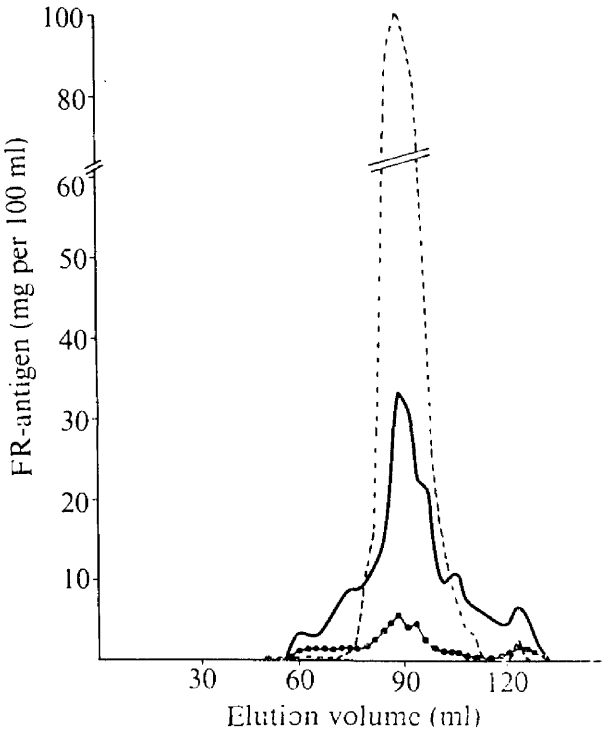

Fig. 1 Elution pattern of FR-antigen following agarose gel filtration of a β alanine precipitate of plasma obtained from one patient (W.McG.) before starting treatment (- - - - -) and after 6 (———) and 24 h (●) therapy.

preferential proteolytic digestion of the complexes may have occurred. As there is marked plasminogen depletion during ancrod therapy[5], plasmin may be responsible for this proteolysis. Ancrod can produce Aα digestion *in vitro*[10], but the concentration of ancrod required is much greater than that present therapeutically.

Soluble complexes deficient in intact Aα chain could be produced by the repolymerisation of early degradation products[2] or directly by the lysis of insoluble fibrin[7]. It has also been suggested that soluble fibrin monomer is lysed by plasmin in preference to fibrinogen *in vitro*[11]. If this occurred *in vivo* rapidly enough to prevent the formation of insoluble fibrin, soluble complexes similar to those demonstrated would be formed. Further digestion by plasmin would produce uncomplexed fibrin degradation products (FDP).

FDP in ancrod-treated patients may, therefore, be derived from soluble rather than insoluble fibrin. A similar sug-

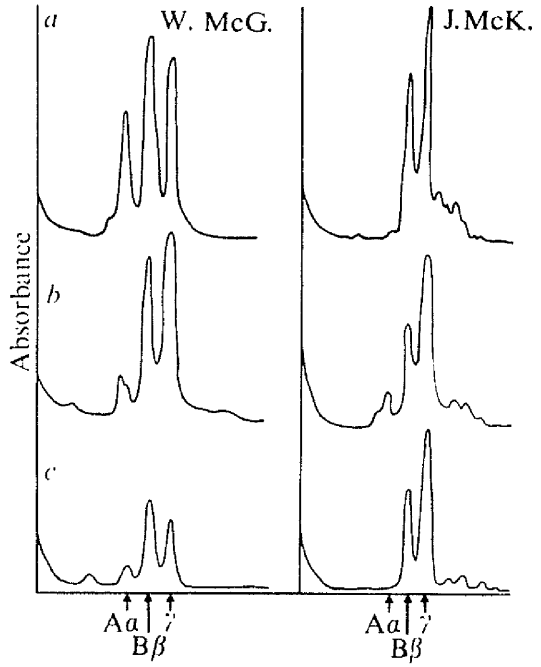

Fig. 2 Densitometric scans of polyacrylamide gel electrophoreses of selected fractions (*a*, main peak fraction at 90 ml; *b*, intermediate zone fraction at 80 ml; *c*, void volume fraction at 60 ml) from agarose gel filtration of the 6 h samples from two patients (W.McG. and J.McK.). The migration position of the Aα, Bβ and γ chains of standard fibrinogen are indicated.

gestion has been made based on studies of the cross-linkage of the FDP (ref. 12). Microclots would not form within the circulation, explaining the benign defibrination state produced by ancrod infusion. The preferential proteolysis of soluble fibrin could represent an important pathway for the production of FDP *in vivo*.

We thank Mr D. W. Short for permission to study three patients with peripheral arterial disease, and Berk Pharmaceuticals Ltd for the gift of Arvin. C.R.M.P. is a Wellcome Research Fellow.

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Received April 22; accepted May 5, 1975.

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tive cholecystectomy with either a subcostal-oblique or midline incision. Informed consent was obtained. There were no complications attributable to the investigations.

After *premedication* with diazepam (0·15–0·20 mg/kg) and droperidol (0·035–0·07 mg/kg) administered intramuscularly approximately an hour before the investigation, *anæsthesia* was induced with a sleep-dose of thiopentone. Succinylcholine (1·5 mg/kg) was then administered and intubation, using a wide-bore, cuffed endotracheal tube, was performed after ventilating the lungs with oxygen. The patient was ventilated mechanically by the anæsthetist and anæsthesia was maintained with fentanyl (0·003 mg/kg). Further doses of fentanyl (0·02–0·04 mg) and diazepam (2–4 mg) were given if required. During the postoperative course, the patient was supervised in an intensive-care unit for 2 to 3 hours before returning to the surgical ward.

Arterial blood samples, taken by needle puncture, were drawn in 5 ml syringes, the dead-space being filled with heparin. The syringes were stored on ice, the blood being analysed within half an hour after sampling. Oxygen and carbon dioxide tensions were determined with standard electrodes (E 5046, E 5036, Radiometer, Copenhagen), calibrated with water-saturated, temperature-regulated gases.

Pulmonary photoscanning was done in half the material, using a gamma-camera (Nuclear-Chicago PHO gamma scintillation camera). The patient was given 1·5 mCi of the isotope ^{99}mTc ferric hydroxide (Radiochemical Centre, Amersham) intravenously into a cubital vein, before scanning, which was then performed in three projections—frontal, lateral, and dorsal.

The statistical analysis consisted of the calculation of means and standard errors. Student's *t*-test was used to assess the significance of differences between the results, both by testing dependent paired data and independent mean values.

Procedure

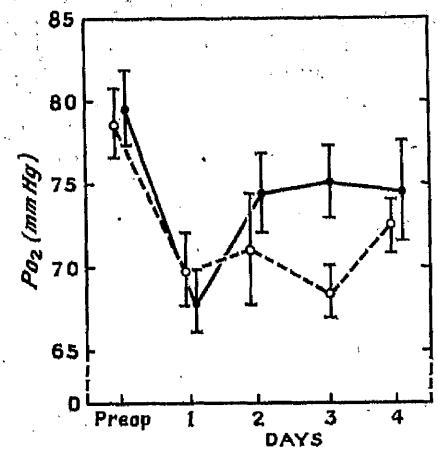
The patients were randomised into two groups, each containing 24 subjects, with the same distributions of subcostal-oblique and midline incisions. The patients in one group (heparin group) were given 5000 units of sodium heparin (10 000 units/ml, Vitrum, Stockholm) subcutaneously into the thigh every 12 hours for 5 days, starting 2–3 hours before operation. The patients in the other group (control group) were given 0·5 ml of 0·85% saline at corresponding intervals. Arterial blood samples were drawn preoperatively and daily for 4 days during the postoperative period. Preoperatively and on the 3rd or 4th postoperative day, a pulmonary photoscan and a plain chest X-ray were obtained in 24 patients—12 from each group.

Results

The mean age in the heparin group was 53 years (range 26–70 years) and in the control group 54 years (range 30–74 years).

The arterial oxygen tension was the same in both groups preoperatively, and during the 1st postoperative day it fell significantly ($P<0·001$) and to the same extent (see accompanying figure). During the 2nd postoperative day the oxygen tension rose in the heparin group to values which no longer differed significantly from the preoperative level. In the control group a significant reduction ($P<0·01$) persisted until the 4th postoperative day. The oxygen tension was lower in the control group than in the heparin-treated patients during the 2nd and 3rd postoperative days, the difference reaching a significant level ($P<0·01$) on day 3.

The arterial carbon-dioxide tension did not differ between the groups preoperatively (heparin-treated subjects: 36·2±0·8 mm Hg, mean and S.E.; controls: 31·6±0·8 mm Hg); neither did it vary significantly between days, the variation between days amounting to less than 2 mm Hg in both groups.



Arterial oxygen tension (PO_2) during the perioperative period.

●=heparin-treated subjects, o=control group (Mean±S.E., n=24 in each group). Note the faster recovery of Po_2 in the heparin group.

In no patient were there any clinical signs of pulmonary embolism during the postoperative period (no chest pain, dyspnœa, cyanosis, hæmoptysis). The chest X-ray obtained postoperatively was normal in all 24 patients examined and the photoscan was normal in 23 of 24 cases. In 1 patient, belonging to the heparin group, the photoscan showed defects of uncertain origin.

Discussion

A reduced ventilatory capacity and decreased arterial oxygen tension are common findings after a high laparotomy.[12–14] The increased intra-abdominal pressure pushes the diaphragm cranially, thereby reducing the lung volume and increasing respiratory work. Reflex inhibition of the abdominal muscle function may also occur.[15] The reduced lung volume promotes airway closure and veno-arterial shunting.[16] The postoperative pain, sedation, mucus remaining in the airways, and persisting bronchial spasm are other factors which reduce ventilatory efficiency and increase the incidence of atelectasis and infection.[17] Ventilation/perfusion disturbances, including shunting, may develop and reduce arterial oxygen tension still further.[14 18]

Another cause of hypoxæmia may be microthromboembolisation in the lung vessels, resulting in release of smooth-muscle active substances.[11 19–21] Thus measures to counteract both microthromboembolisation and large pulmonary emboli would appear to reduce the incidence or shorten the period of postoperative hypoxæmia. In the present study, heparin-treated patients had a shorter period of postoperative hypoxæmia than controls. This difference cannot be ascribed to differences in alveolar ventilation since the arterial carbon-dioxide tension was substantially the same in the two groups. In view of the well-known effects of heparin on coagulation, the most reasonable explanation for the shortened hypoxæmia is a reduced incidence of embolisation. Since no convincing evidence of large pulmonary emboli was obtained in this study in either group, the heparin prophylaxis presumably consisted in counteracting microembolisation.

The anticoagulant action of heparin is not completely understood. Presumably heparin interferes at several stages with the coagulation.[22] Eika et al.[23] found that as little as 0·003–0·01 units of heparin per ml blood prolonged the thrombin-time. Biggs et al.[24] and Wessler and Yin[25] have suggested that low levels of heparin enhance the effect of naturally occurring inhibitors of activated factor X (anti-Xa). Yin et al.[26] have demonstrated that heparin together with factor Xa inhibitor forms a potent antiplatelet-aggregant.

Considering the results, it appears that low-dose heparin treatment may shorten the period of post-operative hypoxæmia, probably by counteracting both large pulmonary emboli, as shown previously, and microthromboembolism.

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SOLUBLE FIBRINOGEN/FIBRIN COMPLEXES IN PRE-ECLAMPSIA

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Summary Significantly increased concentrations of soluble fibrinogen/fibrin complexes were found in plasma samples from ten normal pregnant women when compared with ten non-pregnant age-matched controls. In ten women with pre-eclampsia mean soluble complex concentration was more than three times that in the age, parity, and gestation matched pregnant controls. Soluble fibrinogen/fibrin complexes are also found in the plasma of patients in various hypercoagulable and thrombotic states, including disseminated intravascular coagulation. These findings provide additional evidence that pre-eclampsia is associated with disseminated intravascular coagulation.

Introduction

THERE is histological evidence of fibrin deposition on the vascular endothelium in cases of severe pre-eclampsia,[1 2] and in addition there are changes in both the blood-coagulation and fibrinolytic systems, which are significantly greater than those occurring in normal pregnancy.[3–5] These features suggest that disseminated intravascular coagulation (D.I.C.) is associated with pre-eclampsia. Soluble complexes of fibrinogen/fibrin-related antigen (F.R. antigen) were found in plasma samples from patients with various hypercoagulable and thrombotic conditions[6 7] and were demonstrated in the plasma of a patient with severe eclampsia and coma associated with the coagulation changes of D.I.C.[8] This study was undertaken to see whether soluble complexes could be detected in pre-eclampsia.

Patients, Materials, and Methods

Three groups were studied—ten patients with pre-eclampsia (group I), ten normal, pregnant women (group II), and ten normal, non-pregnant women (group III).

Pre-eclampsia was diagnosed in patients developing hypertension, with a diastolic blood-pressure greater than 90 mm Hg on at least two occasions, and proteinuria in excess of 0·5 g per 24 hours. All patients were normotensive and did not have detectable proteinuria at their first antenatal visit. The signs developed after the 24th week of pregnancy and resolved after delivery. The two pregnant groups were matched for age, parity, and gestation. When the plasma samples were taken the diastolic blood-pressure was 101±9 mm Hg (mean±1 S.D.) in group I and 73±8 mm Hg in group II ($P<0·001$). Proteinuria in group I ranged from 0·75 to 12·0 g/24 h (5·3±4·1 g/24 h). No patient in group II had detectable proteinuria. In addition œdema was present in all patients in group I and in none of the control group II. Group III consisted of ten normal, nulliparous women, who were not taking oral contraceptives. They were matched for age with women in groups I and II.

Blood-samples were collected by careful venepuncture and anticoagulated with sodium citrate (final concentration 0·013 mol/l). ε-aminocaproic acid and aprotinin were added to prevent in-vitro proteolysis. 6% agarose-gel filtration was performed using 'Biogel' A5 M mesh size 200–400 (Bio-rad Laboratories Ltd.)[6 8] The F.R.-antigen concentration of eluant fractions was measured by radial immunodiffusion. Details of the method have been published elsewhere.[9] Statistical analysis was performed using Student's *t* test.

Results

Representative examples of F.R.-antigen curves from each of the three groups (matched samples) are shown in fig. 1. F.R.-antigenic material was eluted earlier in normal pregnancy (group II) at 81·5±3·5 ml than in the non-pregnant control group (group III) at 90·5±2·0 ml. In pre-eclampsia (group I) the F.R.-antigenic material was eluted at 63±2·5 ml. There are significant differences between the results for groups I and II ($P<0·001$).

Soluble fibrinogen/fibrin complex concentration was estimated by measuring the areas under the elution curves as shown in fig. 2. As any pure protein will elute from the column with small "trails" on both ascending and descending limbs, approximately 2–3% of area "m"

will always represent uncomplexed fibrinogen. The results for the three groups are shown in fig. 3. Area "m" represented 2·7±0·4% of the total area in group III, 4·7±0·9% in group II, and 16·0±6·4% in group I.

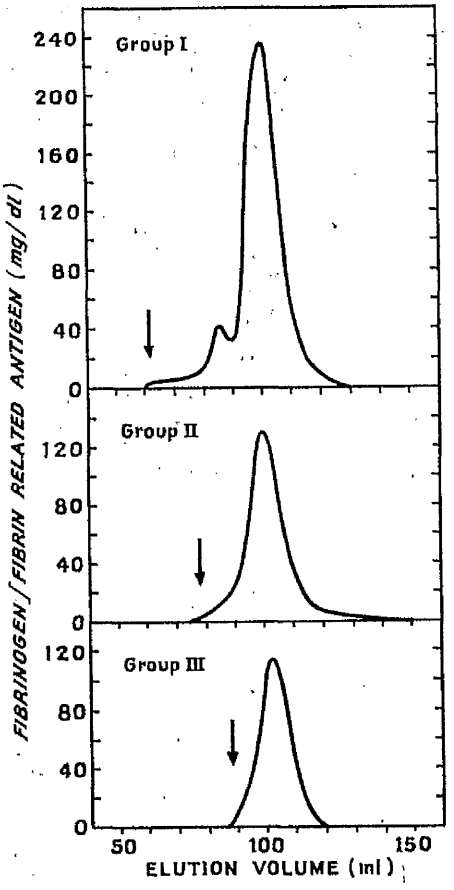

Fig. 1—Representative F.R.-antigen elution curves from matched samples.

Group I (pre-eclamptic patient), group II (normal pregnant control), and group III (normal, non-pregnant control). Arrow indicates the volume at which F.R.-antigen was first eluted (concentration >0·5 mg/dl).

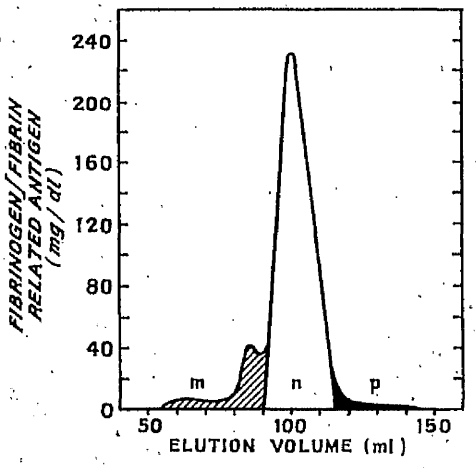

Fig. 2—Elution curve showing areas "m", "n", "p", which were calculated by extending the ascending and descending limbs of the main peak to the baseline.

Area "m" consists mainly of soluble F.R.-antigen complexes, although a small amount of normal fibrinogen was also present (see text). Areas were expressed as a percentage of total area.

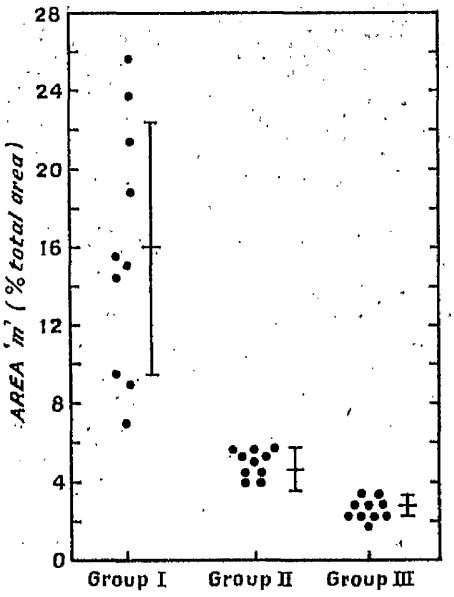

Fig. 3—Results of measurements of area "m" in groups I, II and III.
Mean ±1 S.D. is shown.

These differences are statistically significant (group I *vs.* group II, $P<0.001$; group II *vs.* group III, $P<0.001$).

Discussion

Most blood coagulation factors increase during normal pregnancy and this together with a depression of fibrinolytic activity has led to the hypothesis that normal pregnancy represents a hypercoagulable state.[10] In this study we found a small, but significant increase in soluble fibrinogen/fibrin complex concentration in association with normal pregnancy. This accords with the observations of Hafter et al., who used optical density rather than F.R.-antigen curves.[11]

Pre-eclampsia was, however, invariably associated with a further, highly significant increase in soluble complex concentration. In addition the more seriously ill the patient, as measured by standard clinical criteria, the greater was the concentration of soluble complex.

The exact nature of soluble fibrinogen/fibrin complexes is still uncertain. In-vitro experiments have shown that they may contain units of fibrin monomer, fibrinogen, and fibrinogen/fibrin degradation products.[12] The action of thrombin (or a thrombin-like enzyme) seems to be essential for their production,[6 7 12] and they are found in plasma samples from patients with hypercoagulable or thrombotic disease.[6–8]

Our results, therefore, strongly support the concept of hypercoagulability in normal pregnancy with some conversion of fibrinogen to fibrin occurring within the circulation. The higher concentration of soluble complexes found in each pre-eclamptic patient suggests that the conversion of fibrinogen to fibrin may be occurring at an increased rate. Alternatively, clearance of soluble complexes from the circulation could have been inhibited. In such circumstances it is possible that deposition of insoluble fibrin has occurred.

Our observations produce further evidence to support the hypothesis that pre-eclampsia is associated with D.I.C. Although the D.I.C. may be secondary to an unknown primary pathology, it would explain many of the

signs and symptoms of the disease. It has often been suggested that a controlled trial of anticoagulant therapy, possibly with antiplatelet agents, would be useful in pre-eclampsia. The technique we used could be valuable in monitoring the effects of such therapy.

We thank the Wellcome Trust and the Medical Research Council for financial assistance.

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ACUTE LETHAL CARDITIS CAUSED BY HIGH-DOSE COMBINATION CHEMOTHERAPY
A Unique Clinical and Pathological Entity

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Summary An acute lethal myopericarditis has been observed in four out of fifteen patients receiving high-dose combination chemotherapy which includes cyclophosphamide 45 mg/kg/day for four days. In all cases the myopericarditis occurred 5–9 days after the initiation of chemotherapy, with dyspnœa, tachycardia, orthostatic hypotension, fluid retention, decreased voltage on electrocardiography, and pericardial effusion documented by echocardiogram, and progressed in 2 to 6 days to a fatal low-output state despite vigorous treatment. In three of the four patients, necropsy was permitted and revealed the unique pathological finding of fibrin microthrombi in capillaries, fibrin strands in the interstitium, and fibrin strands within the heart-muscle cells.

Introduction

HIGH-DOSE chemotherapy utilising primarily cyclophosphamide (CYT) has been used in varying doses and combinations as a preparatory regimen before bone-marrow transplantation. Buckner[1,2] has reported fatal myocardial failure in two patients shortly after they had received 240 mg/kg and 200 mg/kg of CYT given in each case over 4 days. Santos[3–5] has described fatal myocardial necrosis in one patient who received 270 mg/kg of CYT over 10 days. Initial studies at the National Cancer Institute employing a 4-day CYT protocol (total dose = 180 mg/kg) suggested that this dose provided inadequate immunosuppression and antileukæmic effect in patients with refractory leukæmia. In an effort to gain more immunosuppressive and antineoplastic effects, Graw and associates[6] developed a 4-day combination chemotherapy protocol utilising bis-chlorethyl nitrosourea (B), cytosine arabinoside (A), cyclophosphamide (C), and 6-thioguanine (T) (BACT). Of fifteen patients treated on this protocol, four have died of an acute lethal myopericarditis which appears to be a unique clinical and pathological entity.

Materials and Methods

The basic techniques employed for bone-marrow transplantation have previously been reported.[6,7] Three of the four patients in this report were given the 4-day BACT regimen consisting of CYT 45 mg/kg/day (total dose 180 mg/kg); 6-thioguanine (6-T.G.) 100 mg/m² every 12 hours for 3½ days (total dose 700 mg/m²); cytosine arabinoside (Ara-C) 100 mg/m² every 12 hours for 3½ days (total dose 700 mg/m²); and bis-chlorethyl nitrosourea (B.C.N.U.) given as a single dose of 200 mg/m² on the 3rd day. The fourth patient received the 6-day BACT regimen, a modification of the above with CYT given for 6 days (total dose 270 mg/kg), cytosine arabinoside, and 6-thioguanine given for 5½ days each (total dose of 1100 mg/m²), and the nitrosourea as a single dose of 200 mg/m² on day 3. 24 hours after the last dose of chemotherapy, all four patients received an infusion of either allogenic or autologous bone-marrow. The patients were evaluated clinically by members of the transplantation team and cardiac consultants. Electrocardiograms and echocardiograms were reviewed by the cardiac consultants. The necropsy protocols and pertinent histological slides of three patients were reviewed by one of us (V.F.). Paraffin blocks from the formalin-fixed hearts were recut. Sections were stained with hæmatoxylin and eosin, celestine blue-B and eosin, phosphotungstic-acid hæmatoxylin (P.T.A.H.), methyl-green/pyronin-Y, and the periodic-acid/Schiff (P.A.S.) method with and without prior digestion with diastase. Thin transmural sections of the left ventricle were obtained at necropsy and fixed overnight in cold, 3% phosphate-buffered glutaraldehyde. Small blocks from these sections were post-fixed in 1% phosphate-buffered osmium tetroxide, dehydrated in a graded series of alcohols, and embedded in 'Maraglas'. For light microscopy, semifine sections (½-1 μm thick) from the maraglas-embedded tissues were stained with alkaline toluidine-blue. For electron microscopy, ultrathin sections from these blocks were stained with uranyl acetate and lead citrate.

Case-records

FIRST CASE

The patient was a 17-year-old White male with Burkitt's lymphoma which was progressive despite four courses of combination chemotherapy using CYT 1000 mg/m² intravenously (i.v.), vincristine 2 mg i.v., and methotrexate 30 mg intrathecally and 60 mg i.v. in each course, and 2000 rads of radiation to the whole abdomen. He was therefore admitted to the hospital for high-dose ablative chemotherapy and infusion of his autologous bone-marrow which had been harvested and frozen

SOLUBLE FIBRINOGEN-FIBRIN COMPLEXES IN PRE-ECLAMPSIA AND OTHER CLINICAL CONDITIONS WHICH MAY BE ASSOCIATED WITH INTRAVASCULAR COAGULATION

by

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VOLUME II

A thesis in two volumes submitted for the degree of Doctor of Philosophy to the University of Glasgow.

February, 1977

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Figure 1

A simple model of the fibrinogen molecule showing the three pairs of polypeptide chains (Aα , Bβ , γ). The length of the chain is approximately proportional to its molecular weight. The fibrinopeptides are indicated by the solid dots (●). The chains are held together by disulphide bonds, which are placed in an arbitrary fashion in the diagram. No attempt is made to indicate the actual shape of the molecule (adapted from Marder, Budzyński and James, 1972).

C = carboxy-terminal end of the polypeptide chain.

N = amino-terminal end of the polypeptide chain.

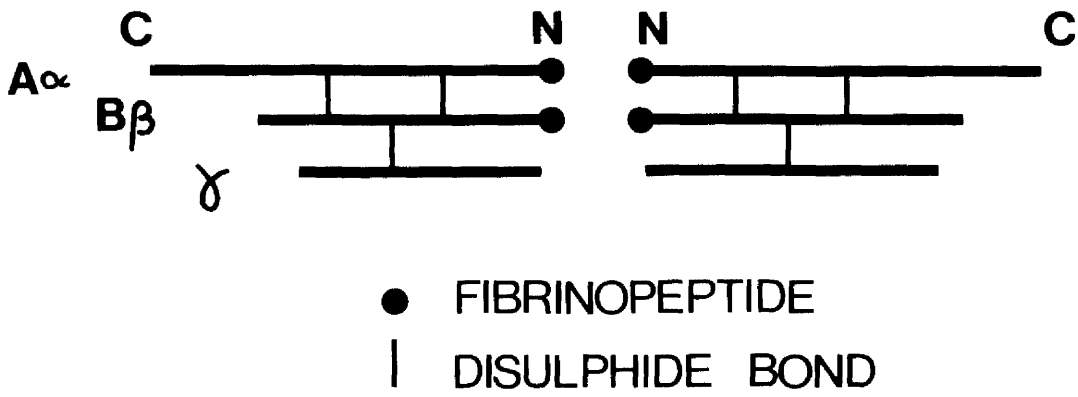


Figure 1

Figure 2

A model of the coagulation "cascade" based on that proposed by Macfarlane (1964). The Roman Numerical System for the classification of the coagulation factors is used. The small letter "a" after the number indicates the activated factor (e.g. IIa = thrombin). The solid arrow indicates "transformation", while the broken arrow indicates "action".

Ia = non-crosslinked fibrin.

Ib = crosslinked fibrin.

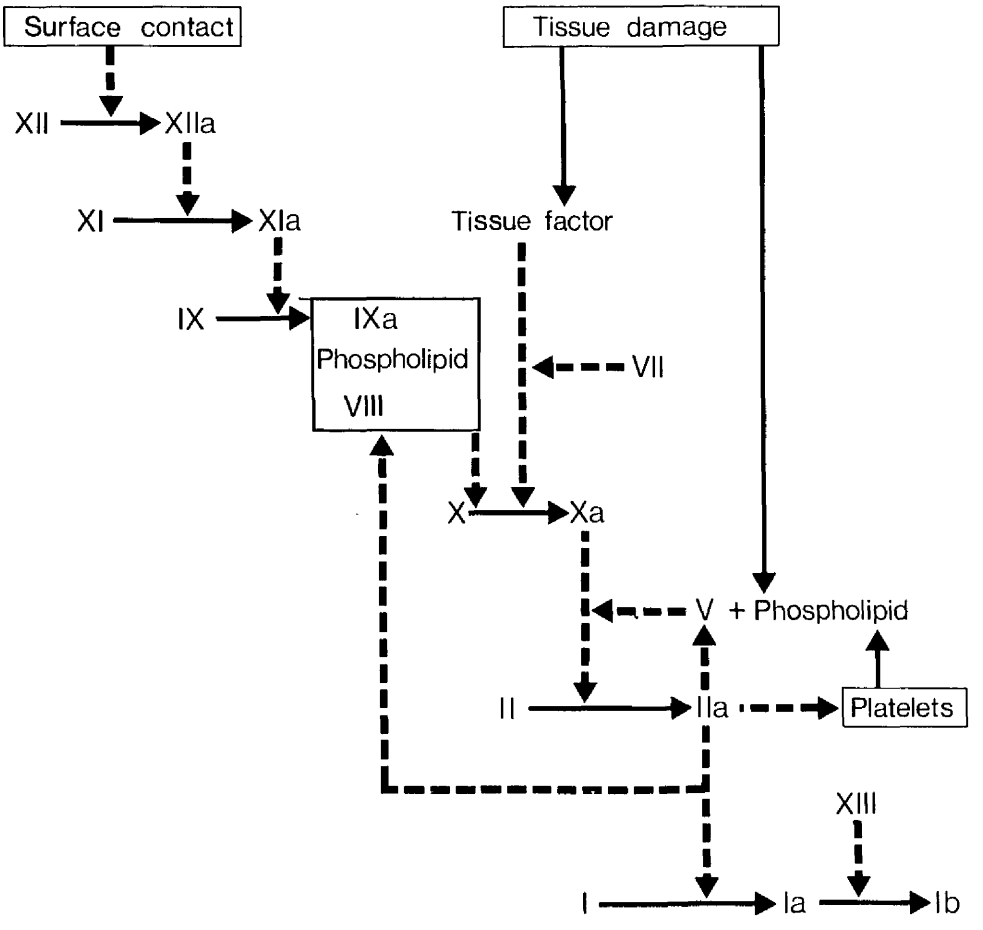

Surface contact
Tissue damage
XII
XIIa
XI
XIa
Tissue factor
IX
IXa
Phospholipid
VIII
VII
X
Xa
V + Phospholipid
II
IIa
Platelets
XIII
I
Ia
Ib

Figure 2

Figure 3

The three stage reaction involved in the conversion of soluble fibrinogen to insoluble fibrin, adapted from that proposed by Laskowski, Rakowitz and Scheraga (1952). Thrombin is only required for step i. Steps ii and iii are reversible.

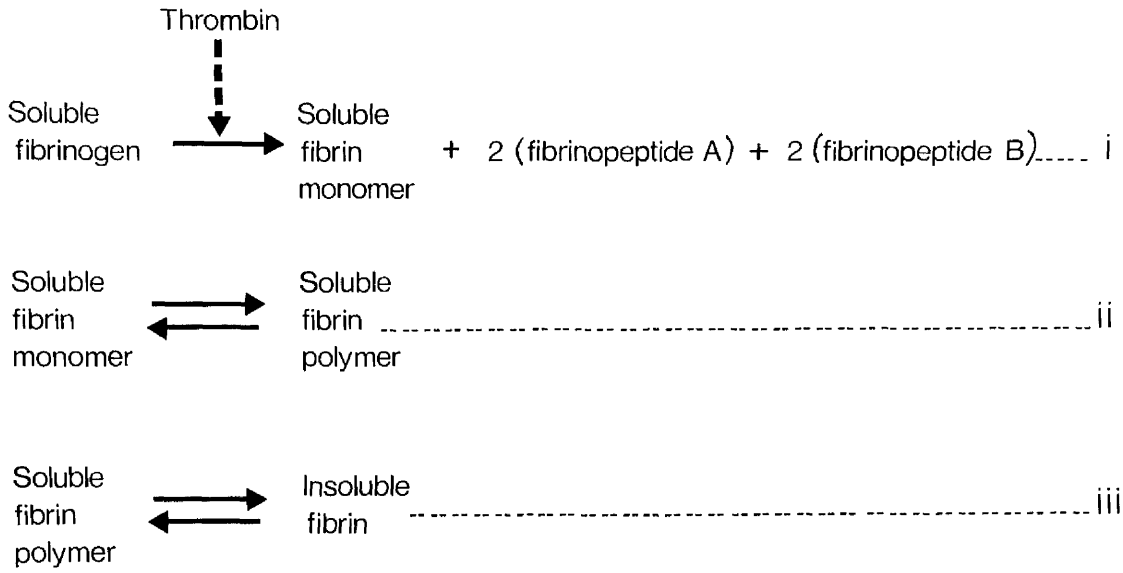

Thrombin
Soluble fibrinogen
Soluble fibrin monomer
+ 2 (fibrinopeptide A) + 2 (fibrinopeptide B)
i
Soluble fibrin monomer
Soluble fibrin polymer
ii
Soluble fibrin polymer
Insoluble fibrin
iii

Figure 3

Figure 4

A model of fibrin polymerisation adapted from that proposed by Blombäck, Hogg, Gårlund et al. (1976). Rectangular boxes are used as building units and these are joined in a double molecule having a two-fold axis of symmetry. (One shaded box and one unshaded box to each molecule). The binding sites AA' - aa' are thought to be situated at the opposite ends of the broad sides of the boxes, while the binding sites BB' - bb' are located at opposite ends of the narrow sides of the boxes (shown as the "top" and "bottom" of the boxes in this diagram). The open headed arrows (—▷) indicate the position of the binding sites which are not visible in the diagram. A polymer arises through "end-to-end" polymerisation involving binding of the sites a and A on one molecule to the complementary sites A' and a' on the adjacent molecule (➤). In a similar manner, "side-to-side" polymerisation takes place between b and B sites on one molecule and the complementary B' and b' sites on an adjacent molecule (➤). Branching of the initial polymer can therefore take place.

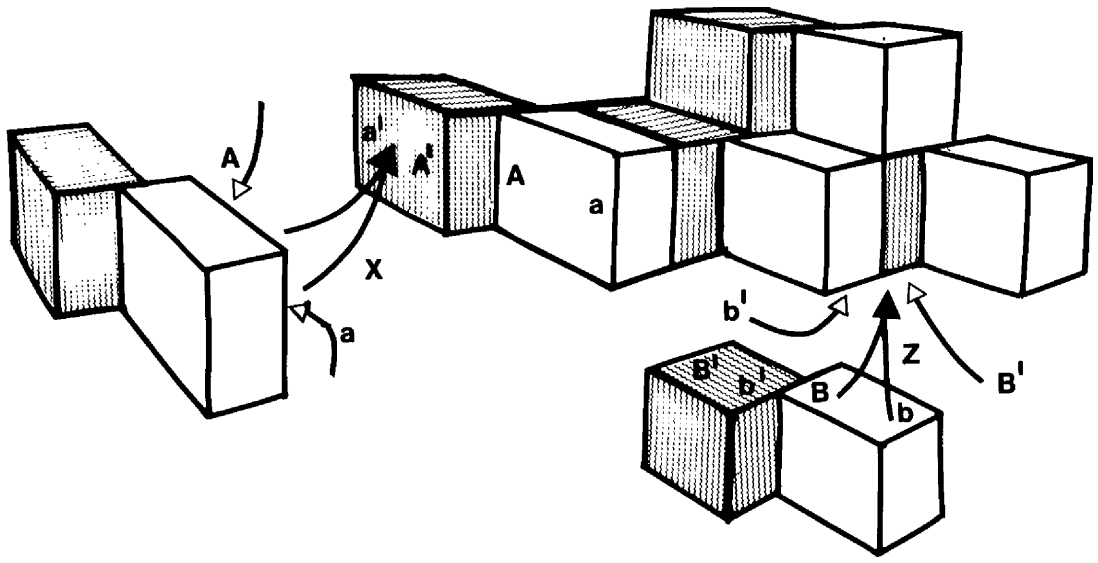


Figure 4

Figure 5

A diagramatic representation of the formation of fibrinogen degradation products using the model of fibrinogen shown in Figure 1. (The disulphide bonds are omitted in this diagram). The length of the chain is approximately proportional to its molecular weight and so shortening of the chain indicates digestion. The early fibrinogen X fragment is considered to be clottable by thrombin, the other fragments are unclottable (adapted from Marder, Budzyński and James, 1972 and Budzyński, Marder and Shainoff, 1974).

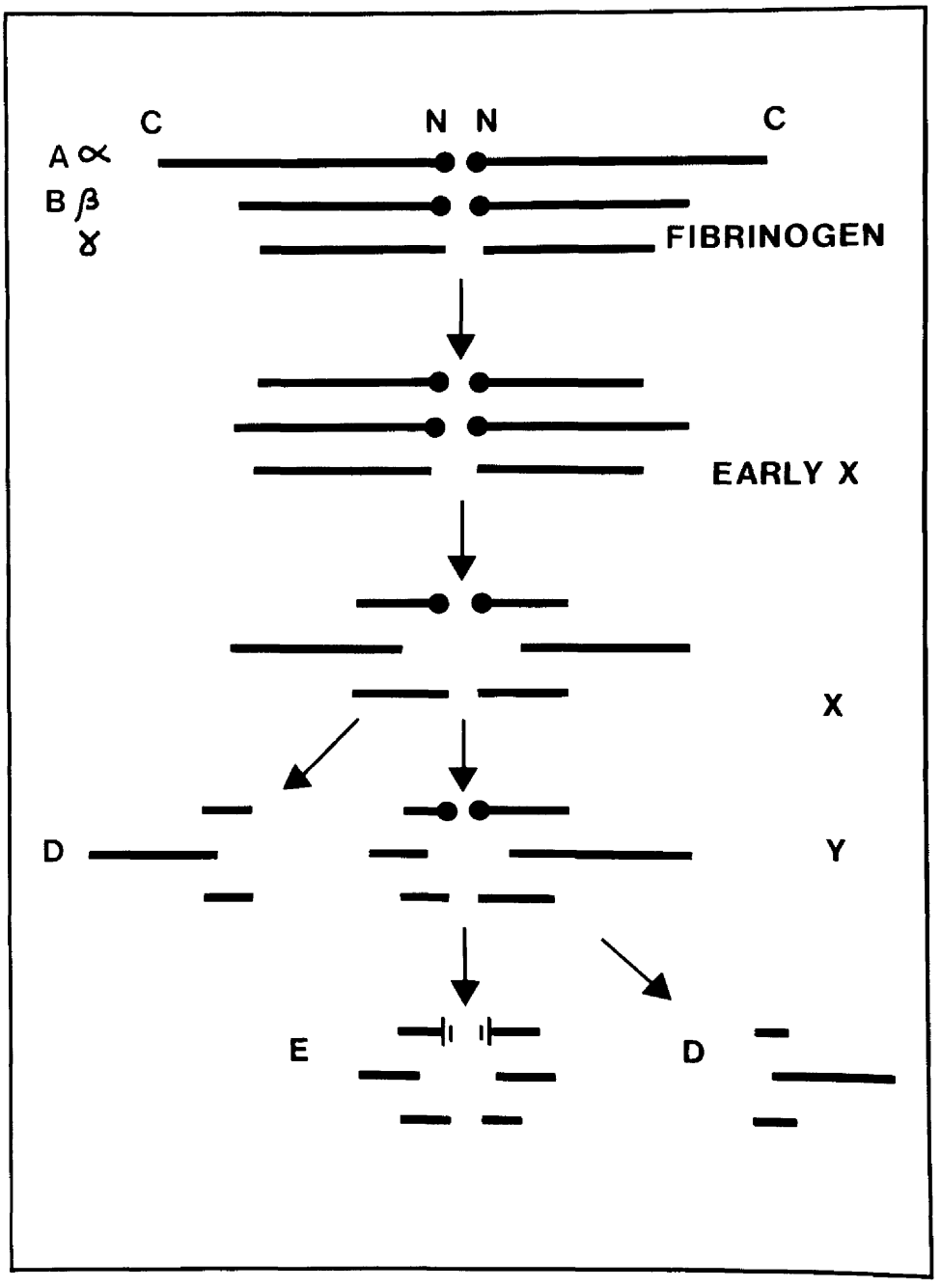


Figure 5

Figure 6

The basic fibrinogenolytic/fibrinolytic pathway is shown. The solid arrow indicates "transformation" and the broken arrow indicates "action" as in Figure 2.

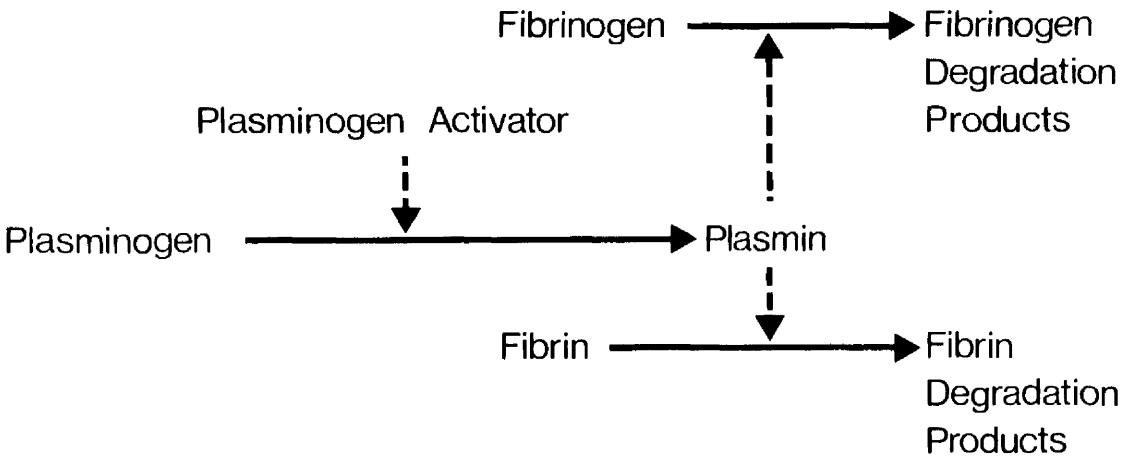

Fibrinogen
Fibrinogen
Degradation
Products
Plasminogen Activator
Plasminogen
Plasmin
Fibrin
Fibrin
Degradation
Products

Figure 6

Figure 7

A compartmental model of the synthesis, metabolism and distribution of fibrinogen and its derivatives (adapted from that of Reeve and Franks, 1974). The compartments boxed with solid lines (—) and the open arrows (⟹) indicate the "fibrinogen core system", which is thought to be the physiological pathway. The other pathways (solid arrows ⟶) are thought to operate mainly in pathological conditions. (Much of this diagram is speculative e.g. the existence of interstitial fibrin monomer and Fragment X, which are therefore boxed with broken lines - - -).

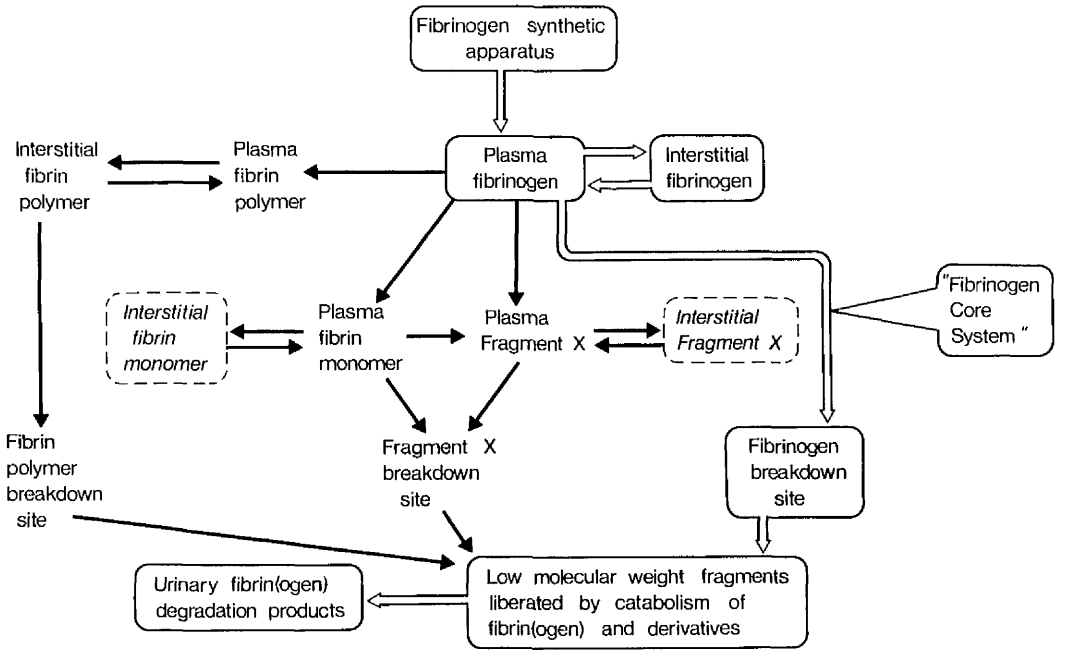
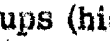
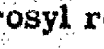

Fibrinogen synthetic apparatus
Interstitial fibrin polymer
Plasma fibrin polymer
Plasma fibrinogen
Interstitial fibrinogen
"Fibrinogen Core System"
Interstitial fibrin monomer
Plasma fibrin monomer
Plasma Fragment X
Interstitial Fragment X
Fibrin polymer breakdown site
Fragment X breakdown site
Fibrinogen breakdown site
Urinary fibrin(ogen) degradation products
Low molecular weight fragments liberated by catabolism of fibrin(ogen) and derivatives

Figure 7

Figure 8a

A model of soluble complex formation and fibrin polymerisation based on hydrogen bond formation.

F: fibrinogen, in which only hydrogen bond acceptor groups (histidyl residues, ) are available.

f: fibrin monomer, formed by the removal of fibrinopeptides from fibrinogen allowing the donor groups (tyrosyl residues, ) to become available.

f-f-f: soluble fibrin polymer (see Figure 3)

f-F: soluble fibrinogen-fibrin dimer.

(adapted from Latallo, 1975)

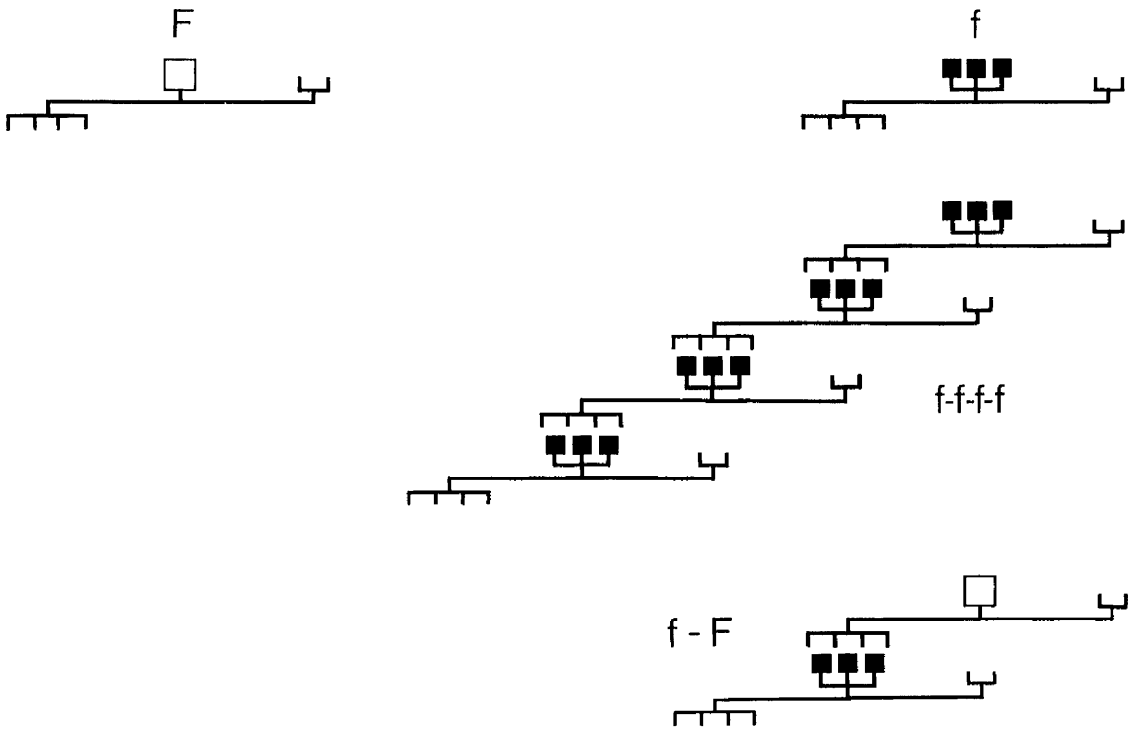


Figure 8a

Figure 8b

It is theoretically possible for soluble complexes to contain fibrinogen-fibrin degradation products, because these degradation products may possess donor or acceptor groups sufficiently similar to those found in fibrinogen and fibrin monomer (Figure 8a) to allow polymerisation. Such complexes are shown involving fibrinogen Fragment Y (Y), fibrin Fragment y (y) and fibrinogen Fragment D (D) (adapted from Latallo, 1975).

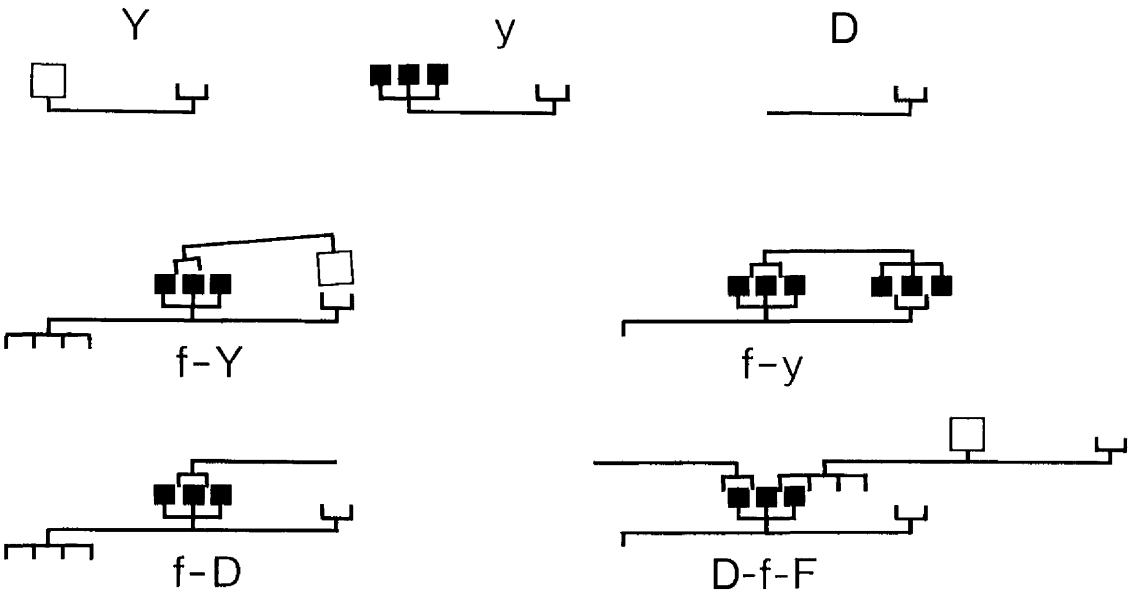


Figure 8b

Figure 9

A comparison of plasma fibrinogen chromatography and ^{125}I fibrinogen scan results on a surgical patient (adapted from the results of Fletcher and Alkjaersig, 1972). Pre-operatively there was no evidence of local thrombosis in the leg veins using the scan technique (Leg scan negative, -ve). The plasma fibrinogen chromatography results for plasma samples are shown by plotting the cumulative effluent (or elution) volume (ml) on the X axis and the fibrinogen-fibrin antigenic concentration (fibrinogen mg/ml) on the Y axis. The solid line and dots indicate the antigenic elution curve while the position of the peak concentration of antigen found when a purified fibrinogen solution is analysed is shown by the vertical dotted line. On the first post-operative day (Day 1) the patient developed evidence on fibrinogen scan of local thrombosis in the leg veins (Leg scan positive, +ve). On Day 1 there was also a "shift to the left" found using the chromatography technique, suggesting the presence of soluble complexes. By the fourth post-operative day (Day 4) a "shift to the right" was found using the chromatography technique suggesting the formation of fibrinogen/fibrin degradation products.

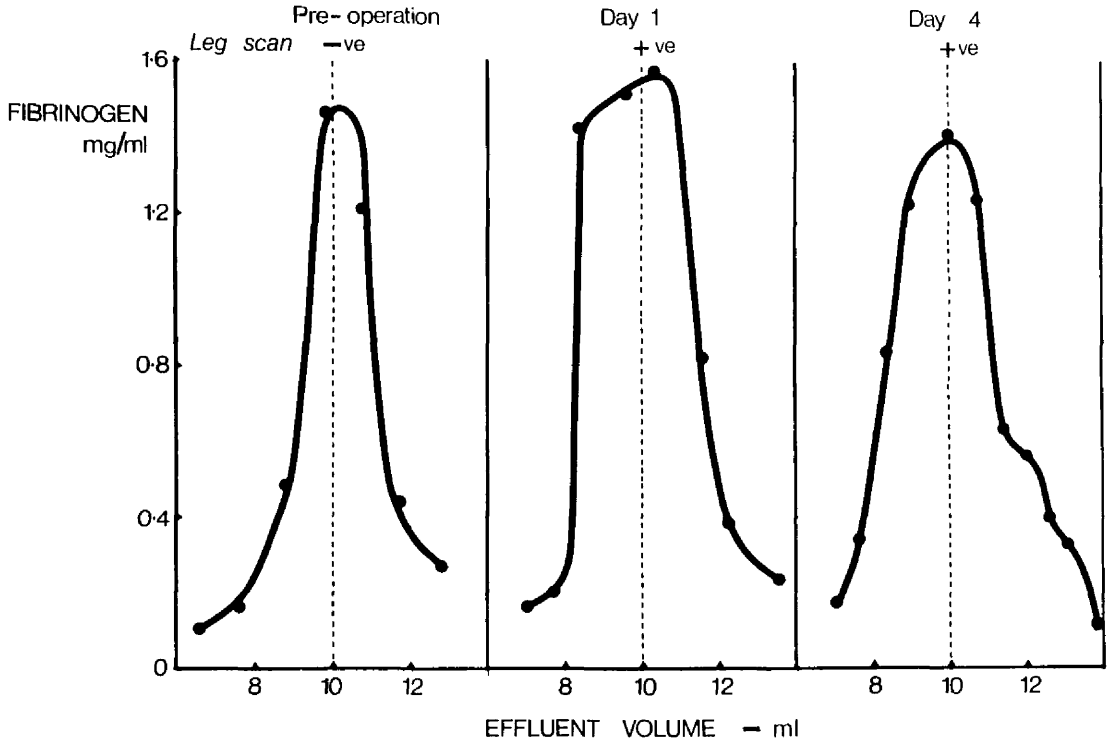

Pre-operation
Leg scan −ve
Day 1
+ve
Day 4
+ve
FIBRINOGEN
mg/ml
1·6
1·2
0·8
0·4
0
8
10
12
8
10
12
8
10
12
EFFLUENT VOLUME − ml

Figure 9

Figure 10

The calculation of the elution volume (V_e) of a purified protein by two different methods. The cumulative volume of buffer (ml) eluting from the gel column from the start of the experiment (i.e. when the protein is applied) is plotted on the X axis and the protein concentration (mg/100 ml) on the Y axis. Using method 1 V_e is measured as the volume at which 50 percent of the peak protein concentration is eluted (in this example the peak protein concentration is 120 mg/100 ml, therefore 50 percent of this is 60 mg/100 ml, giving a V_e of 100 ml for this protein). Using method 2 the ascending limb of the protein peak is extended to cross the baseline. The volume at which the two lines cross is considered as the V_e of the protein (in this example a V_e of 96 ml is obtained).

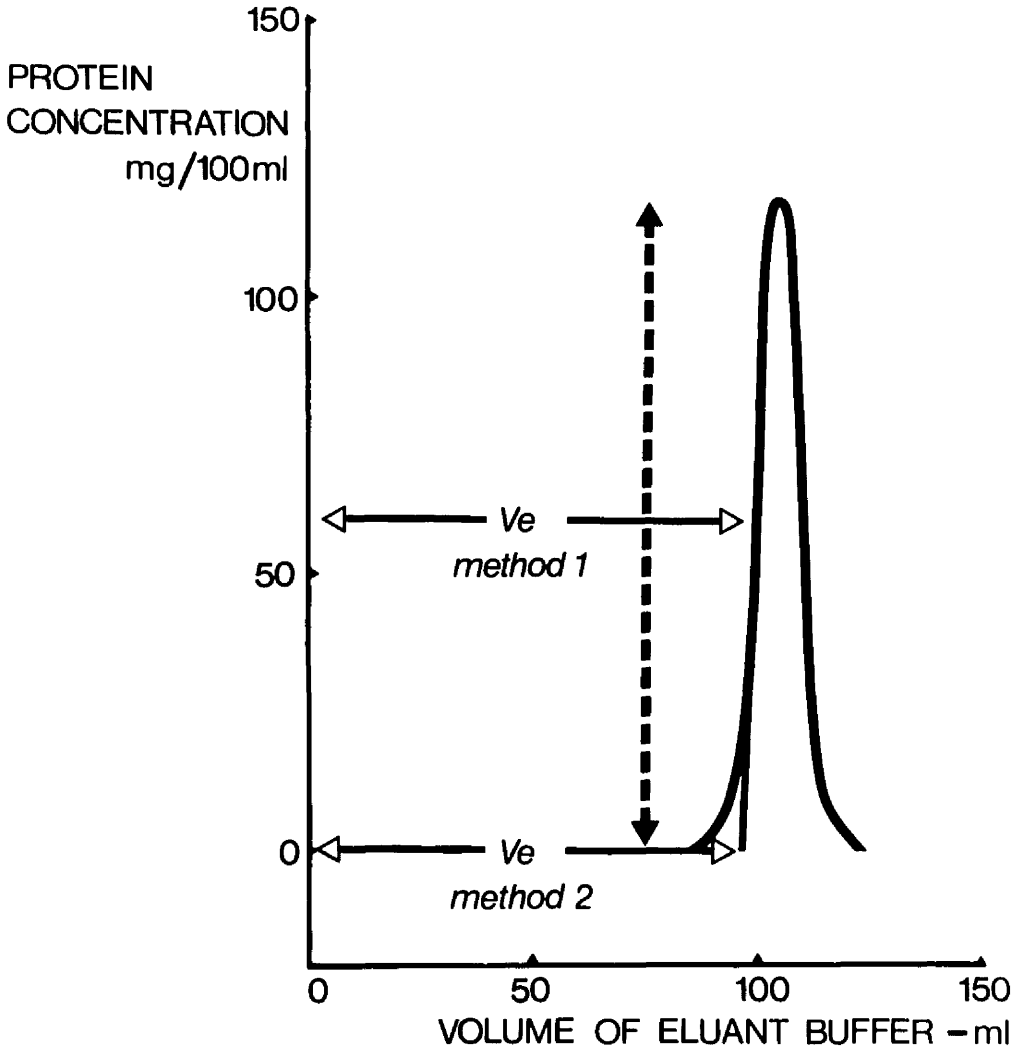

150
PROTEIN
CONCENTRATION
mg/100ml
100
50
0
Ve
method 1
Ve
method 2
0
50
100
150
VOLUME OF ELUANT BUFFER - ml

Figure 10

Figure 11

The formation of the different types of soluble complexes in vivo (adapted from Fletcher and Alkjaersig, 1973). Approximate molecular weights are shown in brackets.

R.E.S. : reticulo-endothelial system

F XIII : Factor XIII or Fibrin-Stabilizing Factor.

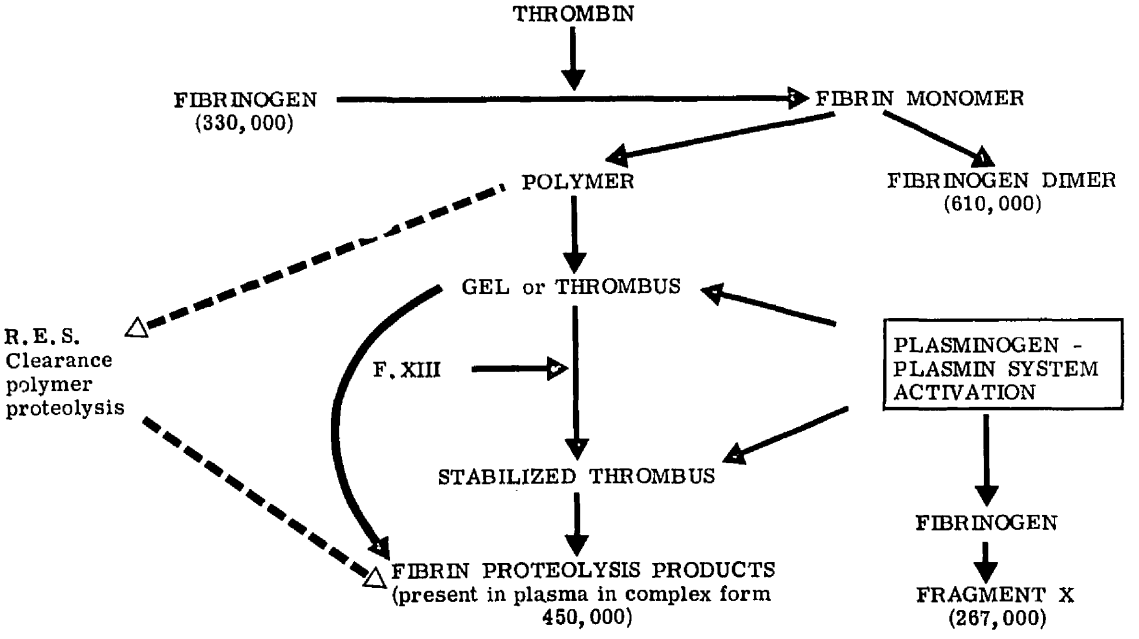

THROMBIN
FIBRINOGEN
(330,000)
FIBRIN MONOMER
POLYMER
FIBRINOGEN DIMER
(610,000)
GEL or THROMBUS
R.E.S.
Clearance
polymer
proteolysis
F.XIII
PLASMINOGEN -
PLASMIN SYSTEM
ACTIVATION
STABILIZED THROMBUS
FIBRINOGEN
FIBRIN PROTEOLYSIS PRODUCTS
(present in plasma in complex form
450,000)
FRAGMENT X
(267,000)

Figure 11

Figure 12

The formation of soluble complexes (normal plasma + lysed fibrin) and fibrinogen degradation products (normal plasma + urokinase) in vitro demonstrated by plasma fibrinogen chromatography using the method of Fletcher and colleagues (adapted from results published by Fletcher and Alkjaersig, 1972). The cumulative effluent (or elution) volume (ml) is plotted on the X axis and the fibrinogen-fibrin related antigen concentration results on the Y axis (fibrinogen, mg/ml). The vertical dotted line indicates the position of the peak concentration of antigen found when a purified fibrinogen solution is analysed. It can be seen that the soluble complexes caused a "shift to the left" and the fibrinogen degradation products caused a "shift to the right".

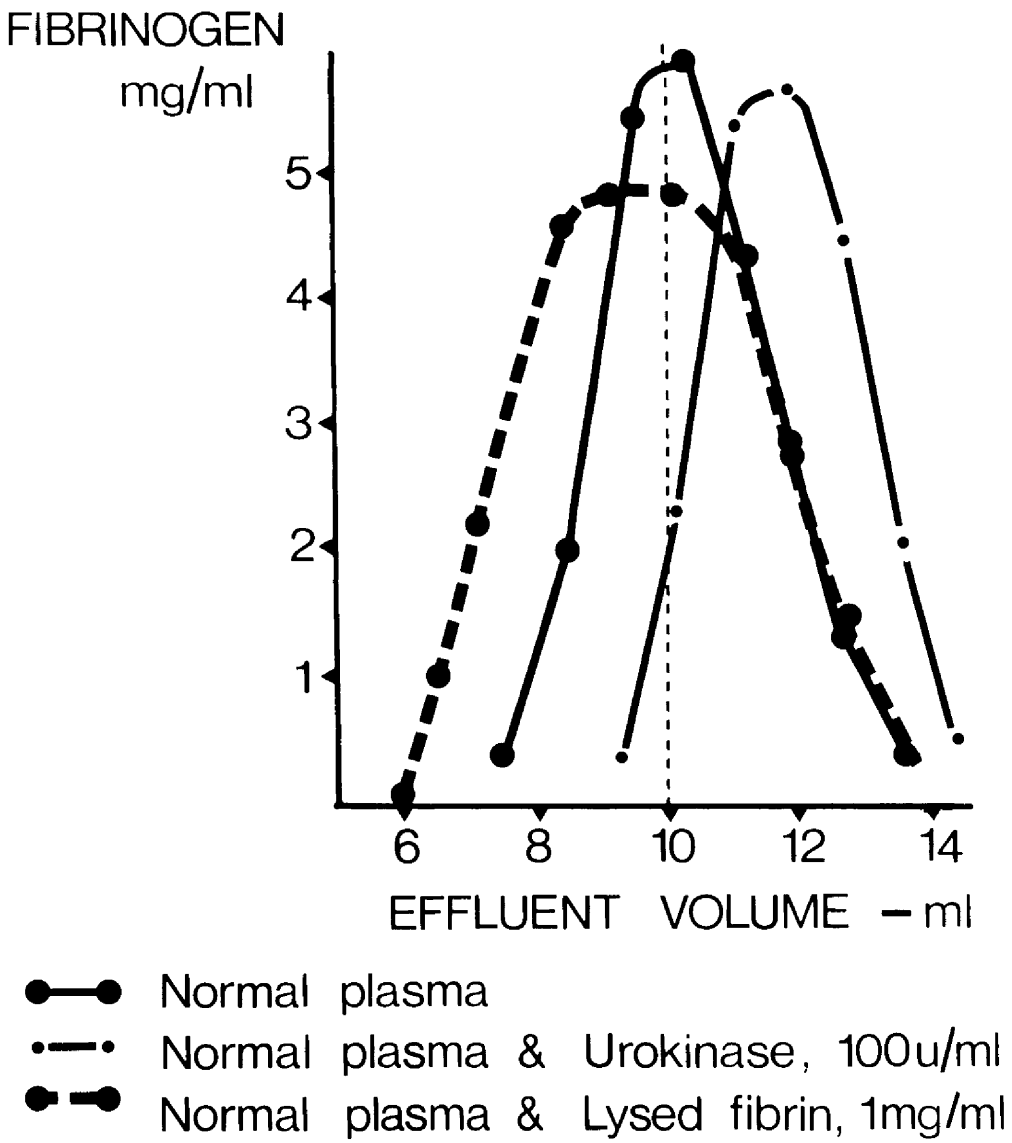

FIBRINOGEN
mg/ml
5
4
3
2
1
6
8
10
12
14
EFFLUENT VOLUME – ml
Normal plasma
Normal plasma & Urokinase, 100u/ml
Normal plasma & Lysed fibrin, 1mg/ml

Figure 12

Figure 13

Plasma fibrinogen concentrations (mg/100 ml) were measured in the same samples by two different methods. The X axis shows the results measured by the radial immunodiffusion method (Mancini, Carbonara and Heremans, 1965) and the Y axis shows the results measured by the thrombin clottable protein method (Ratnoff and Menzie, 1951). The equation of the line calculated by linear regression analysis was:-

$y = 38.4 + 0.6x$

Correlation coefficient $(r) = 0.931$.

$(2 \propto < 0.001)$.

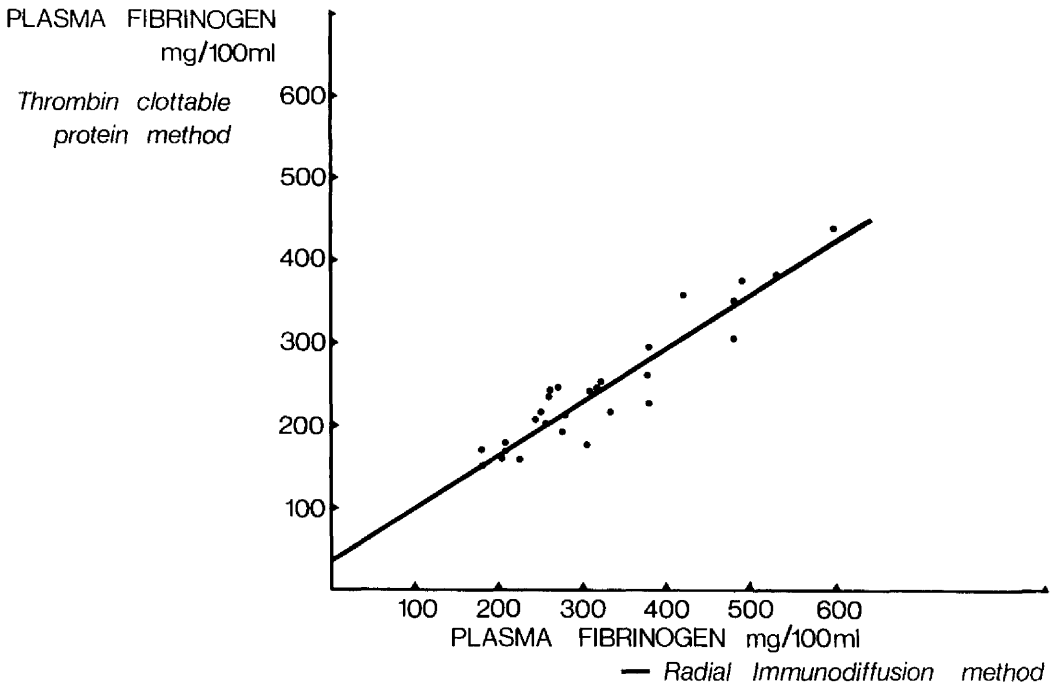

PLASMA FIBRINOGEN
mg/100ml
Thrombin clottable
protein method
600
500
400
300
200
100
100 200 300 400 500 600
PLASMA FIBRINOGEN mg/100ml
— Radial Immunodiffusion method

Figure 13

Figure 14

Diagram of the equipment used for agarose gel filtration. The protein sample was applied to the top of the chromatography column packed with Biogel A5m Buffer from the reservoir was allowed to wash through the packed column at a constant flow rate controlled by the pump. The buffer eluting from the packed column was collected in aliquots in the fraction collector. The direction of flow of buffer is shown by the arrows (⟶). An estimate of the total protein concentration was obtained by continuous monitoring of the optical density (at 280 nm) of the eluant buffer using the spectrophotometer and pen recorder.

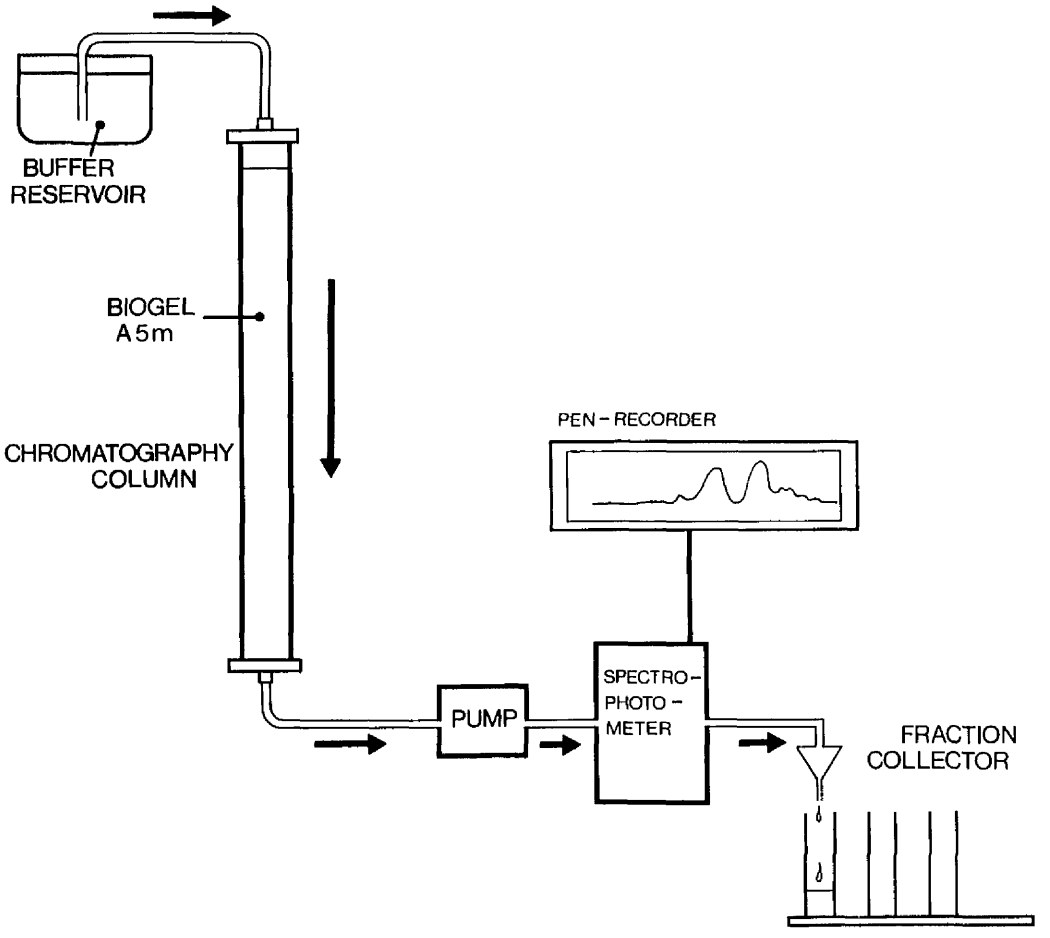

BUFFER
RESERVOIR
BIOGEL
A5m
CHROMATOGRAPHY
COLUMN
PEN-RECORDER
PUMP
SPECTRO-
PHOTO-
METER
FRACTION
COLLECTOR

Figure 14

Figure 15

The method of calculation of the thrombin clotting time (T.C.T.) and the thrombin clottable protein (T.C.P.) ratios. A typical elution curve for a β -alanine precipitate of normal plasma is shown, the eluant fractions being analysed by the thrombin clotting time and thrombin clottable protein tests. The cumulative elution volume (ml) is plotted on the X axis and the results of the T.C.T. test (seconds) on the Y axis. The elution volume over which a positive T.C.P. test was recorded is shown as an inset (▨) above the X axis. To calculate the ratios the following volumes were measured:-

t = the cumulative elution volume at which the first positive T.C.P. test was recorded

τ = the cumulative elution volume at which the first positive T.C.T. test was recorded (i.e. ⟨180 seconds)

s = the cumulative elution volume at which the shortest T.C.T. test was recorded.

$$\text{T.C.T. ratio} = \frac{\tau}{s}$$

$$\text{T.C.P. ratio} = \frac{t}{s}$$

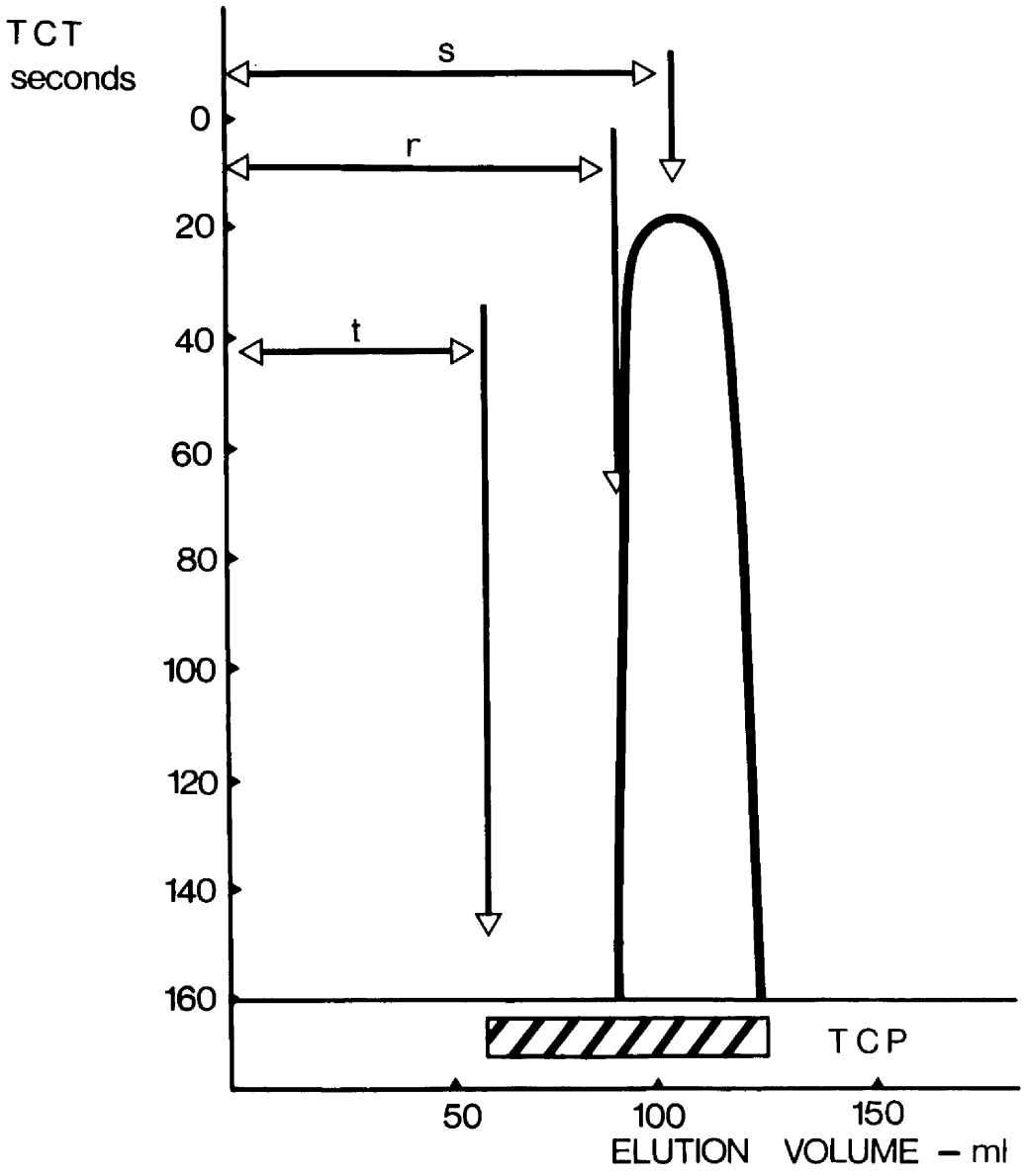

TCT
seconds
0
20
40
60
80
100
120
140
160
s
r
t
TCP
50
100
150
ELUTION VOLUME – ml

Figure 15

Figure 16

In the experiment performed to construct this graph a purified fibrinogen solution was diluted to give concentrations of 40, 30, 20, 10, 5 and 2.5 mg/100 ml. These dilutions were then analysed in duplicate using the modified radial immunodiffusion technique (Mancini, Carbonara and Heremans, 1965) and the diameter of the precipitin rings (D) measured and the mean value used. The fibrinogen-fibrin related (FR-) antigen concentration (mg/100 ml) is plotted on the X axis and D^2 (mm^2) on the Y axis. It can be seen that there was a good positive correlation between the two measurements. The line calculated by linear regression analysis was:-

$y = 19.5 + 2.0x$

correlation coefficient (r) = 0.999

($2 \propto < 0.001$).

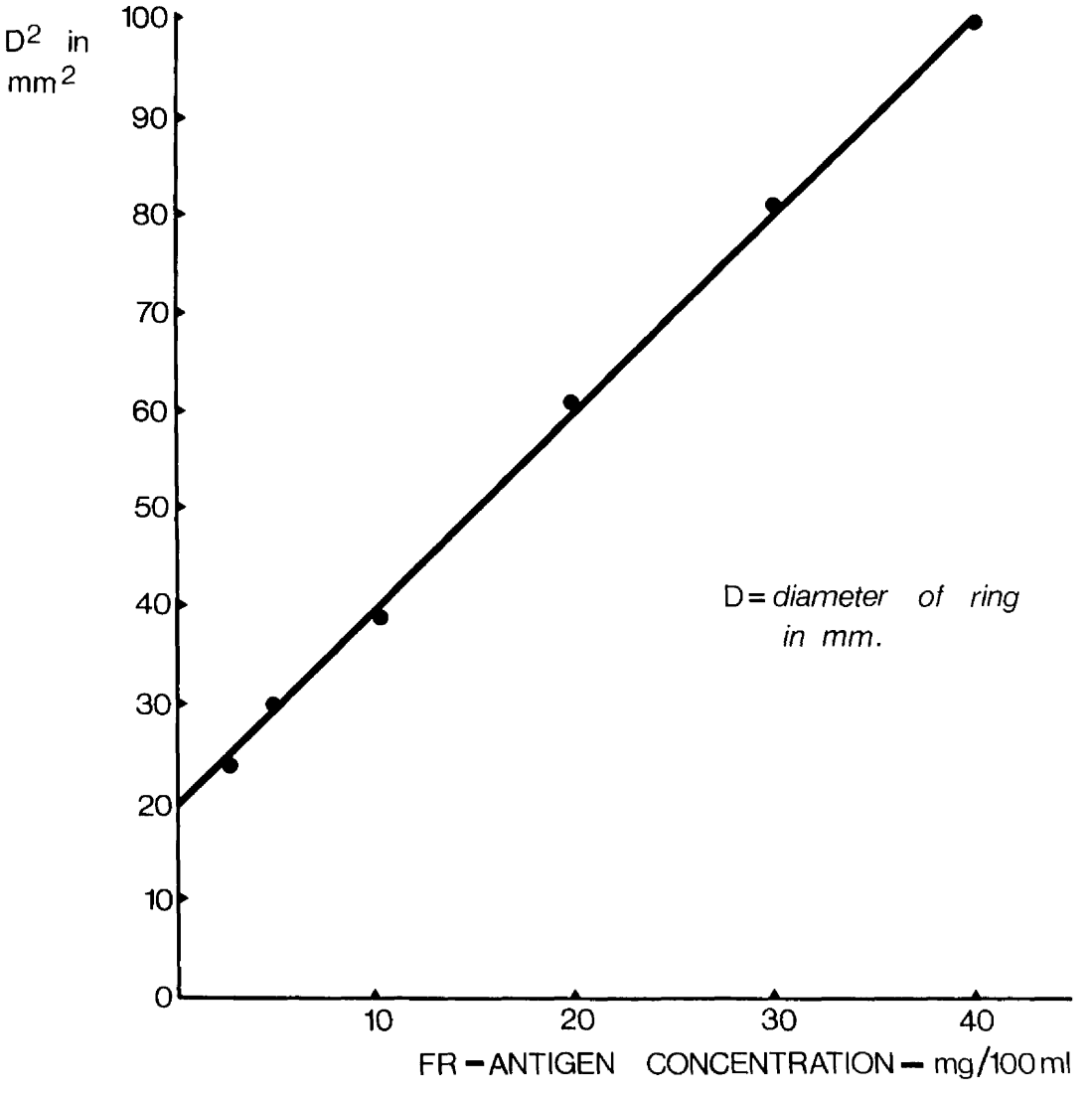
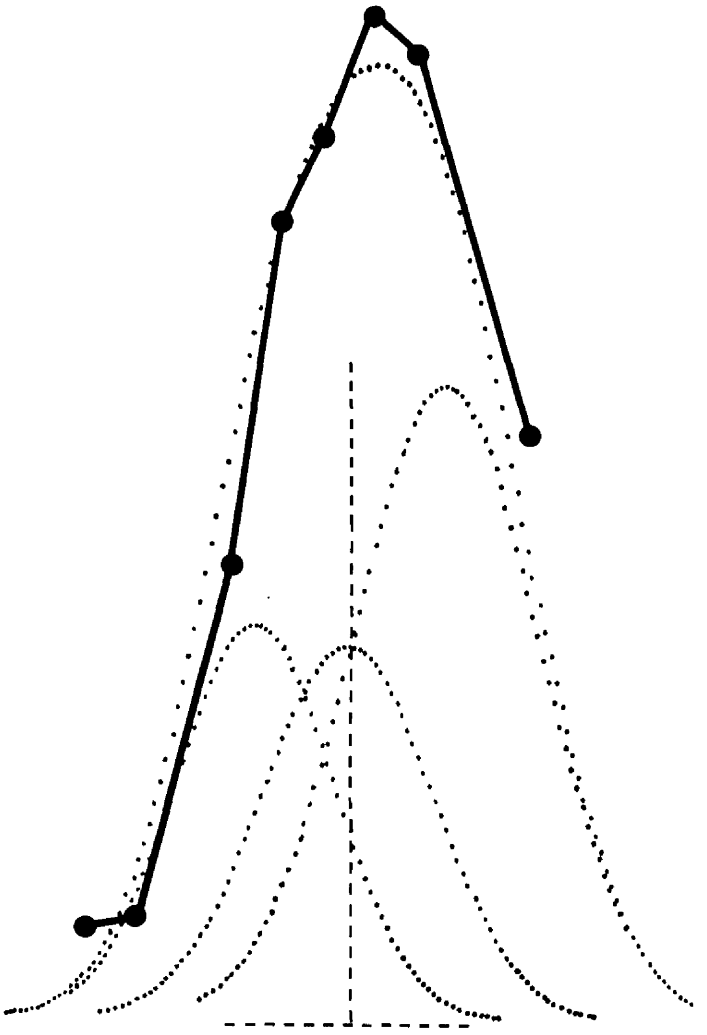

D² in mm²
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
D = diameter of ring in mm.
FR – ANTIGEN CONCENTRATION – mg/100 ml

Figure 16

Figure 17

A computer "print-out" of the fibrinogen chromatography results for a plasma sample from a patient with "post-operative thrombophlebitis" (adapted from Fletcher and Alkjaersig, 1972). The solid black line and solid dots indicate the actual fibrinogen-fibrin related antigenic elution curve. The computer analyses this into three components (polymer, fibrinogen and first derivative) as shown by the dotted line curves. The position of the maximum concentration of fibrinogen-fibrin related antigen, when a purified fibrinogen solution is analysed using the same column system, is shown by the vertical dotted line. The percentage concentration of polymer, fibrinogen and first derivative are calculated and shown at the foot of the diagram.



Polymer 25% fibrinogen 26·4% first derivative 48·4%

Figure 17

Figure 18

The method of calculating soluble complex concentrations used by Hafter, Schneebauer, Tafel et al. (1975). The elution profile of a β-alanine precipitate of plasma is calculated by measuring the optical density (O.D. arbitrary units) of the eluant fractions at 325 nm and subtracting this from the optical density (arbitrary units) at 280 nm. This result is then plotted on the Y axis with the cumulative elution (ml) volume on the X axis. The elution curve is then divided as shown. The void volume area is excluded from the analysis because of the presence of contaminant proteins. The ascending limb of the main peak is extended to the base-line and a vertical line is drawn from the base-line to the top of the main peak. Areas A and $\frac{1}{2}$B are then measured planimetrically and the following formula used to calculate the soluble complex concentration:-

$$\text{Soluble complex concentration (percent total area)} = \frac{A}{A + B} \times 100$$

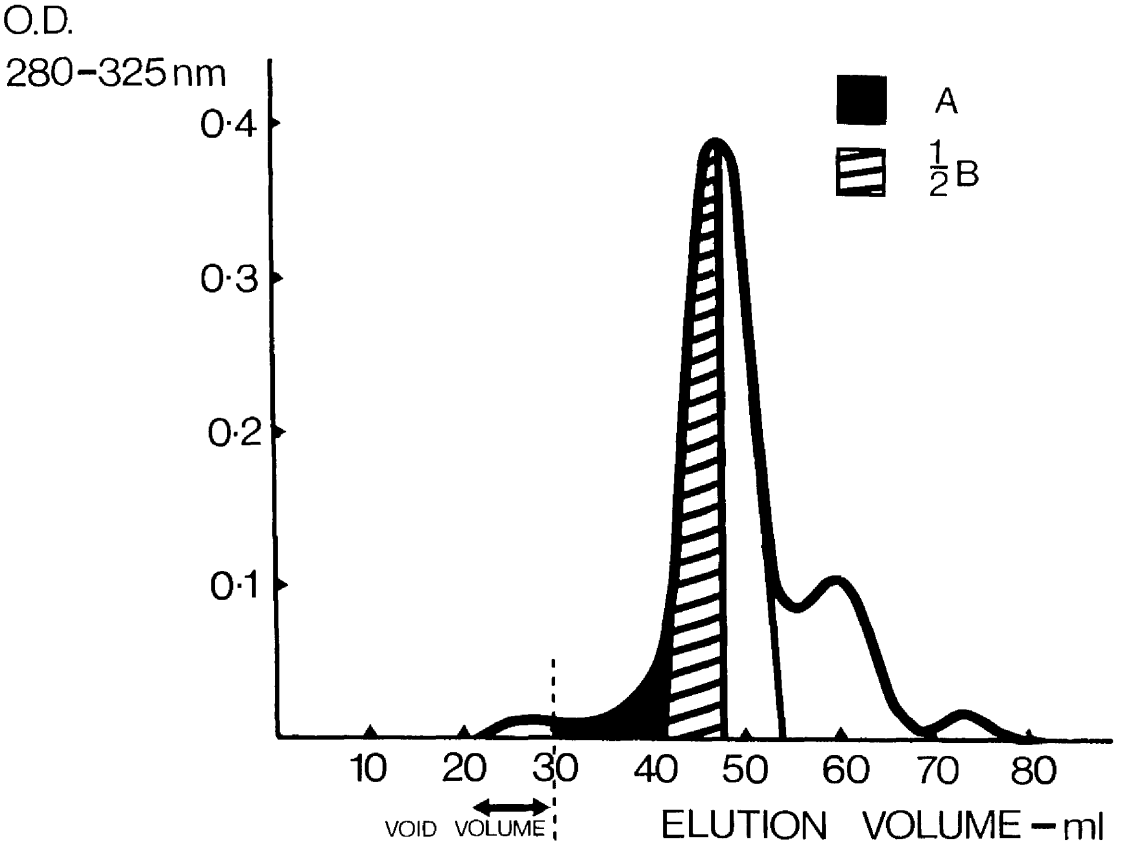

O.D.
280–325 nm
0·4
0·3
0·2
0·1
A
½B
10
20
30
40
50
60
70
80
VOID VOLUME
ELUTION VOLUME – ml

Figure 18

Figure 19

The method used in this thesis for calculation of results (areas m, n and p) from the fibrinogen-fibrin related antigen elution curve. An elution curve from a β-alanine precipitate of plasma is shown. The antigenic concentration (mg/100 ml) measured by the modified radial immunodiffusion technique is shown on the Y axis and the cumulative elution volume (ml) on the X axis. In order to calculate the areas the ascending and descending limbs of the mean peak were extended to the base-line and areas m, n and p measured planimetrically. The soluble complex concentration (area m) was then calculated using the formula:-

$$\text{area m (percent total area)} = \frac{m}{m + n + p} \times 100$$

The fibrinogen-fibrin degradation product concentration (area p) was calculated using the formula:-

$$\text{area p (percent total area)} = \frac{p}{m + n + p} \times 100$$

The "uncomplexed, non-degraded fibrinogen" (area n) was calculated using the formula:-

$$\text{area n (percent total area)} = \frac{n}{m + n + p} \times 100$$

(published in McKillop, Howie, Forbes et al., 1976).

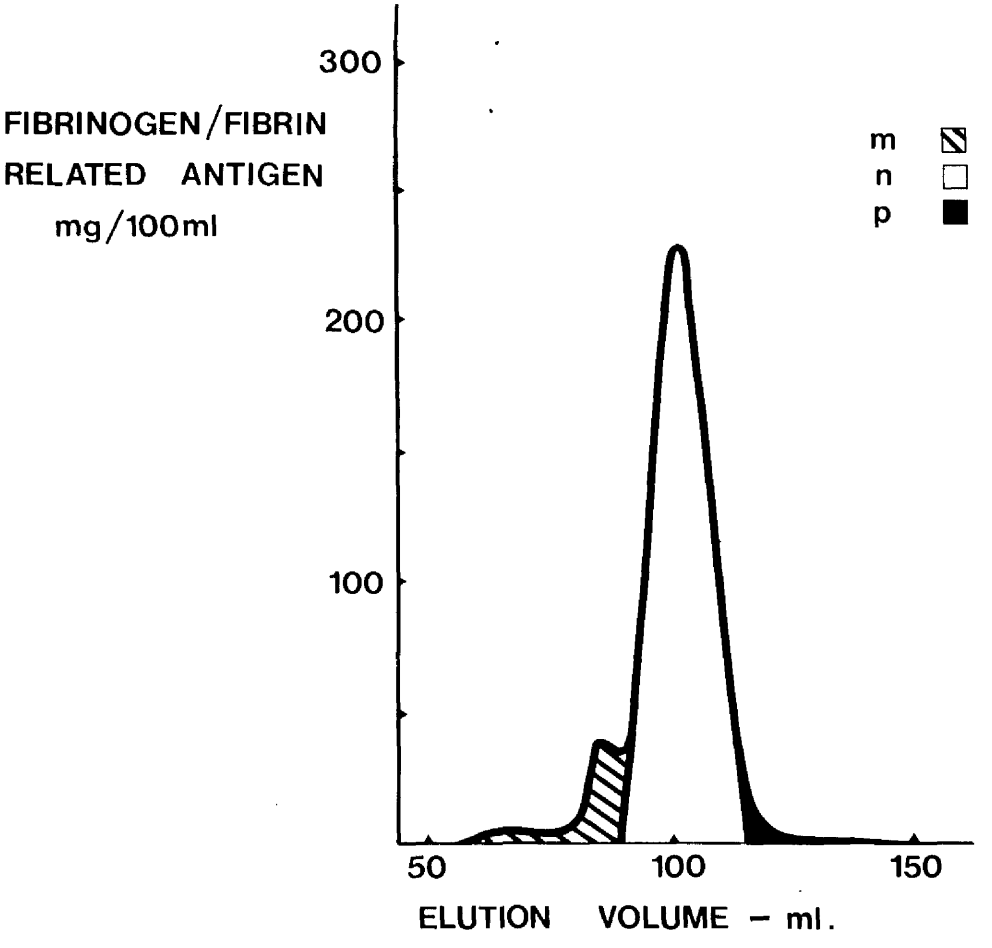

300
FIBRINOGEN/FIBRIN
RELATED ANTIGEN
mg/100ml
200
100
m
n
p
50
100
150
ELUTION VOLUME – ml.

Figure 19

Figure 20

A typical elution curve of a β-alanine precipitate of normal plasma is shown, the eluant fractions being analysed by the modified radial immunodiffusion technique. The cumulative elution volume (ml) is plotted on the X axis and the fibrinogen-fibrin related (FR-) antigen concentration (mg/100 ml) on the Y axis. To calculate the FR-antigen ratio two volumes were measured:-

a = the cumulative elution volume at which FR-antigen was first detected using the radial immunodiffusion technique.

b = the cumulative elution volume at which the peak concentration of FR-antigen was detected using the radial immunodiffusion technique.

The FR-antigen ratio was then calculated using the formula:

$$\text{FR-antigen ratio} = \frac{a}{b}$$

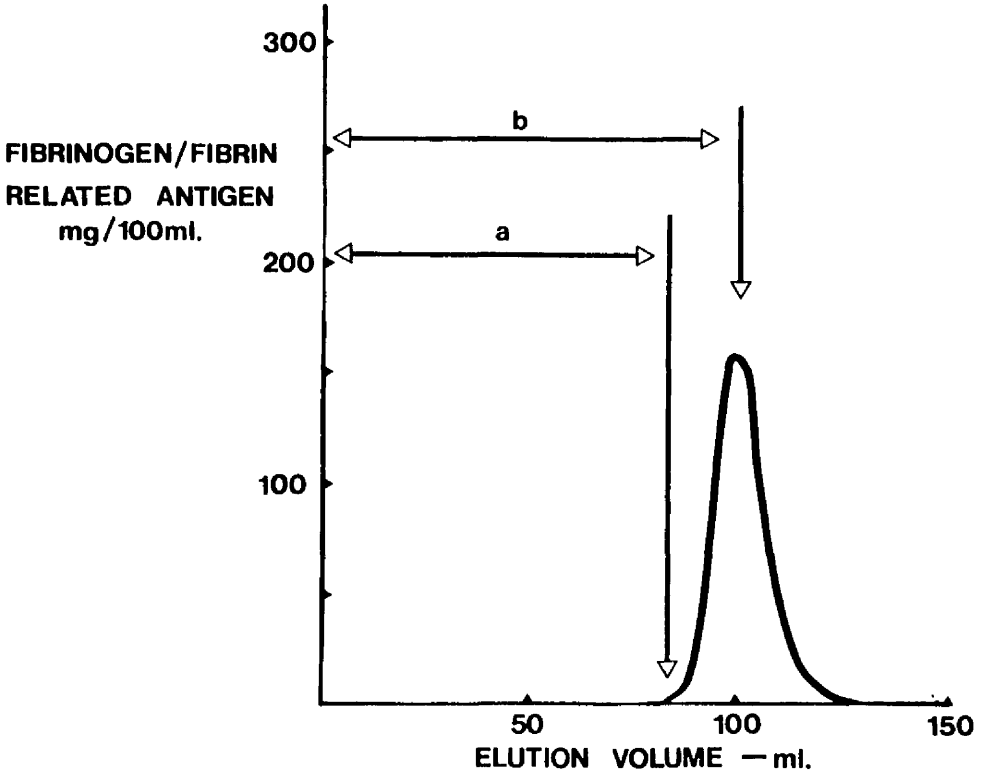

300
200
100
FIBRINOGEN/FIBRIN
RELATED ANTIGEN
mg/100ml.
b
a
50
100
150
ELUTION VOLUME — ml.

Figure 20

Figure 21

The Staphylococcal Clumping Test (SCT) was performed by adding 0.05 ml of reconstituted staphylococcal clumping factor solution to 0.1 ml of the sample. After mixing, 0.5 ml buffer (tris (hydroxymethyl) methylamine 0.05 M; sodium chloride 0.1 M; pH adjusted with 1 M hydrochloric acid to 7.4) was added. The mixture was examined after 30 minutes for evidence of large clumps of staphylococci indicating a positive reaction.

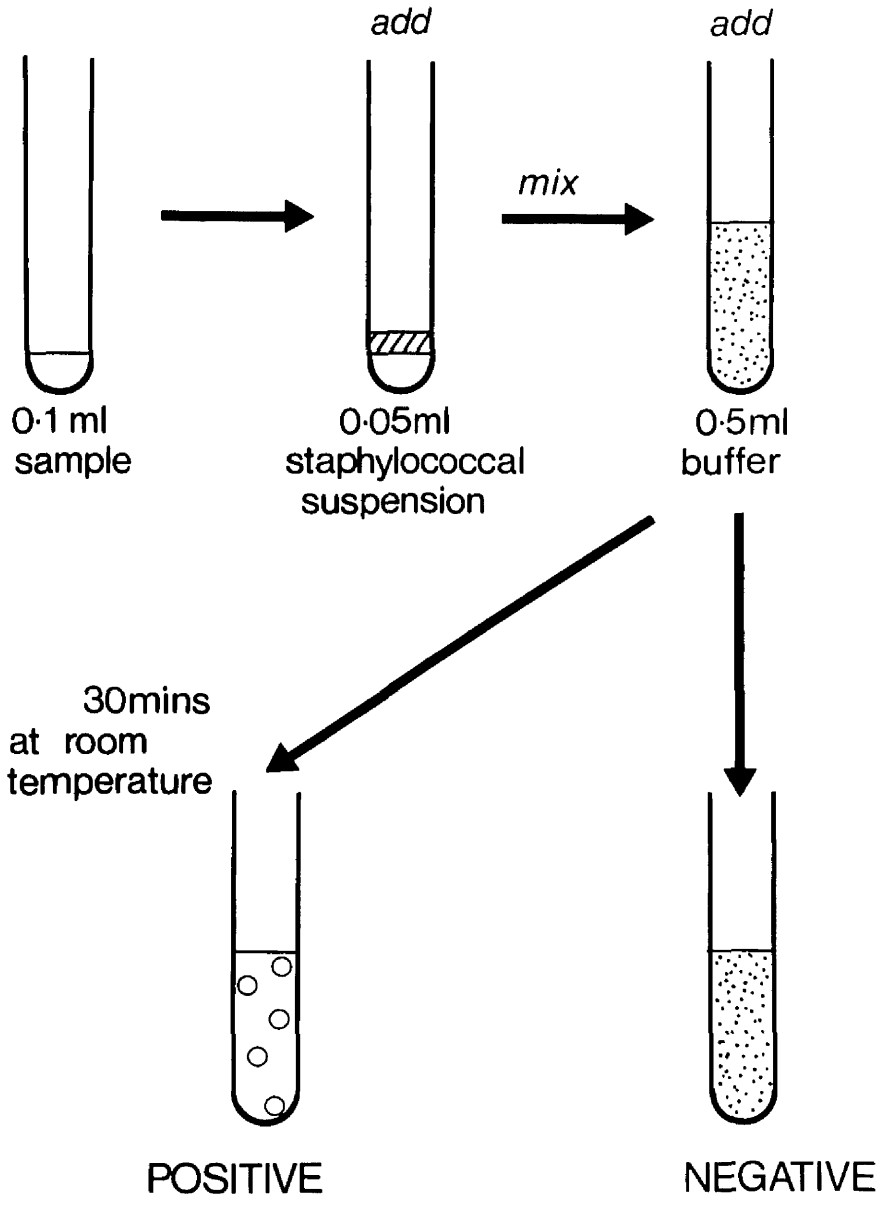

add
add
mix
0·1 ml
sample
0·05ml
staphylococcal
suspension
0·5ml
buffer
30mins
at room
temperature
POSITIVE
NEGATIVE

Figure 21

Figure 22

The elution profiles for four solutes (Blue Dextran, lipopolysaccharide, fibrinogen and albumin, see Table 2). The cumulative elution volume (ml) is shown on the X axis and the total protein concentration, measured by the optical density (O.D. in arbitrary units) at 280 nm, on the Y axis.

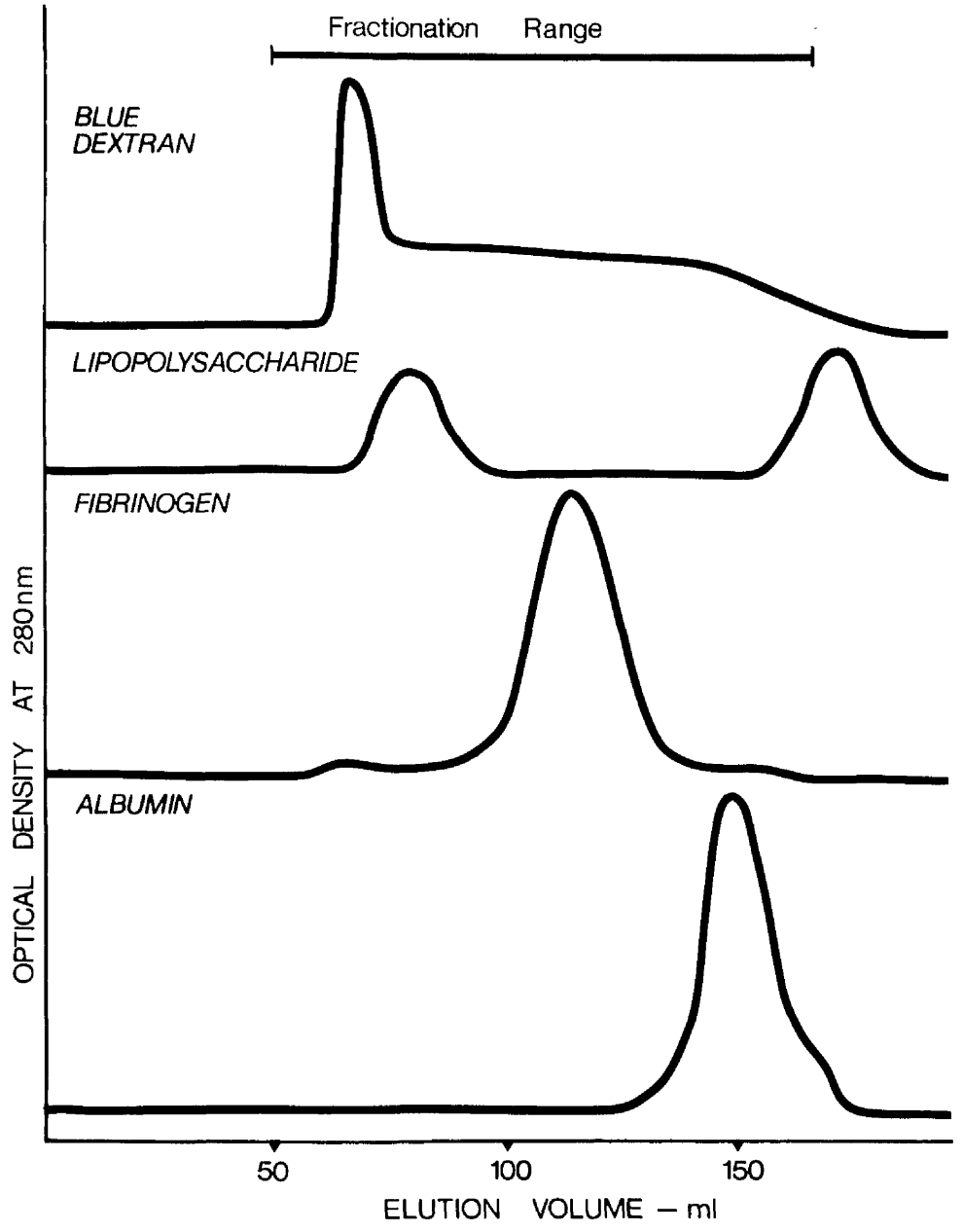

Fractionation Range
BLUE DEXTRAN
LIPOPOLYSACCHARIDE
FIBRINOGEN
ALBUMIN
OPTICAL DENSITY AT 280nm
50
100
150
ELUTION VOLUME — ml

Figure 22

Figure 23

The logarithm to the base 10 of the molecular weight plotted on the X axis and the elution volume (V_e, ml) measured by method 1 (Figure 10) plotted on the Y axis for different solutes. Apart from the results for Bacitracin (molecular weight below the fractionation range of Biogel A5m) there was a good negative correlation between the two results. The equation of the line calculated by linear regression analysis (excluding the Bacitracin results) was:-

$y = 310.6 - 37.8x$

Correlation coefficient (r) = -0.962

($2 \propto < 0.001$)

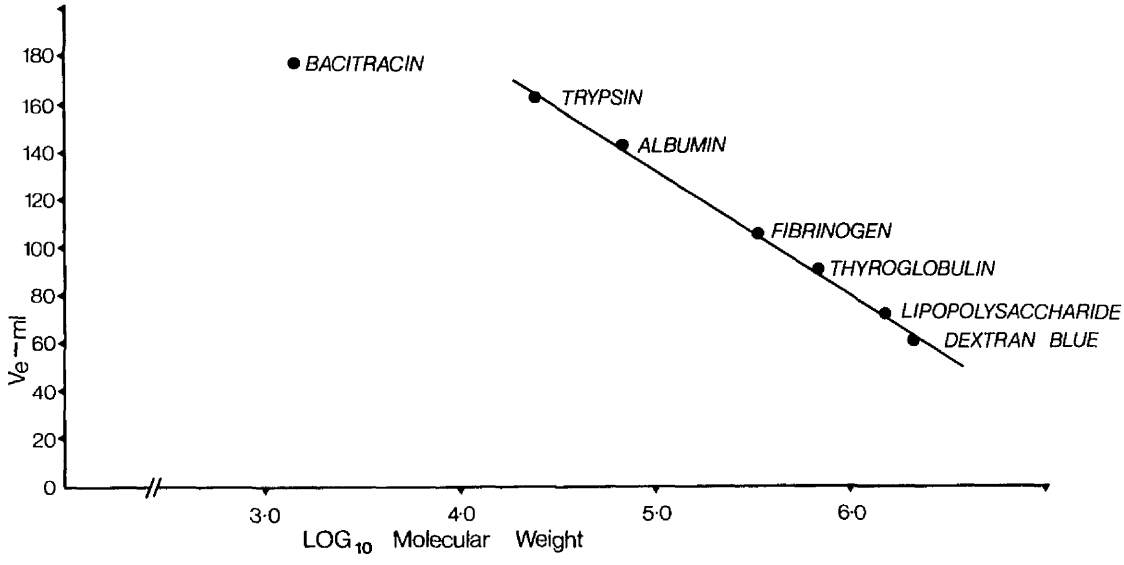

180
160
140
120
100
80
60
40
20
0
Ve - ml
BACITRACIN
TRYPSIN
ALBUMIN
FIBRINOGEN
THYROGLOBULIN
LIPOPOLYSACCHARIDE
DEXTRAN BLUE
3·0
4·0
5·0
6·0
LOG10 Molecular Weight

Figure 23

Figure 24

A comparison of the elution curves found using purified fibrinogen (Kabi) and normal plasma. The cumulative elution volume (ml) is shown on the X axis while the optical density (O.D., arbitrary units) at 280 nm, the fibrinogen-fibrin related (FR-) antigen (mg/100 ml) and the thrombin clotting time (T.C.T., seconds) results are shown on the Y axis.

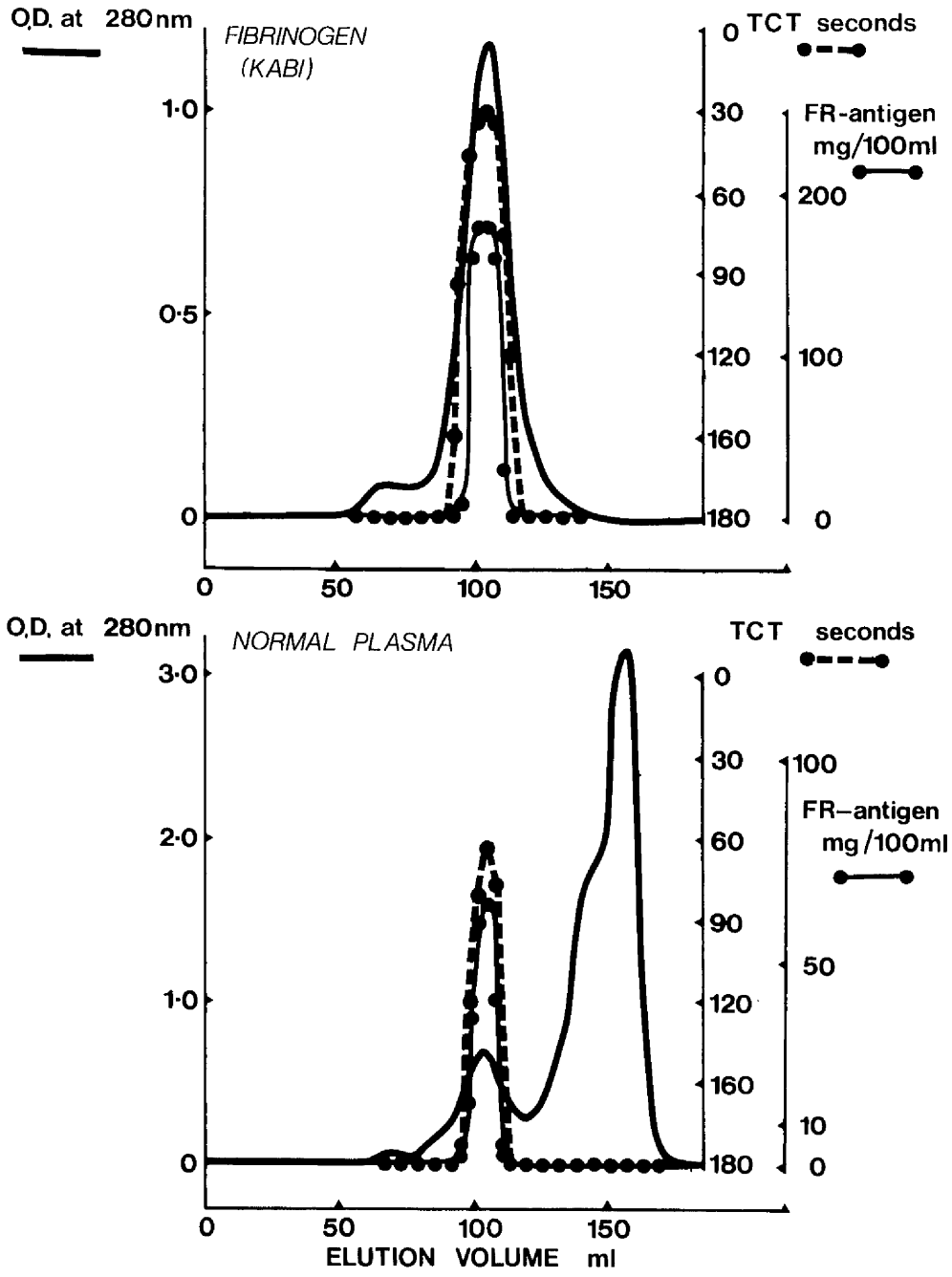

O.D. at 280nm
FIBRINOGEN
(KABI)
1·0
0·5
0
0
30
60
90
120
160
180
TCT seconds
FR-antigen
mg/100ml
200
100
0
0
50
100
150
O.D. at 280nm
NORMAL PLASMA
3·0
2·0
1·0
0
TCT seconds
0
30
60
90
120
160
180
100
FR–antigen
mg/100ml
50
10
0
0
50
100
150
ELUTION VOLUME ml

Figure 24

Figure 25

A comparison of the elution curves found using plasma and serum prepared from the same normal blood sample. The cumulative elution volume (ml) is shown on the X axis and the optical density (O.D., in arbitrary units) at 280 nm and the fibrinogen-fibrin related (FR-) antigen (mg/100 ml) results are shown on the Y axis.

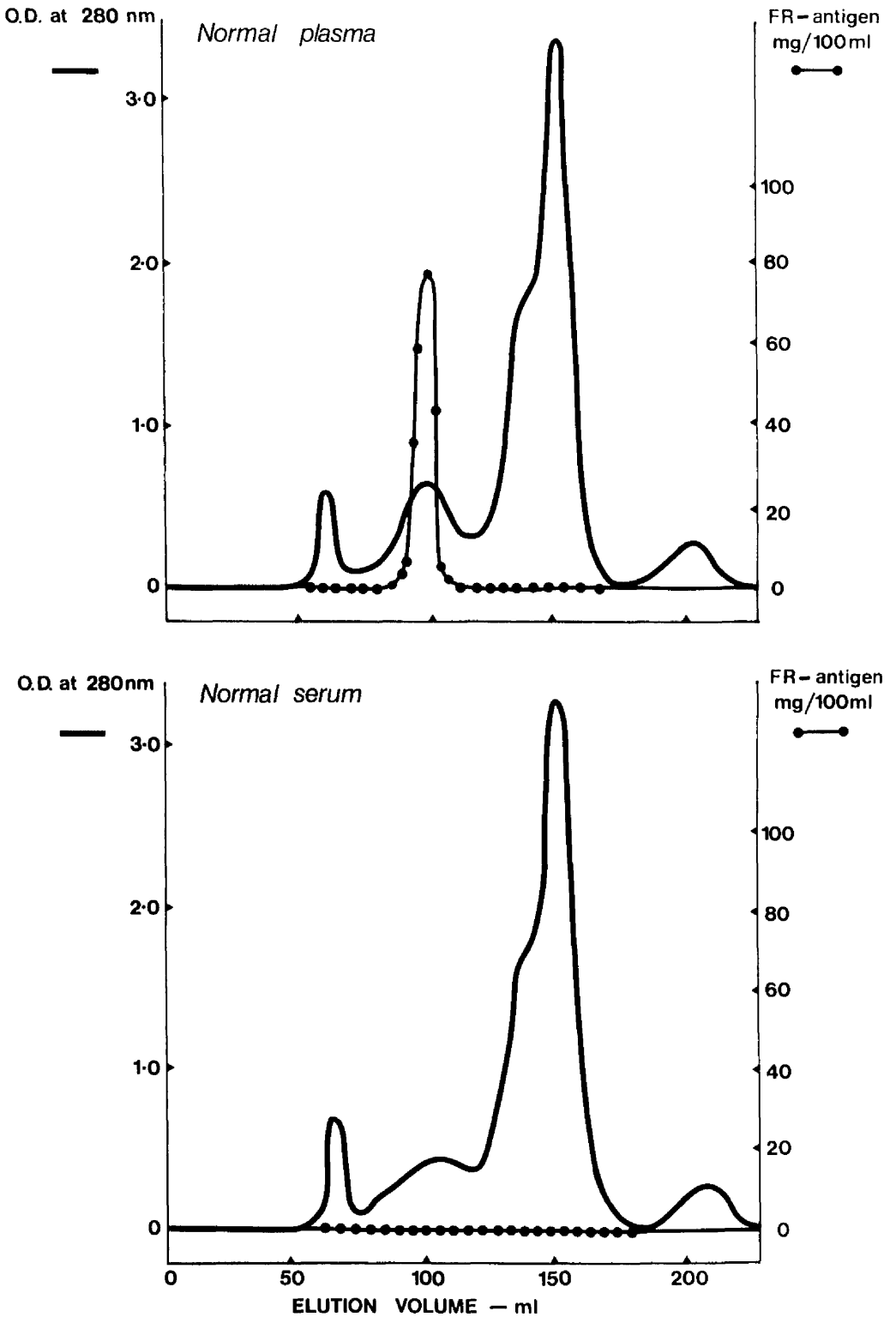

O.D. at 280 nm
Normal plasma
FR-antigen mg/100ml
3·0
2·0
1·0
0
100
80
60
40
20
0
O.D. at 280nm
Normal serum
FR-antigen mg/100ml
3·0
2·0
1·0
0
100
80
60
40
20
0
0
50
100
150
200
ELUTION VOLUME — ml

Figure 25

Figure 26

A comparison of the elution curves found using plasma (2 ml), a β -alanine precipitate of 9 ml of plasma from the same donor and the supernatant (2 ml) from the β -alanine precipitate. The cumulative elution volume (ml) is shown on the X axis and the optical density (O.D., in arbitrary units) at 280 nm, the fibrinogen-fibrin related (FR-) antigen (mg/100 ml) and the thrombin clotting time (T.C.T., seconds) results on the Y axis.

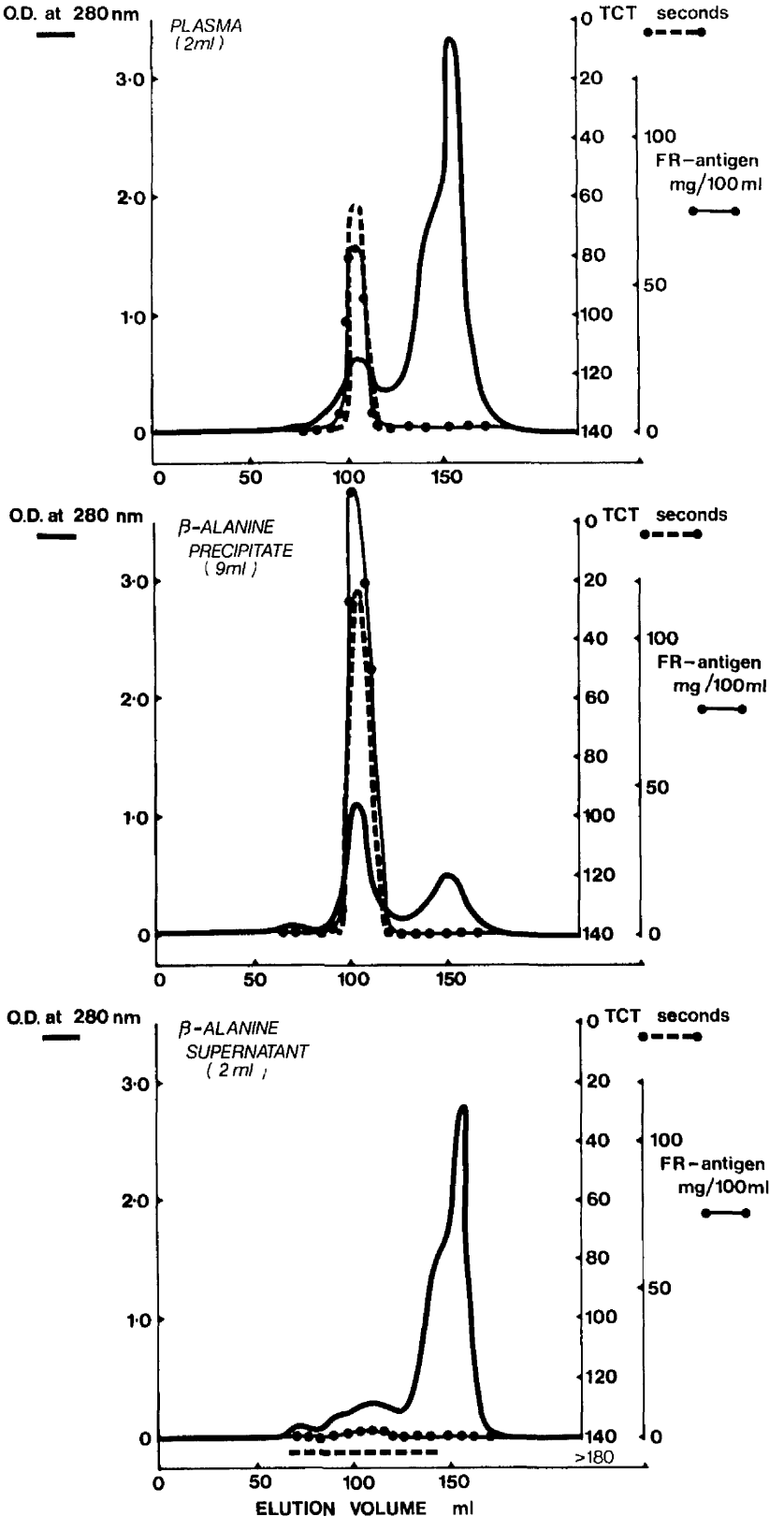

O.D. at 280 nm
PLASMA
(2ml)
TCT seconds
FR-antigen
mg/100 ml
3·0
2·0
1·0
0
20
40
60
80
100
120
140
50
0
50
100
150
O.D. at 280 nm
β-ALANINE
PRECIPITATE
(9ml)
TCT seconds
FR-antigen
mg /100ml
O.D. at 280 nm
β-ALANINE
SUPERNATANT
(2 ml)
TCT seconds
FR-antigen
mg/100ml
>180
ELUTION VOLUME ml

Figure 26

Figure 27

A series of experiments (a-h) is shown in which purified fibrinogen was incubated with increasing concentrations of thrombin (see details in text and Table 4). The cumulative elution volume (ml) is plotted on the X axis and the total protein concentration (optical density, O.D., in arbitrary units, at 280 nm) is shown on the Y axis.

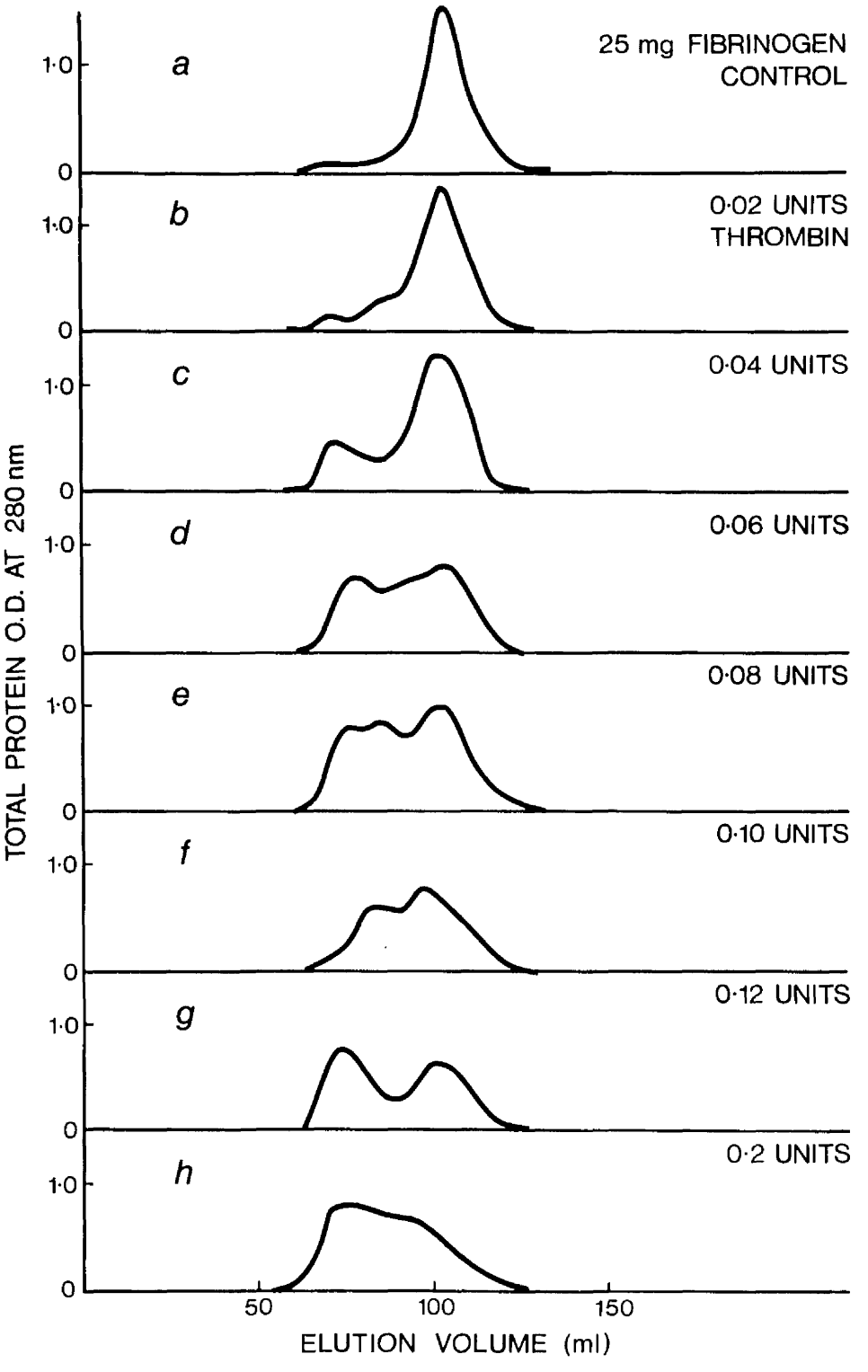

25 mg FIBRINOGEN CONTROL
0·02 UNITS THROMBIN
0·04 UNITS
0·06 UNITS
0·08 UNITS
0·10 UNITS
0·12 UNITS
0·2 UNITS
a
b
c
d
e
f
g
h
1·0
0
50
100
150
TOTAL PROTEIN O.D. AT 280 nm
ELUTION VOLUME (ml)

Figure 27

Figure 28

The same series of experiments (a-h) is shown as in Figure 27. The cumulative elution volume (ml) is plotted on the X axis and the thrombin clotting time (T.C.T., seconds) results are shown on the Y axis.

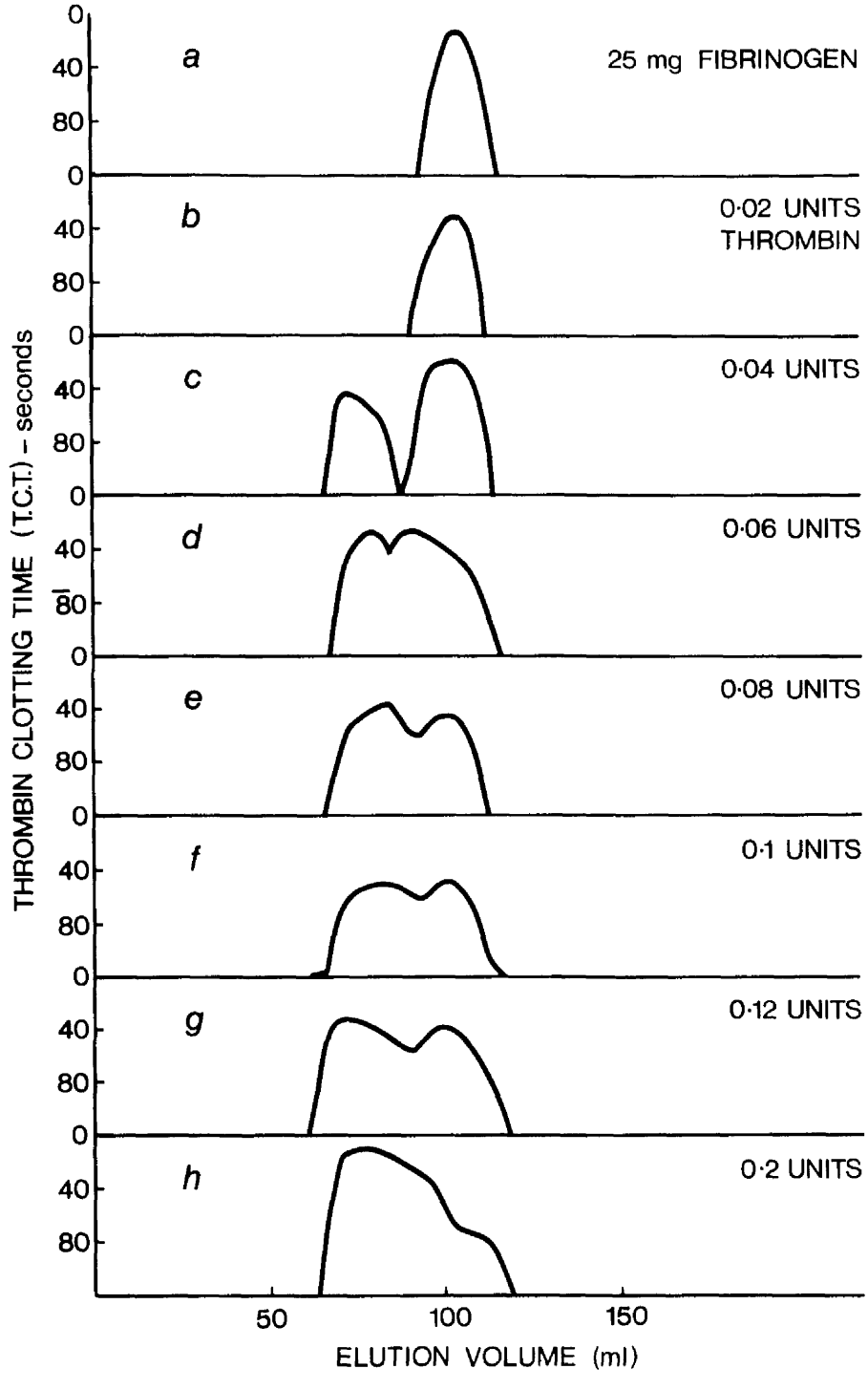

THROMBIN CLOTTING TIME (T.C.T.) - seconds
0
40
80
a
25 mg FIBRINOGEN
b
0·02 UNITS THROMBIN
c
0·04 UNITS
d
0·06 UNITS
e
0·08 UNITS
f
0·1 UNITS
g
0·12 UNITS
h
0·2 UNITS
50
100
150
ELUTION VOLUME (ml)

Figure 28

Figure 29

The same series of experiments (a-h) is shown as in Figures 27 and 28. The cumulative elution volume (ml) is plotted on the X axis and the fibrinogen-fibrin related (FR-) antigen (mg/100 ml) results are shown on the Y axis. In certain elution fractions "clots" formed on standing at room temperature overnight. These are marked "clotted" (◀——▶).

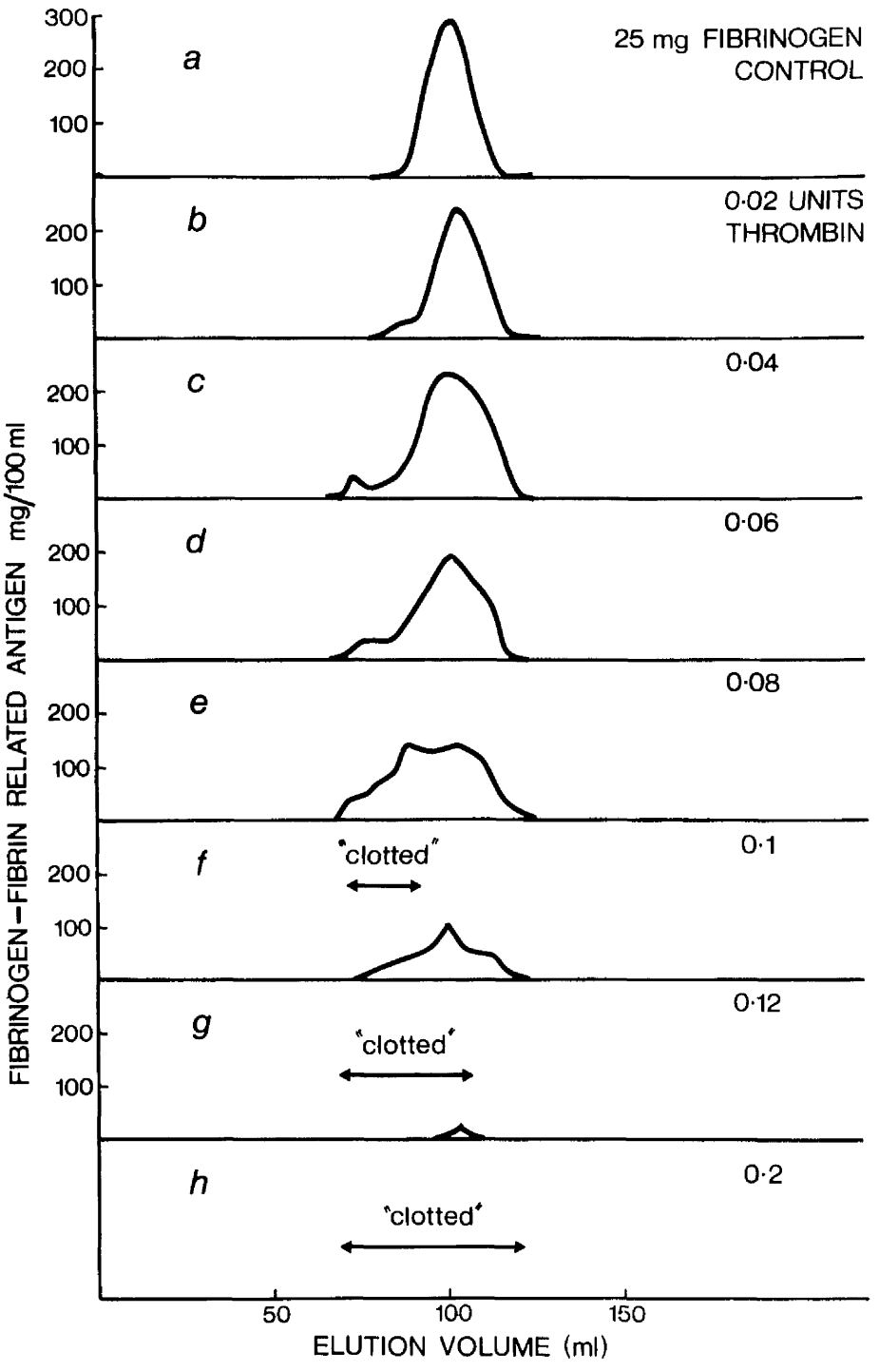

300
200
100
a
25 mg FIBRINOGEN
CONTROL
b
0·02 UNITS
THROMBIN
c
0·04
d
0·06
e
0·08
f
"clotted"
0·1
g
"clotted"
0·12
h
"clotted"
0·2
FIBRINOGEN–FIBRIN RELATED ANTIGEN mg/100 ml
50
100
150
ELUTION VOLUME (ml)

Figure 29

Figure 30

Superimposed thrombin clotting time (T. C. T.) and fibrinogen-fibrin related (FR-) antigen elution curves for four of the experiments shown in Figures 28 and 29 (a, b, c and e). The cumulative elution volume (ml) is shown on the X axis and the results of the T. C. T. (seconds) and FR-antigen (mg/100 ml) assays on the Y axis.

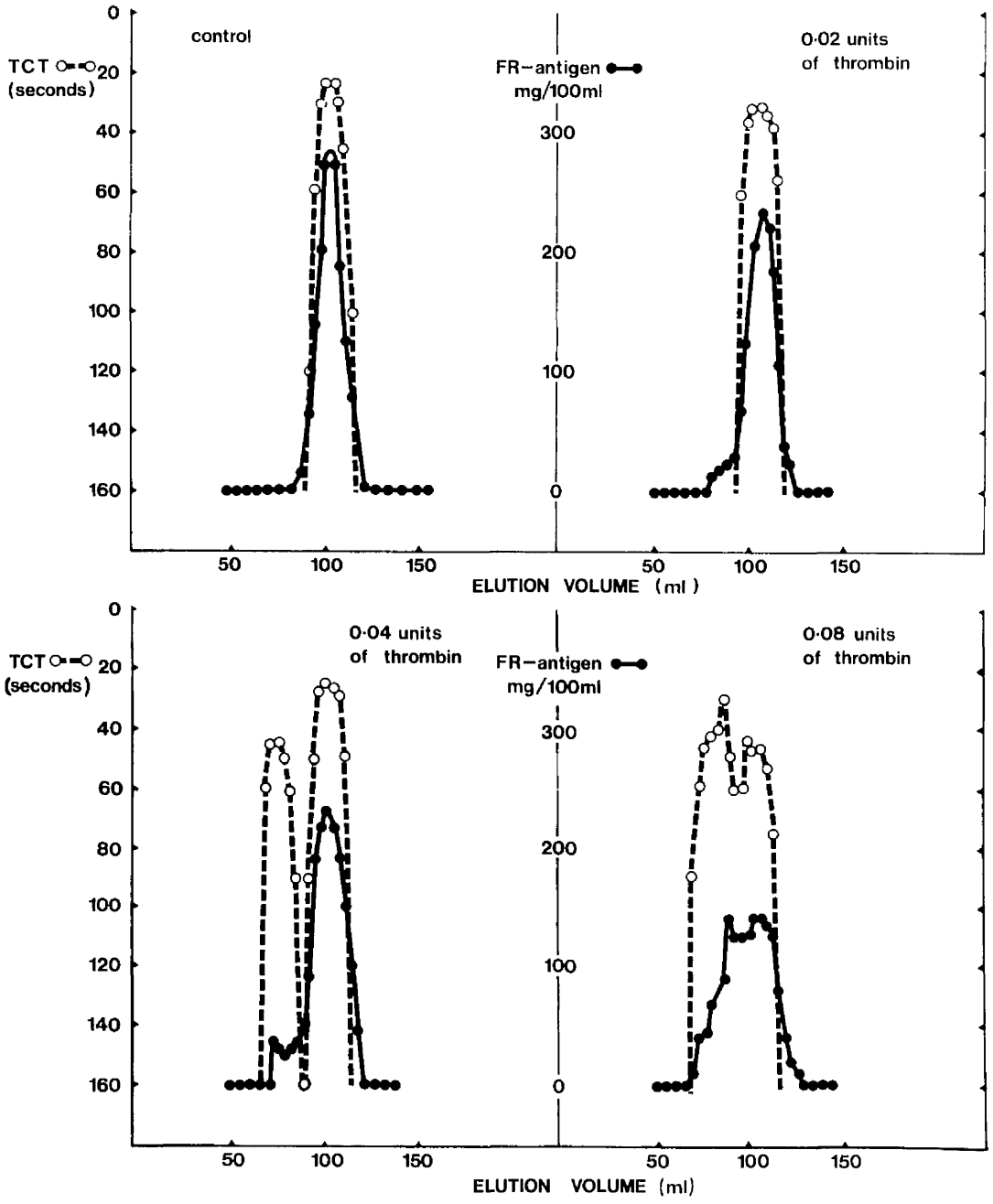

control
0·02 units of thrombin
0·04 units of thrombin
0·08 units of thrombin
TCT (seconds)
FR-antigen mg/100ml
0
20
40
60
80
100
120
140
160
300
200
100
50
100
150
ELUTION VOLUME (ml)

Figure 30

Figure 31

A series of experiments is shown in which purified fibrinogen (Kabi) was incubated with streptokinase (S. K.) for varying lengths of time (see text for details). The cumulative elution volume (ml) is shown on the X axis and the total protein concentration (optical density, O. D., in arbitrary units, at 280 nm) on the Y axis. The expected positions of fibrinogen and the fibrinogen degradation products (X, Y, D and E) are shown at the foot of the diagram (calculated from Marder, Shulman and Carroll, 1969).

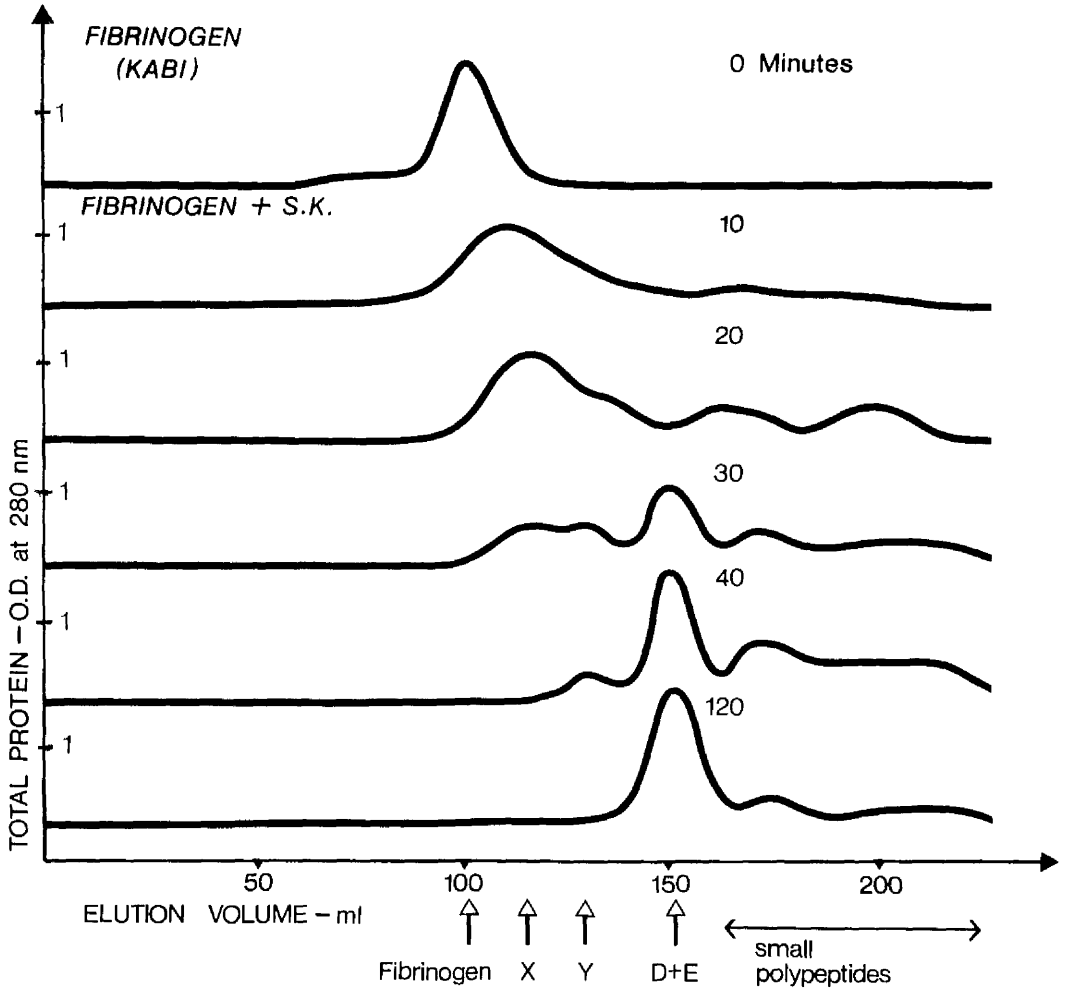

FIBRINOGEN
(KABI)
0 Minutes
1
FIBRINOGEN + S.K.
10
20
30
40
120
TOTAL PROTEIN – O.D. at 280 nm
50
100
150
200
ELUTION VOLUME – ml
Fibrinogen
X
Y
D+E
small
polypeptides

Figure 31

Figure 32

A series of experiments (a-e) is shown in which plasma was incubated with ancrod and/or streptokinase (S.K.) (see details in text and Table 5). The cumulative elution volume (ml) is shown on the X axis and the optical density (O.D., arbitrary units) at 280 nm on the Y axis.

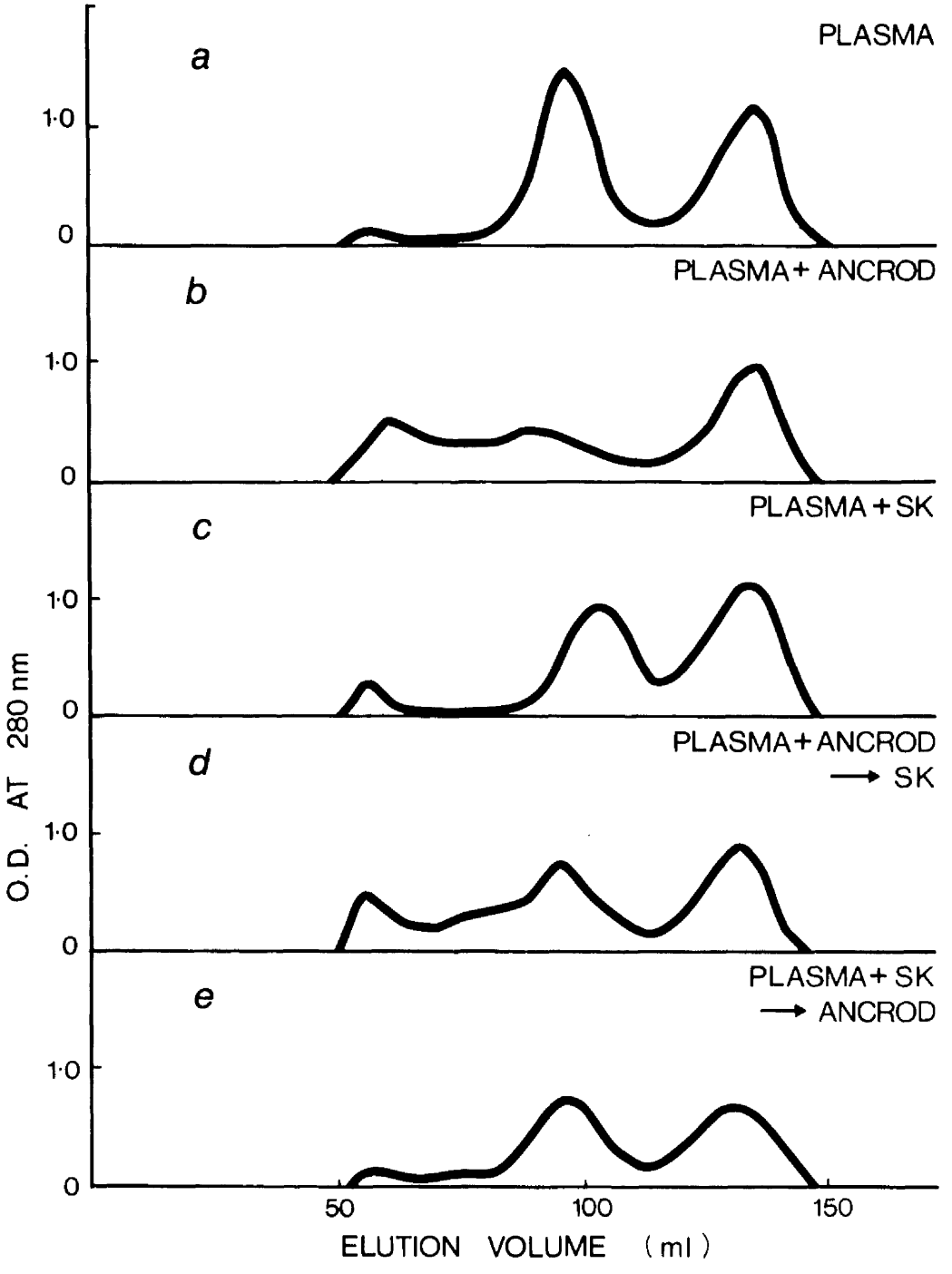

a
PLASMA
1·0
0
b
PLASMA + ANCROD
1·0
0
c
PLASMA + SK
1·0
0
d
PLASMA + ANCROD
→ SK
1·0
0
e
PLASMA + SK
→ ANCROD
1·0
0
O.D. AT 280 nm
50
100
150
ELUTION VOLUME (ml)

Figure 32

Figure 33

The same series of experiments (a-e) is shown as in Figure 32. The cumulative elution volume (ml) is plotted on the X axis and the thrombin clotting time (seconds) results on the Y axis.

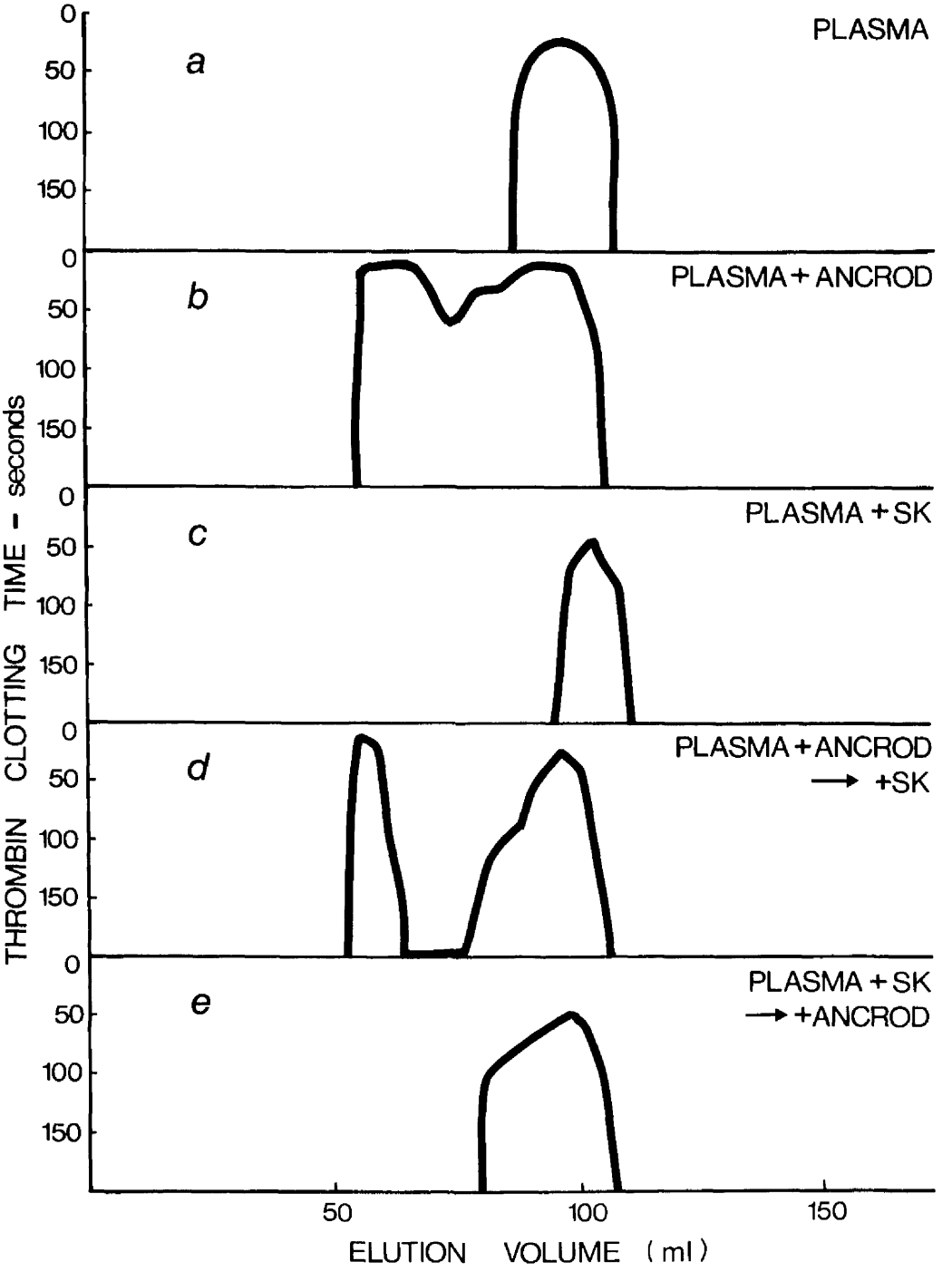

PLASMA
a
PLASMA + ANCROD
b
PLASMA + SK
c
PLASMA + ANCROD
→ +SK
d
PLASMA + SK
→ +ANCROD
e
0
50
100
150
THROMBIN CLOTTING TIME - seconds
50
100
150
ELUTION VOLUME (ml)

Figure 33

Figure 34

The same series of experiments (a-e) is shown as in Figures 32 and 33. The cumulative elution volume (ml) is plotted on the X axis and the fibrinogen-fibrin related antigen (mg/100 ml) results on the Y axis.

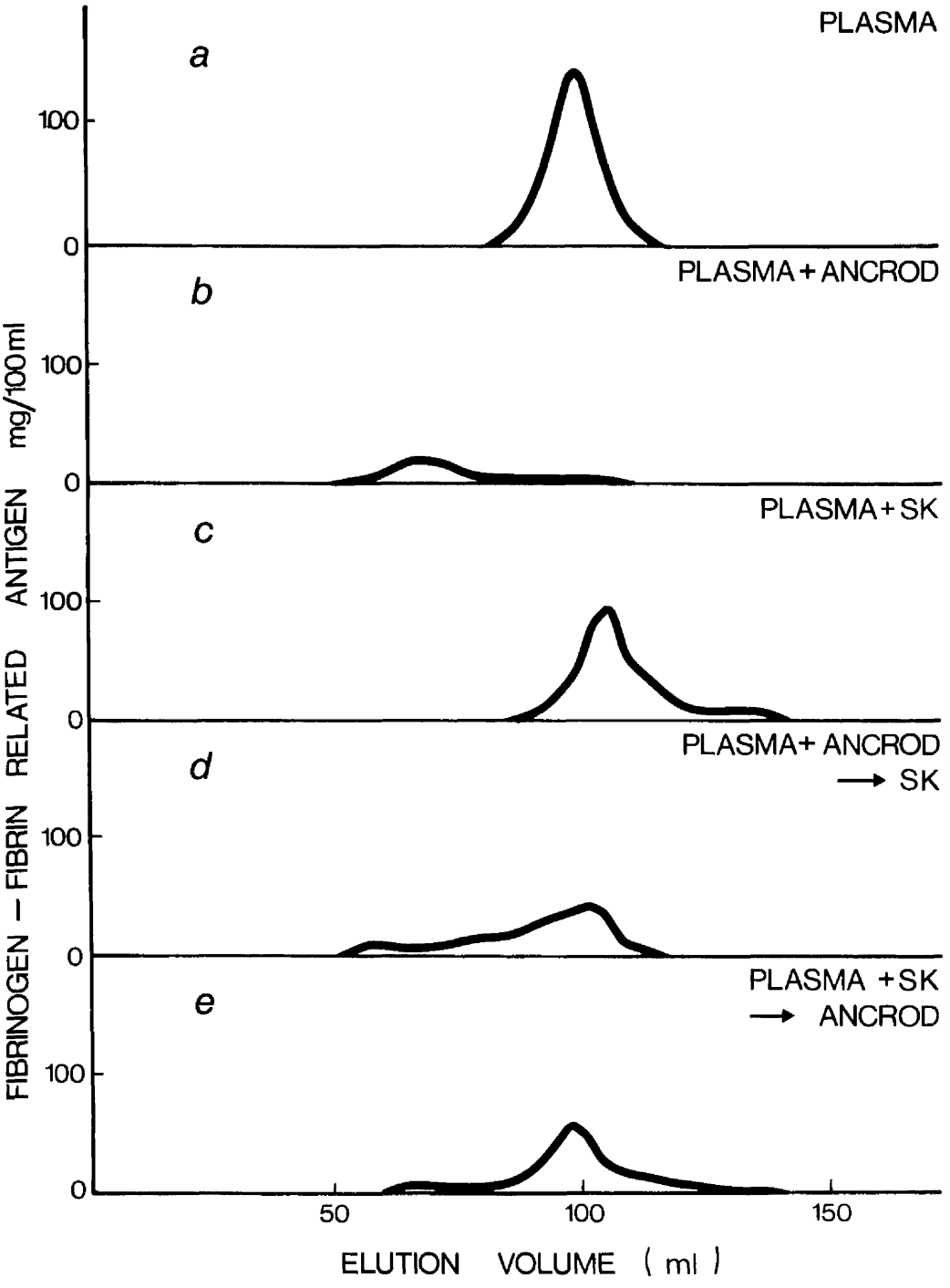

PLASMA
a
100
0
PLASMA + ANCROD
b
100
0
PLASMA + SK
c
100
0
PLASMA + ANCROD
→ SK
d
100
0
PLASMA + SK
→ ANCROD
e
100
0
FIBRINOGEN – FIBRIN RELATED ANTIGEN mg/100ml
50
100
150
ELUTION VOLUME (ml)

Figure 34

Figure 35

Superimposed thrombin clotting time (T. C. T.) and fibrinogen-fibrin related antigen elution curves for the experiments shown in Figures 33 and 34. The cumulative elution volume (ml) is shown on the X axis and the results of the fibrinogen-fibrin related antigen (mg/100 ml) and the T. C. T. (seconds) assays on the Y axis in each case. Note the changes in the fibrinogen-fibrin related antigen scales.

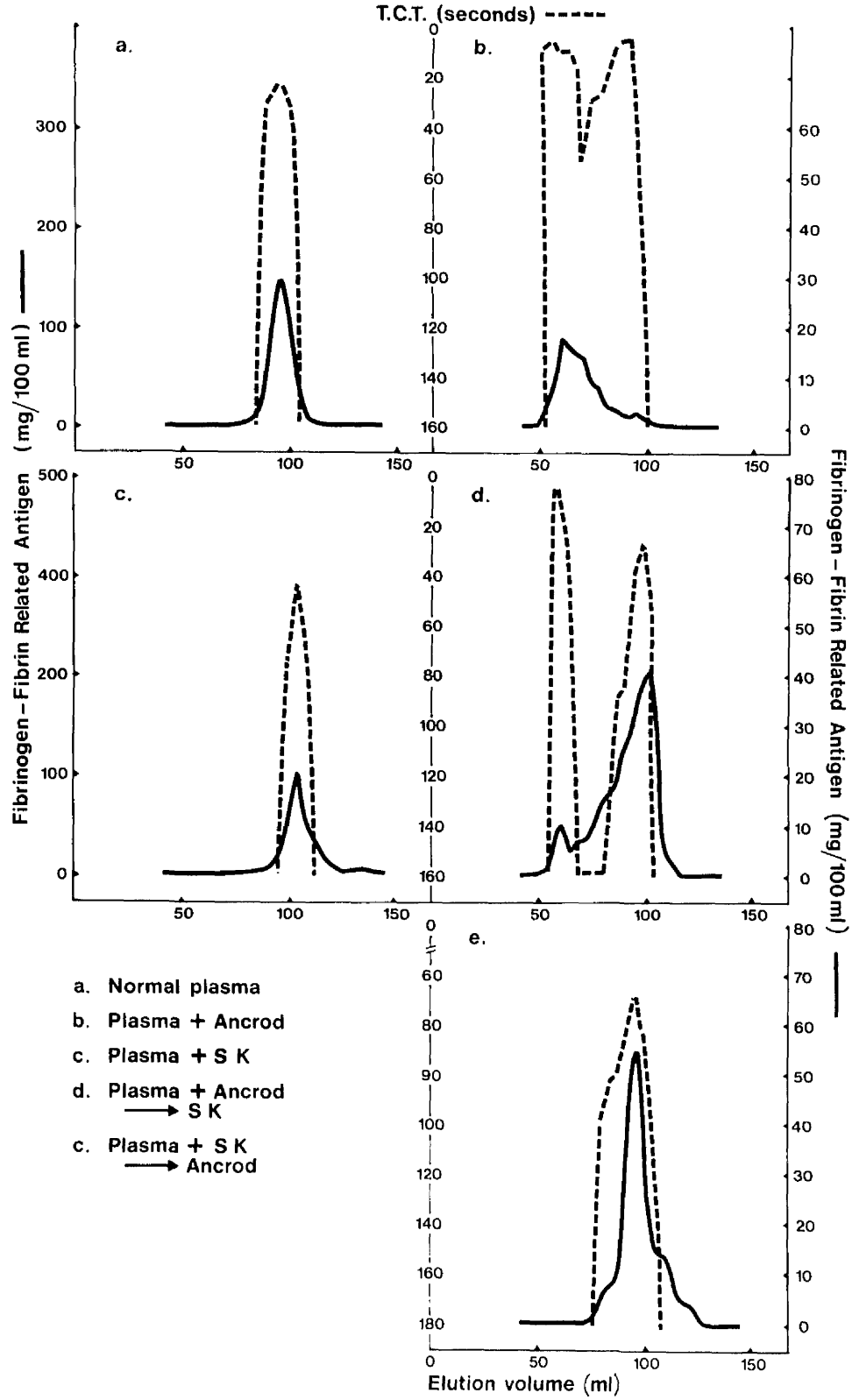

T.C.T. (seconds) -----
a.
b.
c.
d.
e.
Fibrinogen-Fibrin Related Antigen (mg/100 ml) ——
Fibrinogen-Fibrin Related Antigen (mg/100 ml) ——
Elution volume (ml)
a. Normal plasma
b. Plasma + Ancrod
c. Plasma + S K
d. Plasma + Ancrod → S K
c. Plasma + S K → Ancrod

Figure 35

Figure 30

The results of an experiment in which insoluble fibrin (prepared by clotting the fibrinogen in normal plasma with thrombin) was digested with plasmin (see text for details). The cumulative elution volume (ml) is shown on the X axis and the fibrinogen-fibrin (FR-) antigen (mg/100 ml) and thrombin clotting time (T. C. T., seconds) results on the Y axis. The inset above the X axis shows the results of the thrombin clottable protein (T. C. P.) incubation test (▨). The elution volume (V_e) of purified fibrinogen using this column system is indicated.

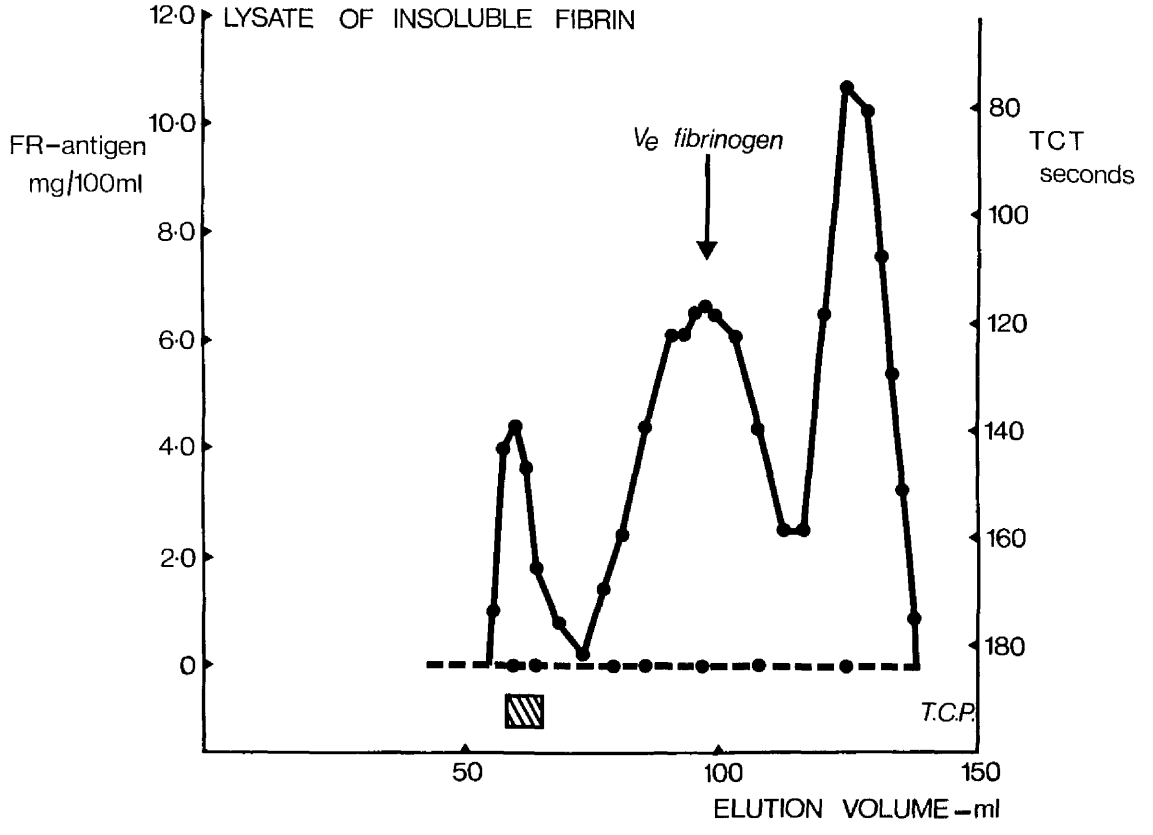

LYSATE OF INSOLUBLE FIBRIN
FR-antigen mg/100ml
12·0
10·0
8·0
6·0
4·0
2·0
0
Ve fibrinogen
TCT seconds
80
100
120
140
160
180
T.C.P.
50
100
150
ELUTION VOLUME – ml

Figure 36

Figure 37

A hypothetical scheme for the action of ancrod on fibrinogen in vivo (adapted from Gaffney and Brasher, 1974). $Polymer_3$ > $Polymer_2$ > $Polymer_1$ in molecular weight. Note that it is suggested that prothrombin is activated by $Polymer_1$.

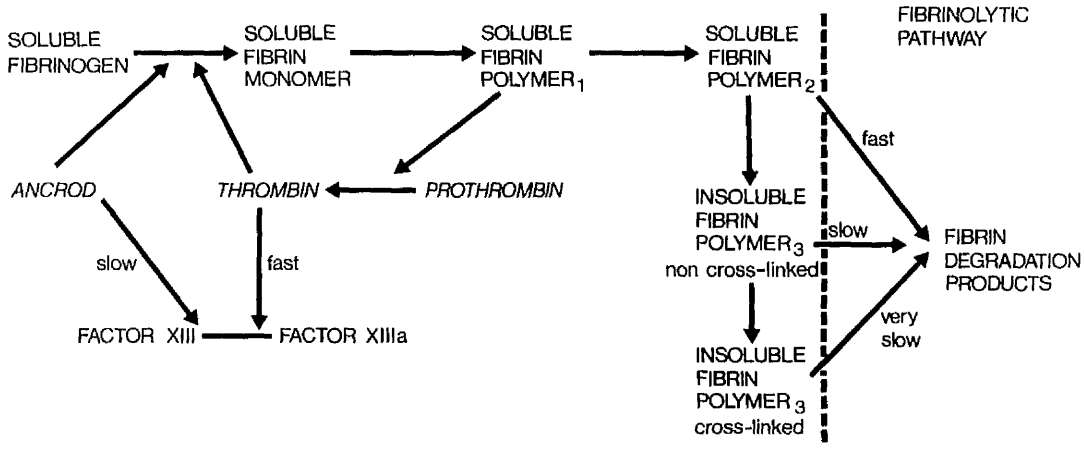

SOLUBLE FIBRINOGEN
SOLUBLE FIBRIN MONOMER
SOLUBLE FIBRIN POLYMER1
SOLUBLE FIBRIN POLYMER2
FIBRINOLYTIC PATHWAY
fast
ANCROD
THROMBIN
PROTHROMBIN
INSOLUBLE FIBRIN POLYMER3 non cross-linked
slow
FIBRIN DEGRADATION PRODUCTS
slow
fast
very slow
FACTOR XIII
FACTOR XIIIa
INSOLUBLE FIBRIN POLYMER3 cross-linked

Figure 37

Figure 38

Plasma fibrinogen chromatography results obtained from blood samples taken from a patient (W. McG.) pre-treatment and after 3, 6 and 24 hours intravenous ancrod infusion. The cumulative elution volume (ml) is shown on the X axis. The total protein concentration, as measured by the optical density (O.D., in arbitrary units) at 280 nm, is shown on the Y axis.

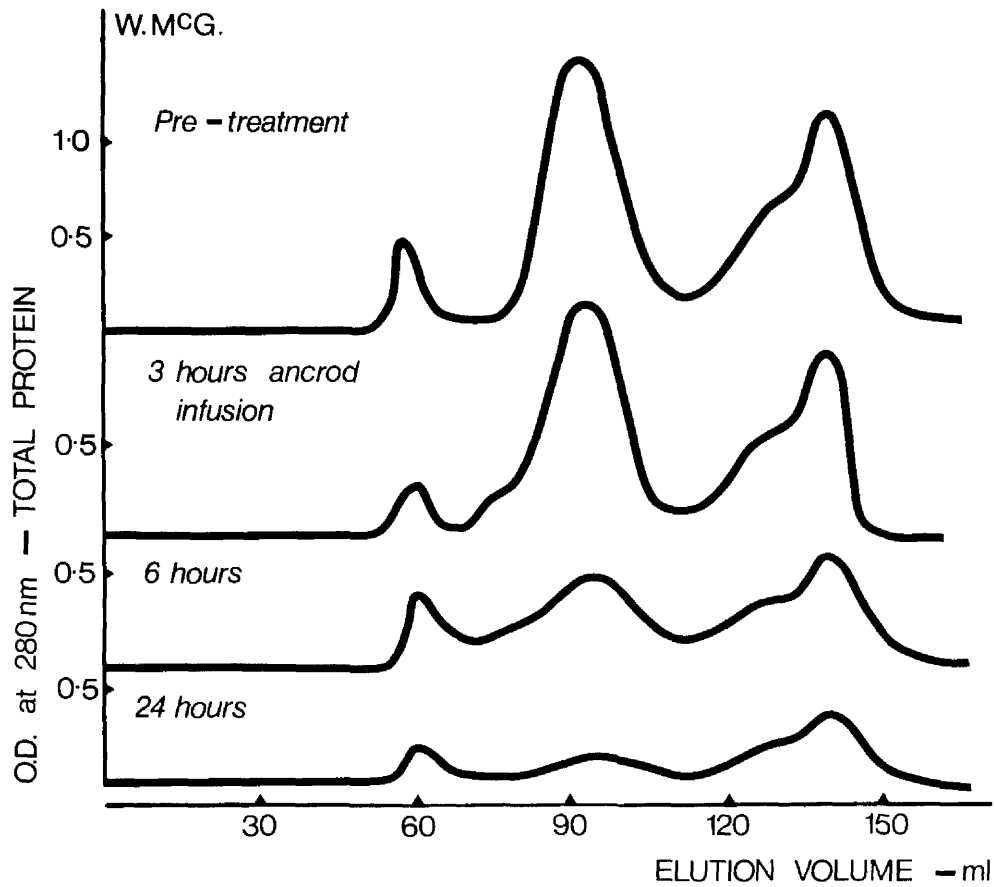

W.McG.
Pre - treatment
1·0
0·5
3 hours ancrod infusion
0·5
6 hours
0·5
24 hours
0·5
O.D. at 280nm — TOTAL PROTEIN
30
60
90
120
150
ELUTION VOLUME — ml

Figure 38

Figure 38a

The fibrinogen-fibrin related antigen elution curves obtained on samples from a patient (W. McG.) taken pre-treatment and after 6 and 24 hours intravenous ancrod infusion. The cumulative elution volume (ml) is shown on the X axis and the fibrinogen-fibrin related antigen (mg/100 ml) results on the Y axis.
(published in McKillop, Edgar, Forbes et al., 1975b and c).

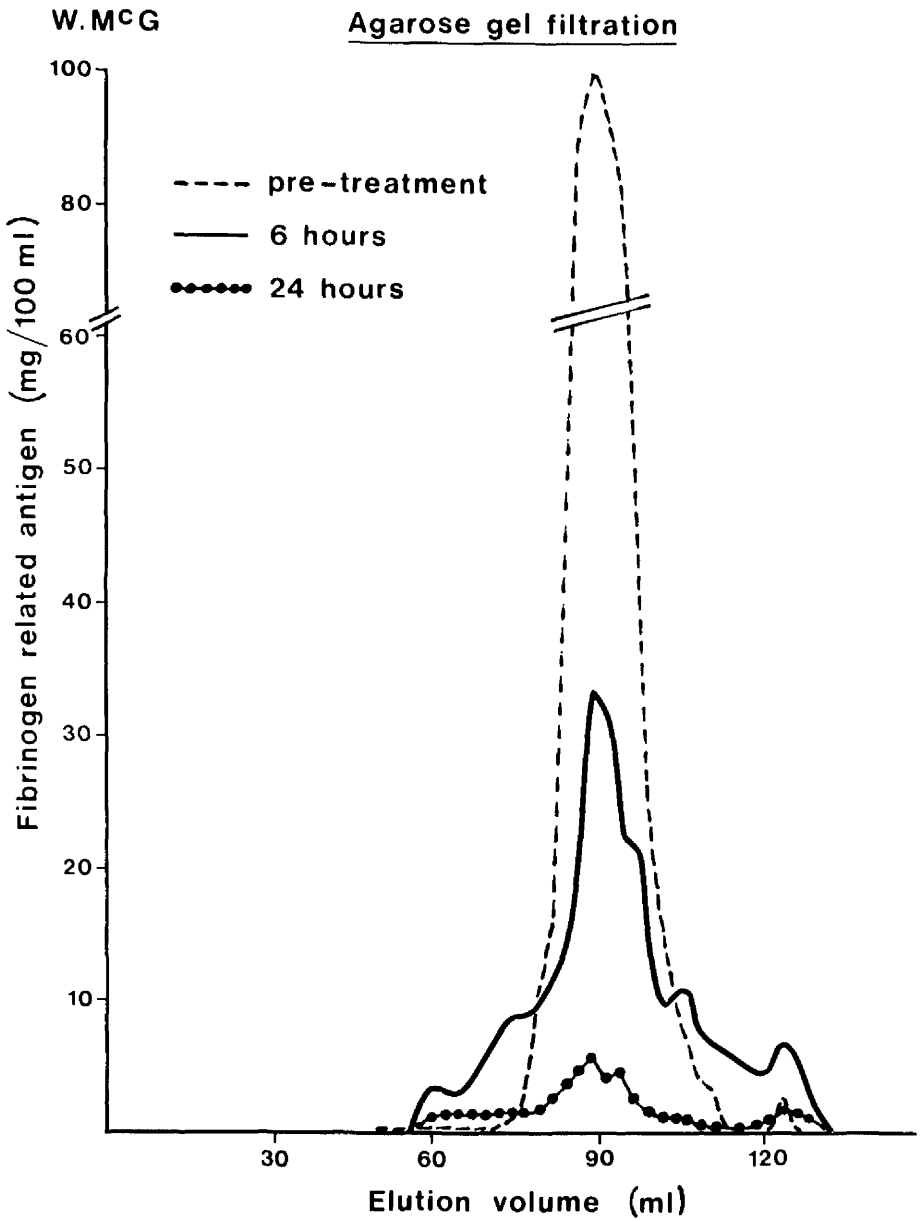

W.McG
Agarose gel filtration
pre-treatment
6 hours
24 hours
Fibrinogen related antigen (mg/100 ml)
100
80
60
50
40
30
20
10
30
60
90
120
Elution volume (ml)

Figure 39a

Figure 39b

The fibrinogen-fibrin related antigen elution curves obtained on samples from a patient (J. McK.) taken pre-treatment and after 6 and 24 hours intravenous ancrod infusion. The cumulative elution volume (ml) is shown on the X axis and the fibrinogen-fibrin related antigen (mg/100 ml) on the Y axis. (published in McKillop, Edgar, Forbes et al., 1975c).

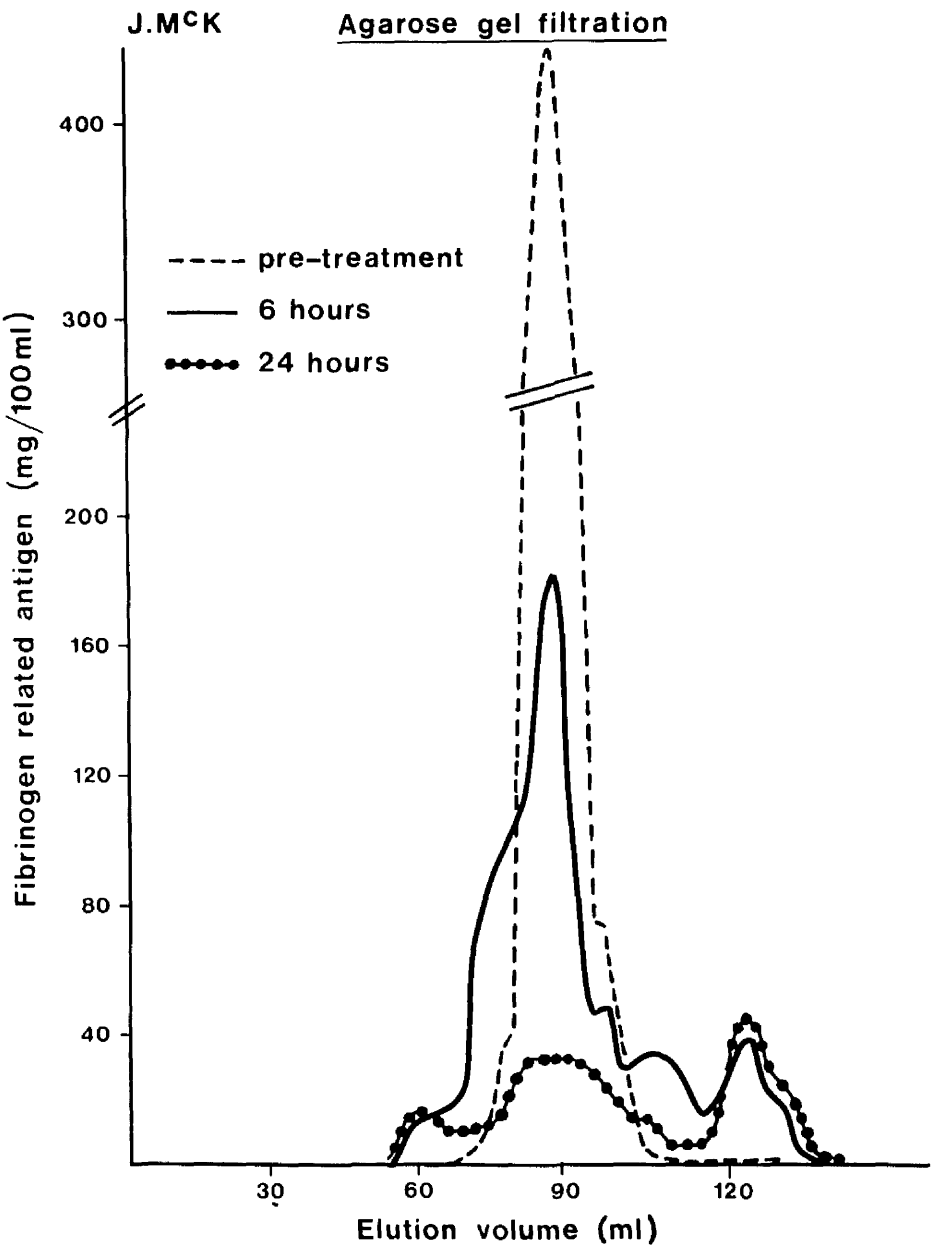

J.McK
Agarose gel filtration
400
300
200
160
120
80
40
pre-treatment
6 hours
24 hours
Fibrinogen related antigen (mg/100ml)
30
60
90
120
Elution volume (ml)

Figure 39b

Figure 40

Superimposed fibrinogen-fibrin related (FR-) antigen and thrombin clotting time (T.C.T.) elution curves on samples obtained from two patients (B.S. and J.R.) after 6 hours intravenous ancrod infusion. The cumulative elution volume (ml) is shown on the X axis and the FR-antigen (mg/100 ml) and T.C.T. (seconds) results on the Y axis. The inset above the X axis shows the results of the thrombin clottable protein (T.C.P.) incubation test (▨).

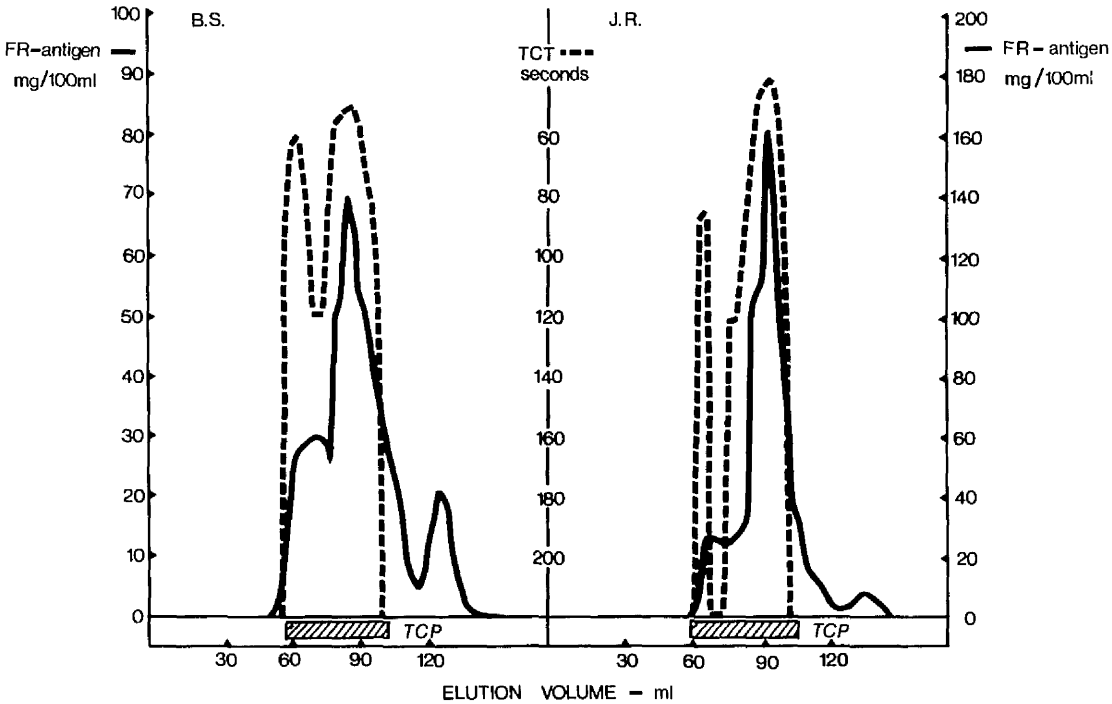

B.S.
J.R.
FR-antigen
mg/100ml
TCT
seconds
FR - antigen
mg/100ml
100
90
80
70
60
50
40
30
20
10
0
60
80
100
120
140
160
180
200
180
160
140
120
100
80
60
40
20
0
TCP
TCP
30
60
90
120
30
60
90
120
ELUTION VOLUME - ml

Figure 40

Figure 41

Changes in area m following the start of intravenous ancrod infusion in 5 patients. The first samples were taken before the time of starting treatment, which is shown by the arrow. The time (in hours) after starting treatment is shown on the X axis and the area m result (percent total area) is shown on the Y axis. The results from each individual patient are connected by the solid lines.

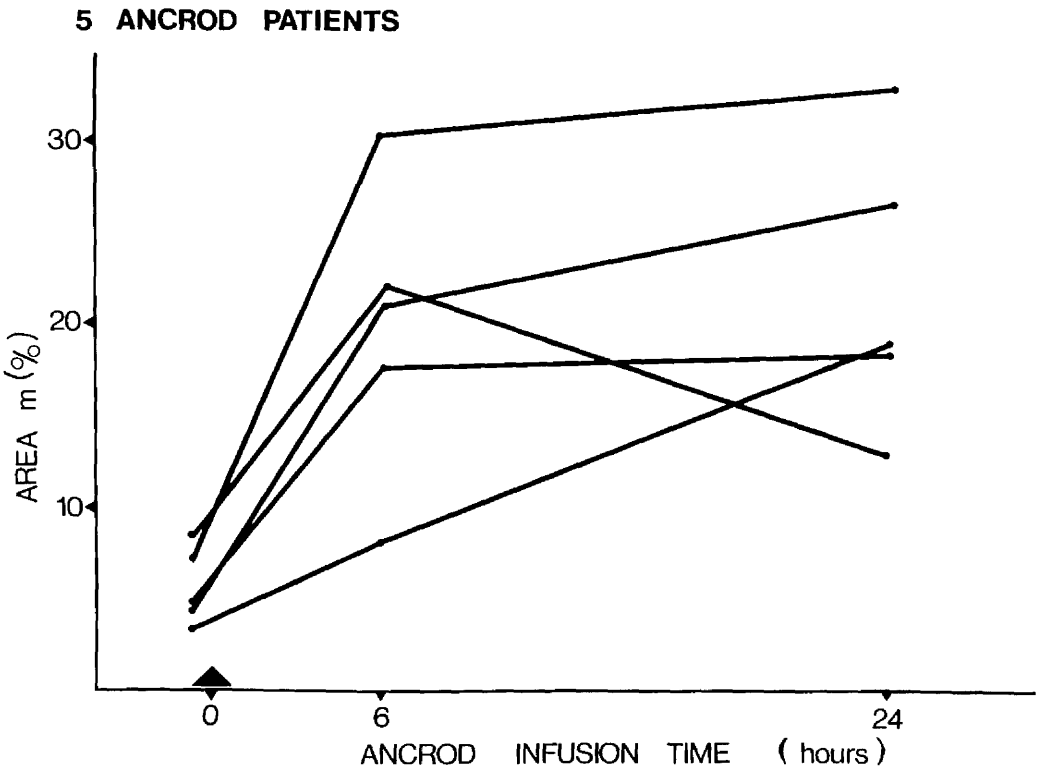

5 ANCROD PATIENTS
30
20
10
AREA m (%)
0
6
24
ANCROD INFUSION TIME (hours)

Figure 41

Figure 42

Changes in area p following the start of anerod infusion in 5 patients. The first samples were taken before the time of starting treatment, which is shown by the arrow. The time (hours) after starting treatment is shown on the X axis and the area p result (percent total area) is shown on the Y axis. The results from each individual patient are connected by the solid lines.

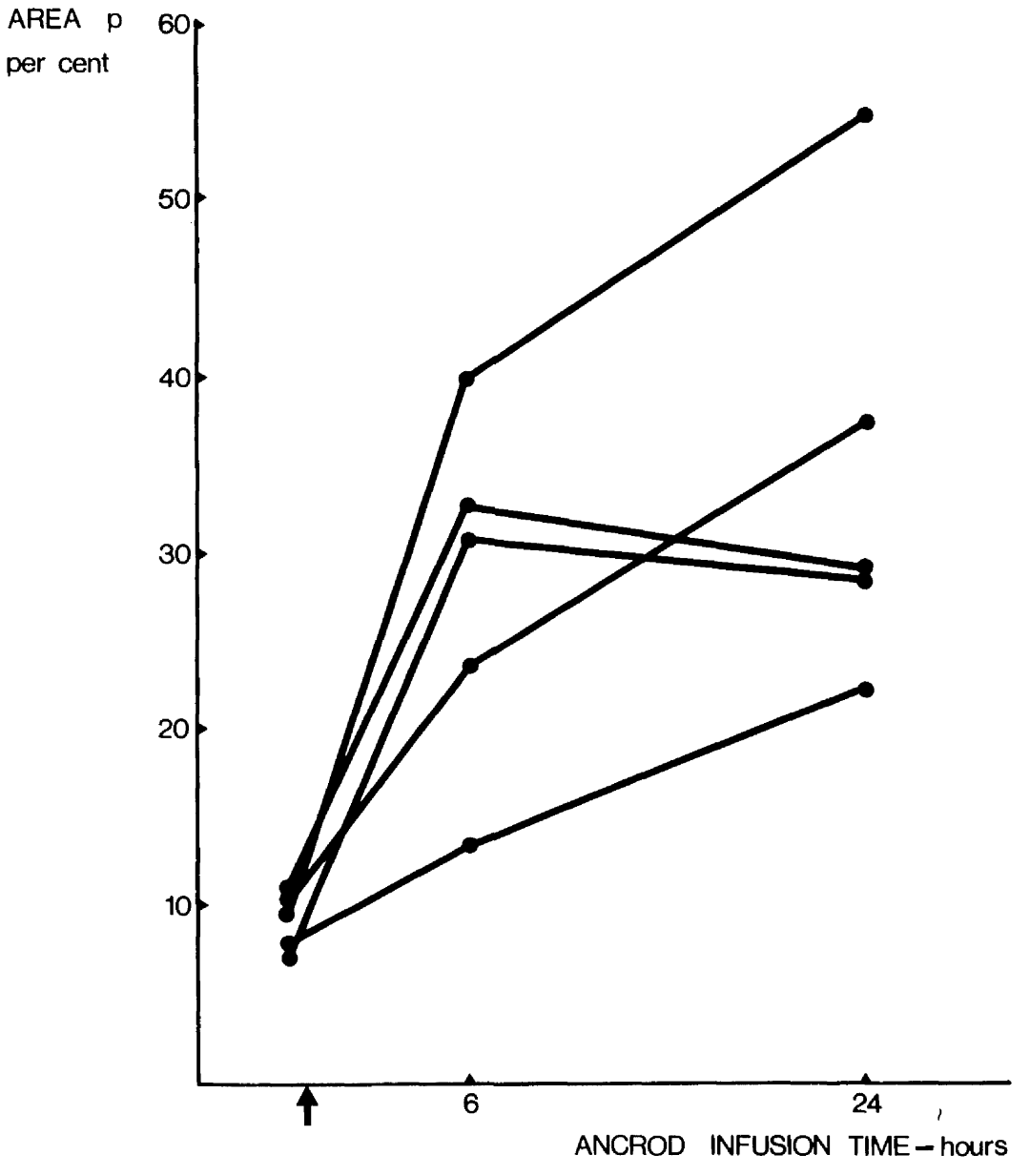

AREA p
per cent
60
50
40
30
20
10
6
24
ANCROD INFUSION TIME – hours

Figure 42

Figures 49, 50 and 51

In these sets of figures the sequential plasma fibrinogen chromatography results are shown for three pre-eclamptic patients (Patient 3 in Figure 49, Patient 7 in Figure 50 and Patient 9 in Figure 51). The results are placed in time sequence starting with the upper left quadrant, followed by the upper right quadrant, then the lower left quadrant and finally the lower right quadrant. The cumulative elution volume (ml) is shown on the X axis in each case. The fibrinogen-fibrin related antigen (mg/100 ml) and thrombin clotting time (T.C.T., seconds) results are shown on the Y axis. The elution volume over which a positive thrombin clottable protein incubation test was found is shown by the shaded area (▨) inset above the X axis. The results of the staphylococcal clumping test titre at the void volume (SCT. V_o) are also shown. The arrow (↓) indicates the elution volume at which fibrinogen-fibrin related antigen could first be detected by the radial immunodiffusion technique. The gestational age (weeks) at the time of blood sampling and the packed gel column system (e.g. B7 (II)) are also shown.

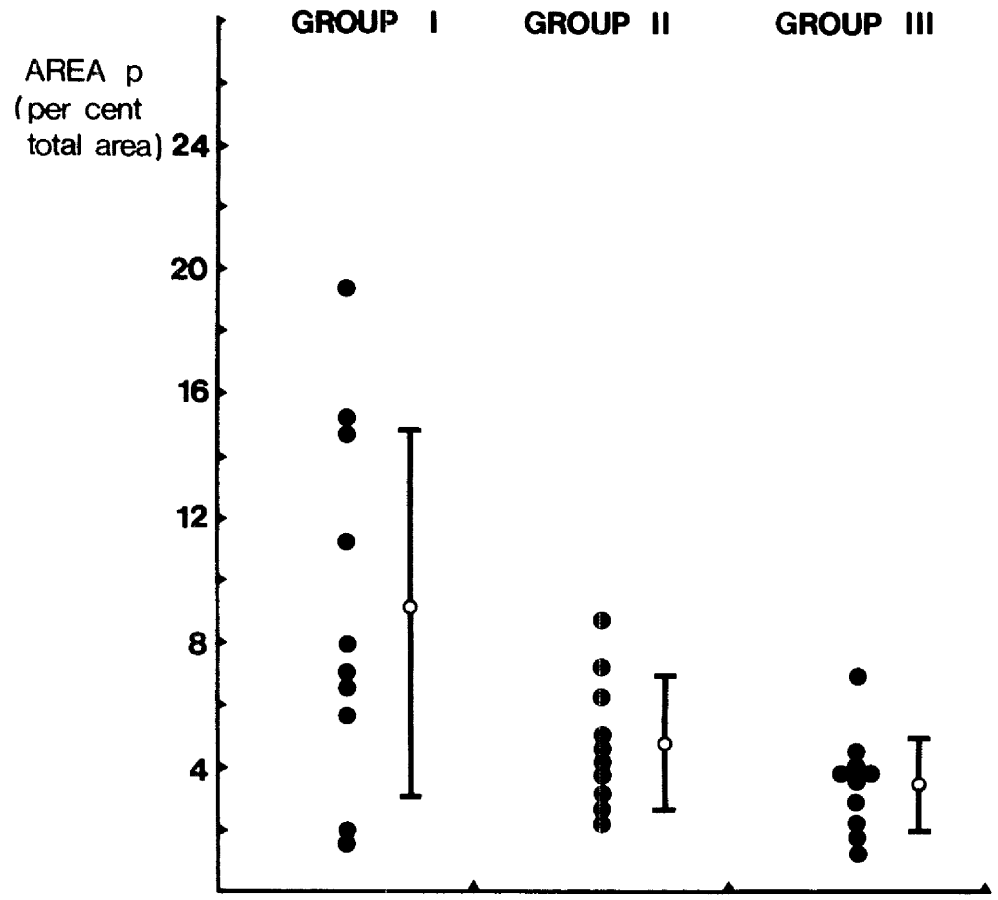

GROUP I
GROUP II
GROUP III
AREA p
(per cent
total area)
24
20
16
12
8
4

Figure 48

Figure 48

The results for area p (percent total area) are shown for the same samples as in Figure 47. The mean $\pm$ single standard deviation is shown to the right of the scatter of results.

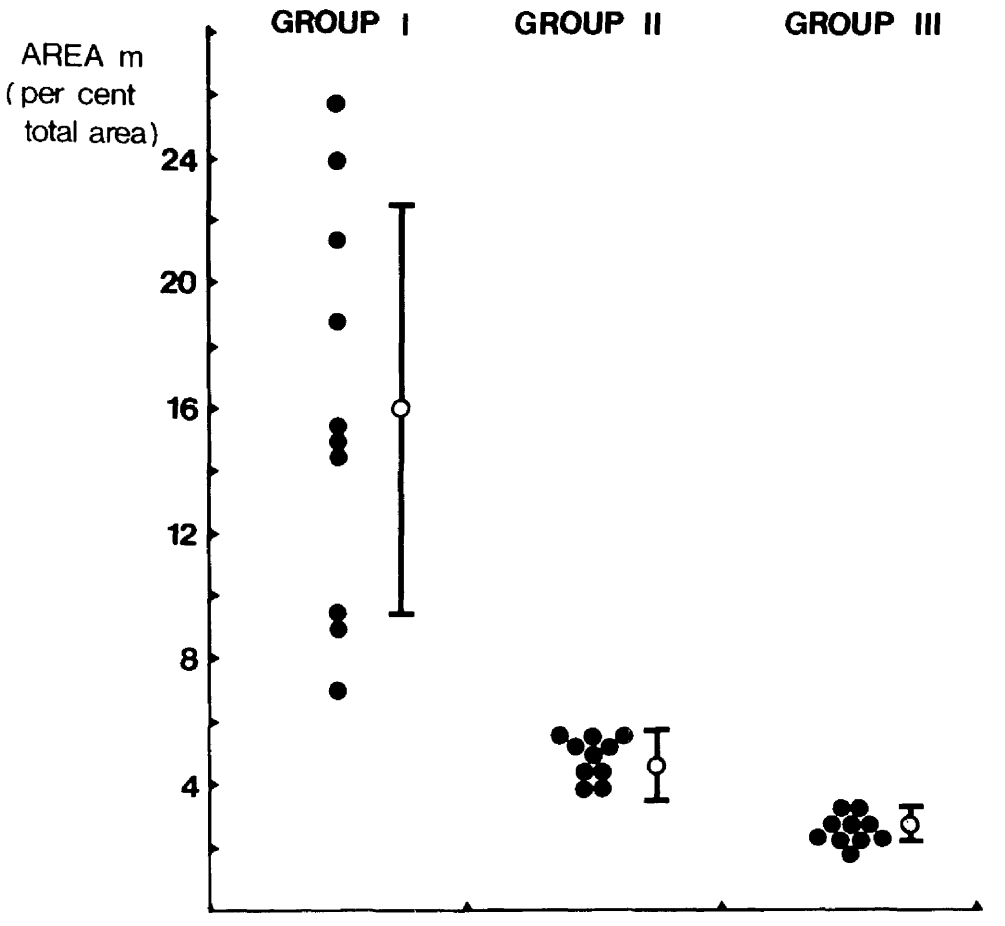

GROUP I
GROUP II
GROUP III
AREA m
(per cent
total area)
24
20
16
12
8
4

Figure 47

Figure 47

The results of area m (percent total area) for samples from 10 pre-eclamptic patients (Group I), 10 matched normal pregnant women (Group II) and 10 normal non-pregnant women (Group III). The mean $\pm$ single standard deviation is shown to the right of the scatter of results. (published in McKillop, Howie, Forbes et al., 1976).

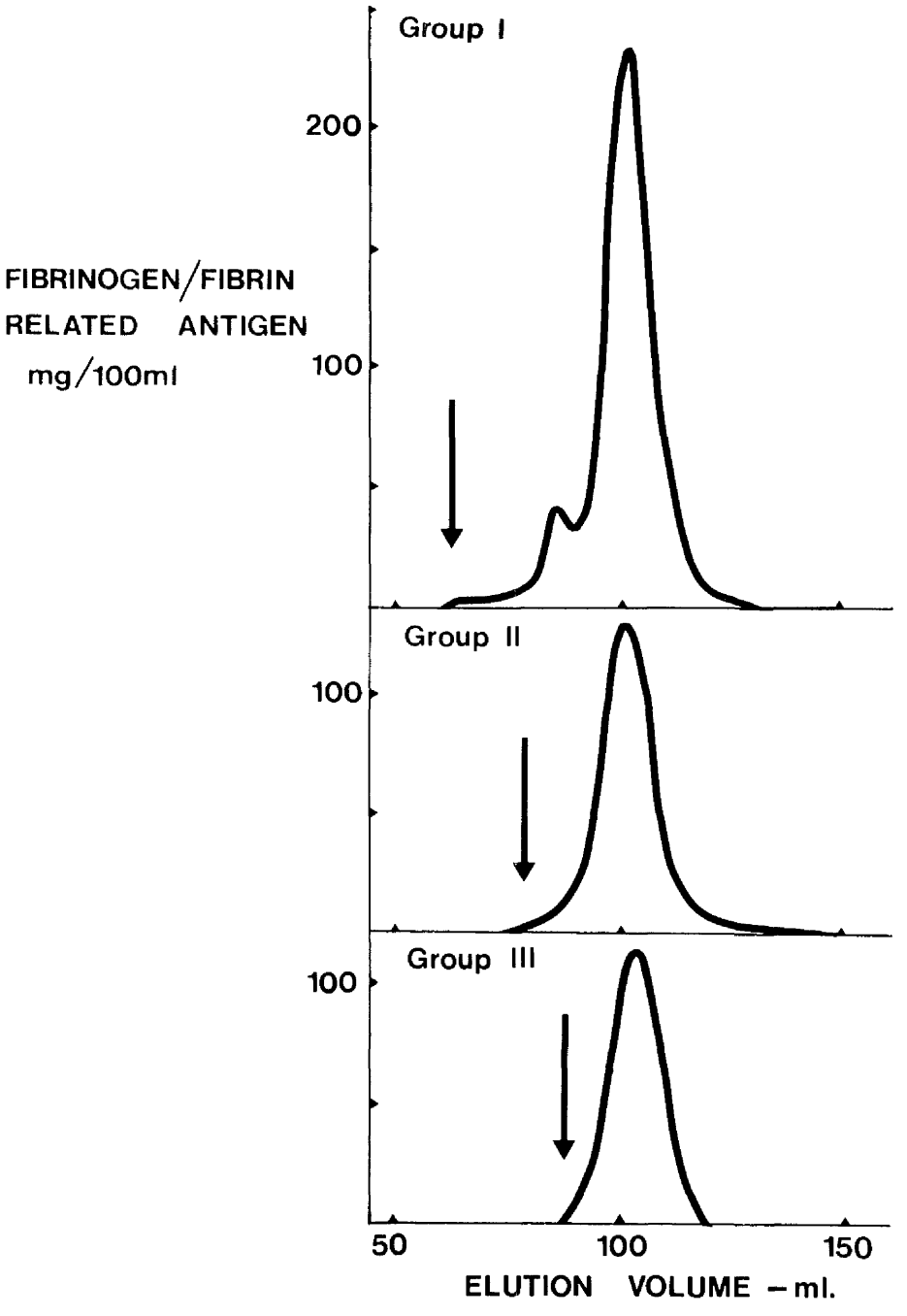

Group I
200
FIBRINOGEN/FIBRIN
RELATED ANTIGEN
mg/100ml
100
Group II
100
Group III
100
50
100
150
ELUTION VOLUME – ml.

Figure 46

Figure 46

The fibrinogen-fibrin related antigen elution curves are shown for a set of matched results for samples from a pre-eclamptic patient (Group I), a normal pregnant control woman (Group II) and a normal non-pregnant control woman (Group III). The cumulative elution volume (ml) is shown on the X axis and the fibrinogen-fibrin related antigen concentration (mg/100 ml) is shown on the Y axis. The arrow (↓) indicates the elution volume at which antigen could first be detected by the radial immunodiffusion technique.
(published in McKillop, Howie, Forbes et al., 1976).

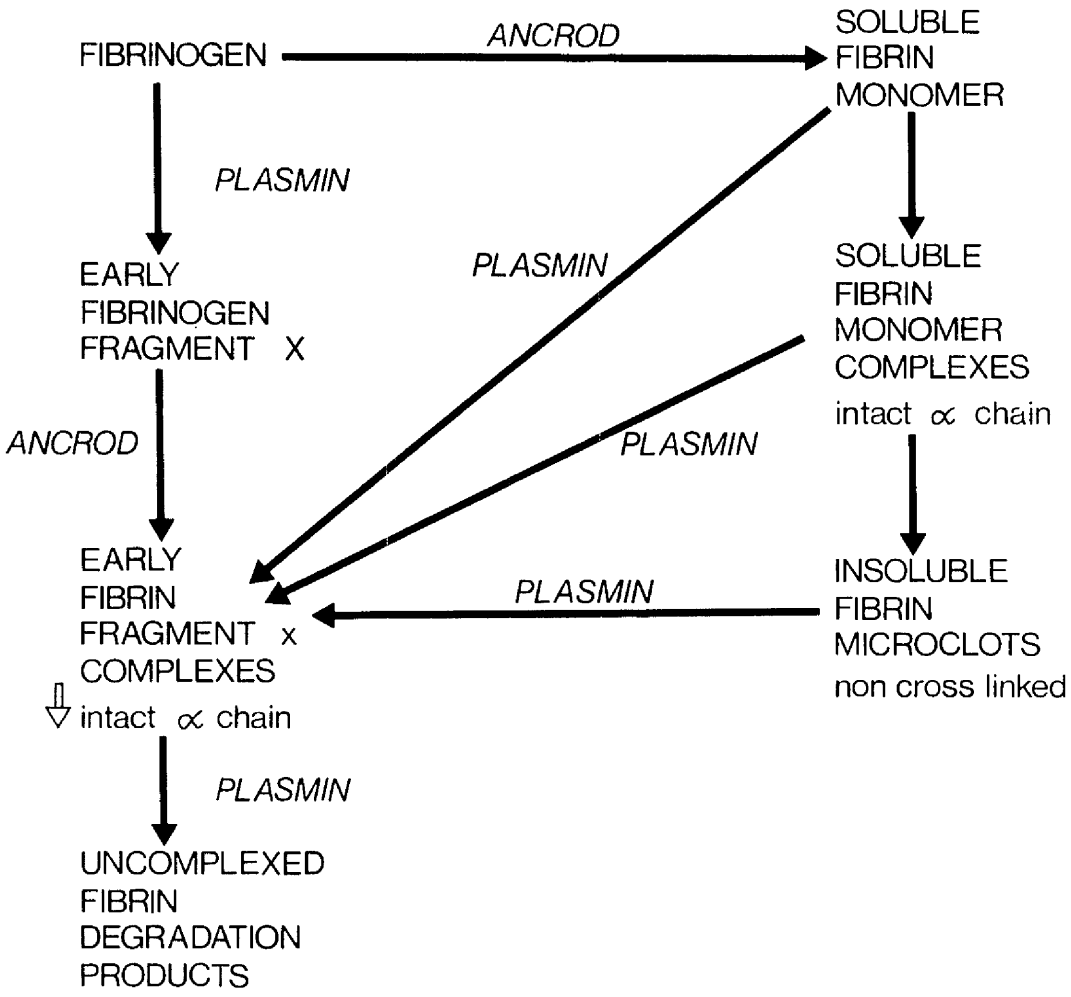

FIBRINOGEN
ANCROD
SOLUBLE FIBRIN MONOMER
PLASMIN
EARLY FIBRINOGEN FRAGMENT X
PLASMIN
SOLUBLE FIBRIN MONOMER COMPLEXES intact ∝ chain
ANCROD
PLASMIN
EARLY FIBRIN FRAGMENT x COMPLEXES intact ∝ chain
PLASMIN
INSOLUBLE FIBRIN MICROCLOTS non cross linked
PLASMIN
UNCOMPLEXED FIBRIN DEGRADATION PRODUCTS

Figure 45

Figure 45

Several possible pathways for the production of soluble complexes deficient in intact α chain during intravenous anerod infusion are shown. Much of this diagram remains hypothetical.

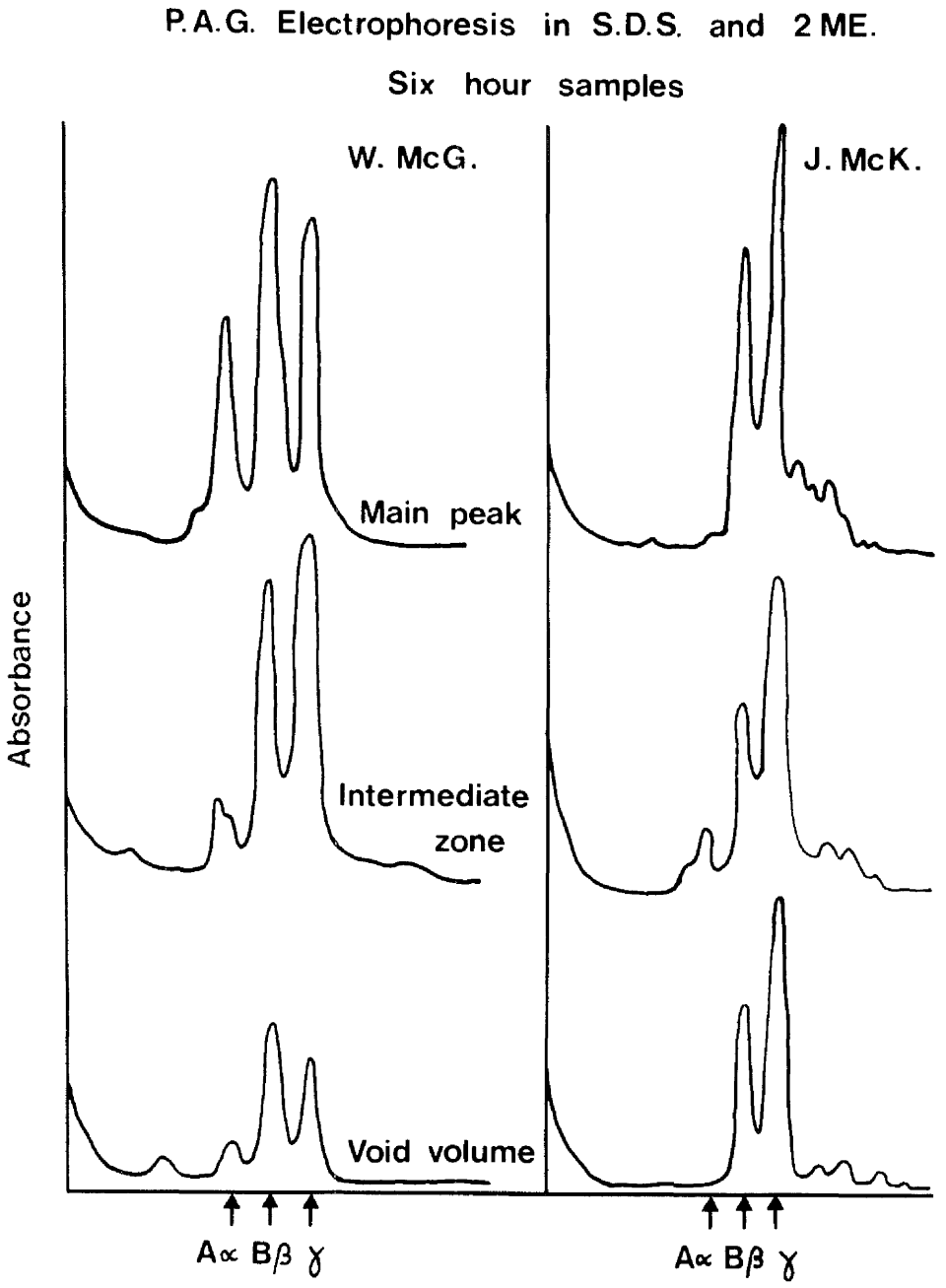

P.A.G. Electrophoresis in S.D.S. and 2 ME.
Six hour samples
W. McG.
J. McK.
Main peak
Intermediate zone
Void volume
Absorbance
Aα Bβ γ
Aα Bβ γ

Figure 44

Figure 44

Polyacrylamide gel electrophoresis results obtained by the method of Weber and Osborn (1969) using the same eluant fractions from plasma samples from the same patients as in Figure 43. Mercaptoethanol (2ME) was added with the sodium dodecyl sulphate (S.D.S.) to cleave the disulphide bonds linking the chains in the fibrinogen-related molecules. The electrophoresis results are shown as densitometric scans with the absorbance measured at 600 nm using a Gilford spectrophotometer. The migration positions of the A α, B β and γ chains of standard fibrinogen are shown. Similar results were obtained with samples from the other patients studied. (published in McKillop, Edgar, Forbes et al., 1975b and c).

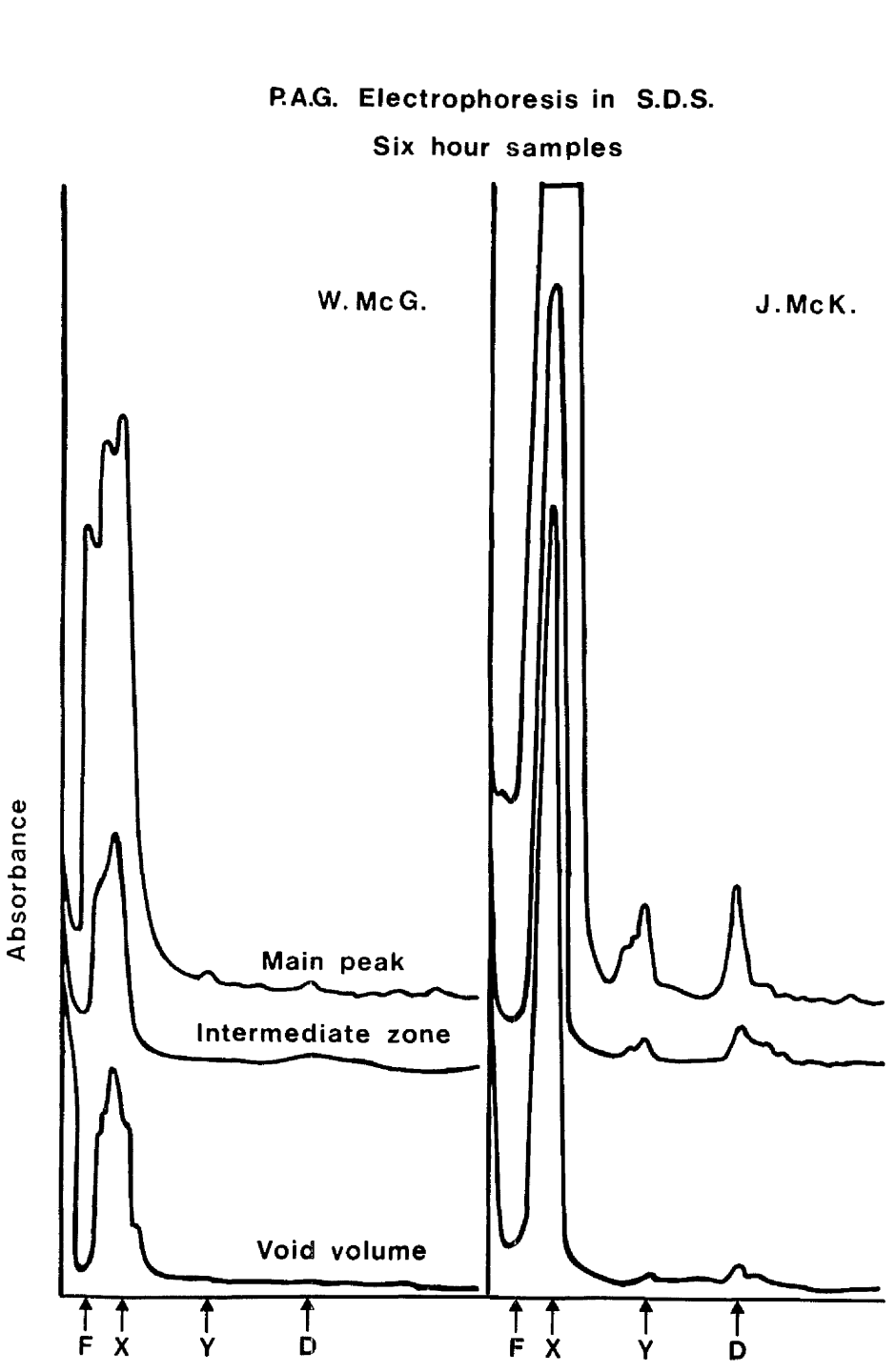

P.A.G. Electrophoresis in S.D.S.
Six hour samples
W. McG.
J. McK.
Absorbance
Main peak
Intermediate zone
Void volume
F
X
Y
D
F
X
Y
D

Figure 43

Figure 43

Polyacrylamide gel electrophoresis results obtained by the method of Weber and Osborn (1969) using various eluant fractions in sodium dodecyl sulphate (S.D.S.). The eluant fractions selected were from the gel filtration experiments with plasma samples obtained from two patients (W.McG. and J.McK.) 6 hours after starting ancrod infusion (see Figure 39a and b). The selected eluant fractions were from:-

(i) the top of the main fibrinogen-fibrin related antigen peak (main peak).

(ii) the void volume.

(iii) an intermediate zone between the void volume and the main peak.

The electrophoresis results are shown as densitometric scans with the absorbance measured at 600 nm using a Gilford spectrophotometer. The migration positions of standard fibrinogen (F) and its degradation products (X, Y, D and E) are shown. Similar results were obtained with samples from the other patients studied.
(published in McKillop, Edgar, Forbes et al., 1975c).

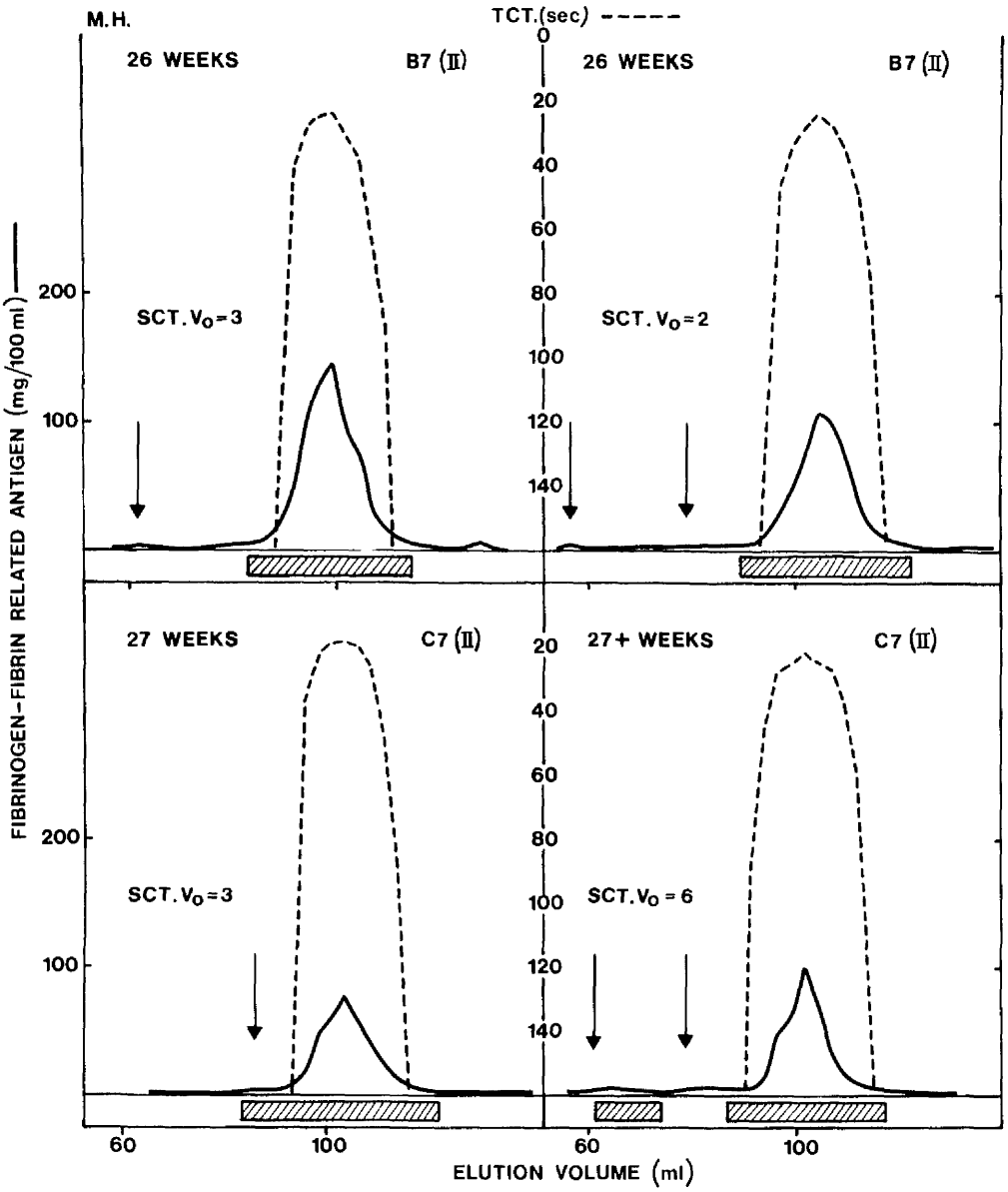


Figure 49a:- Results for Pre-eclamptic Patient 3 (M.H.).

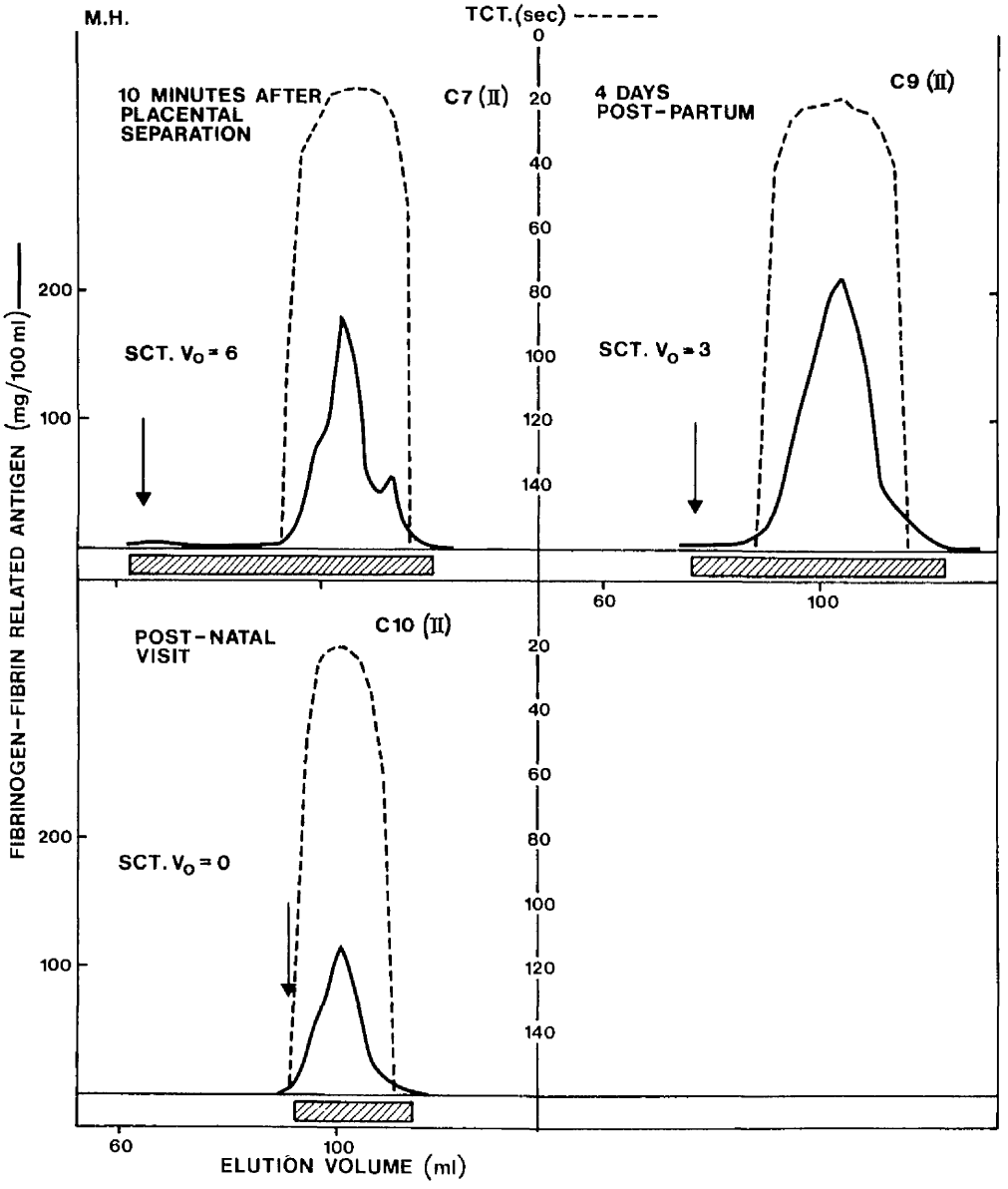


Figure 49b:- Results for Pre-eclamptic Patient 3 (M.H.).

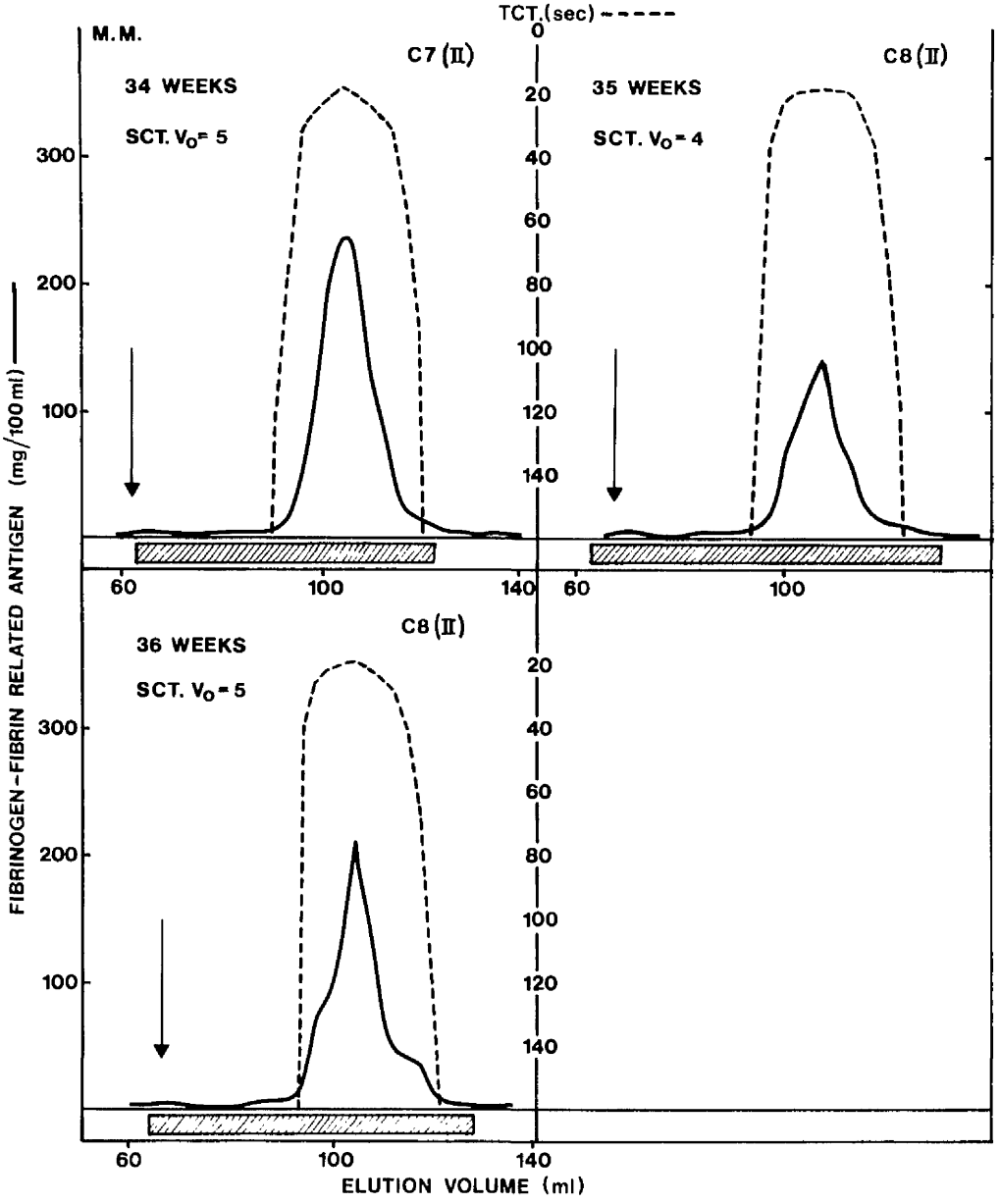


Figure 50a:- Results for Pre-eclamptic Patient 7 (M.M.).

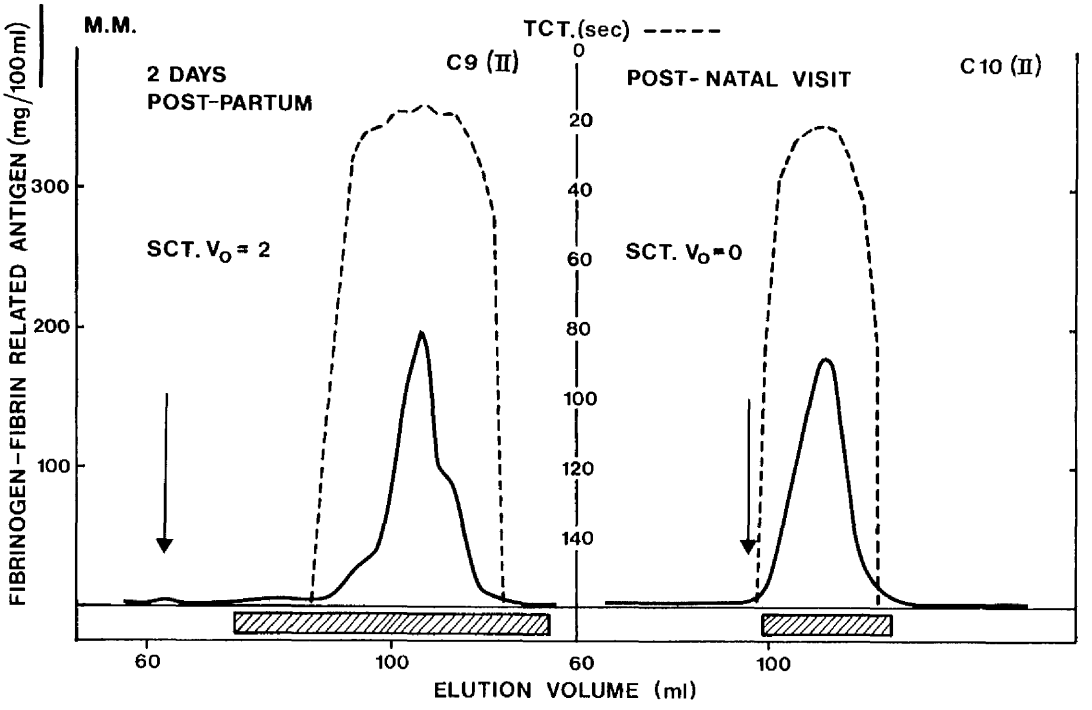


Figure 50b:- Results for Pre-eclamptic Patient 7 (M.M.).

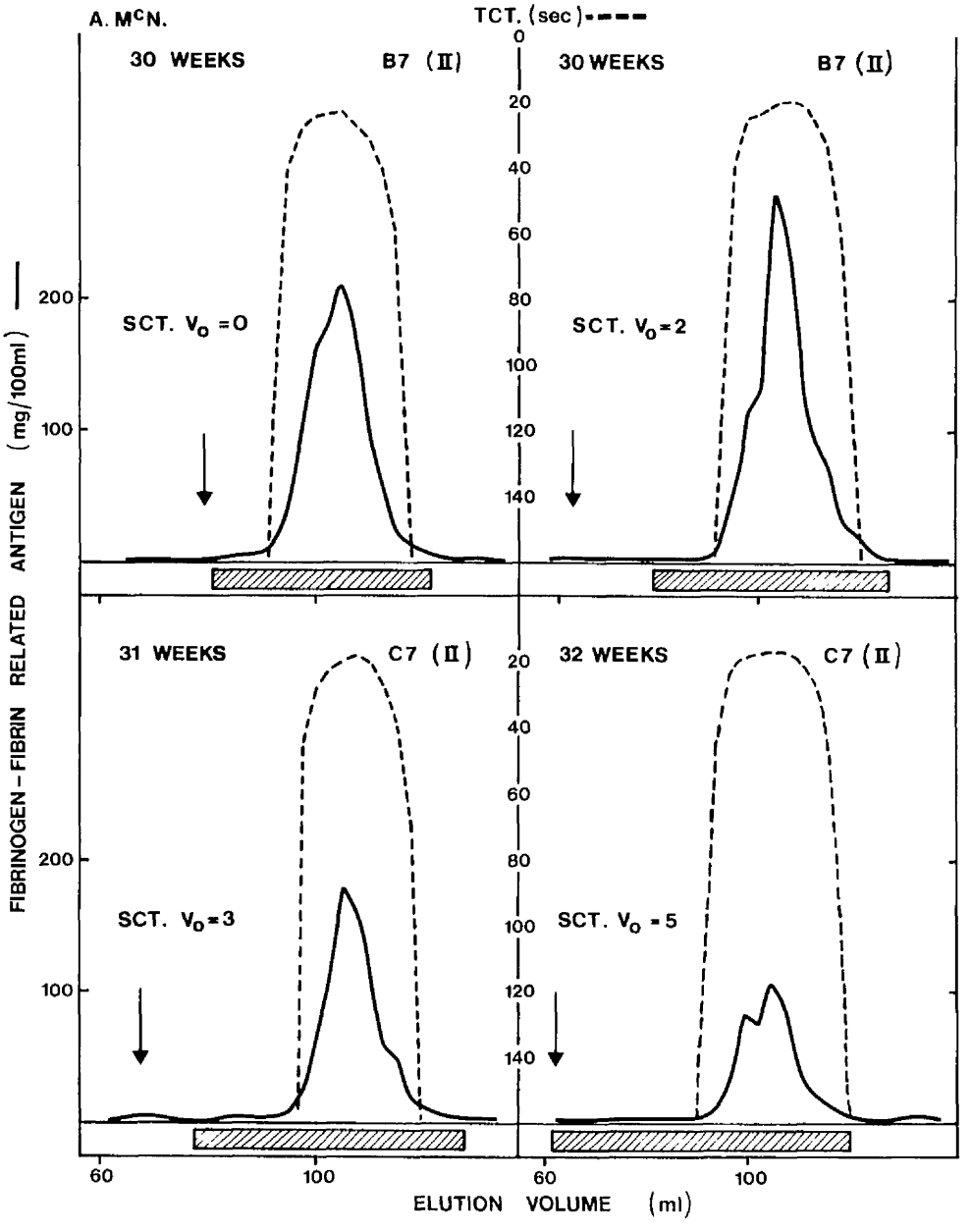


Figure 51a:- Results for Pre-eclamptic Patient 9 (A. McN.).

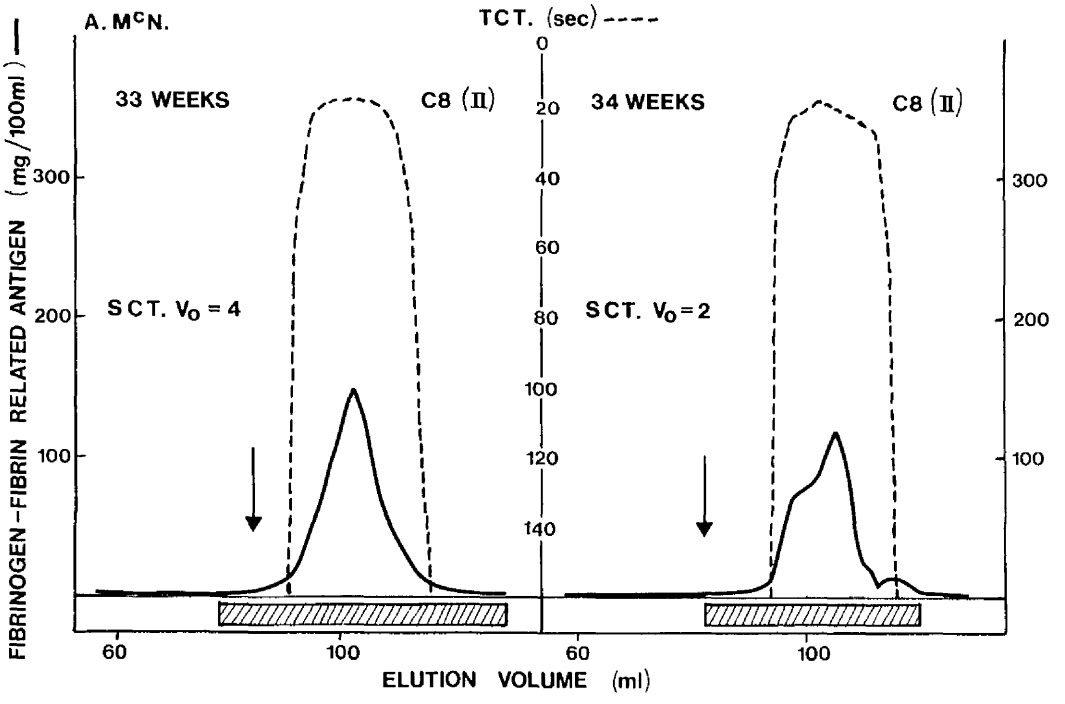


Figure 51b:- Results for Pre-eclamptic Patient 9 (A.McN.).

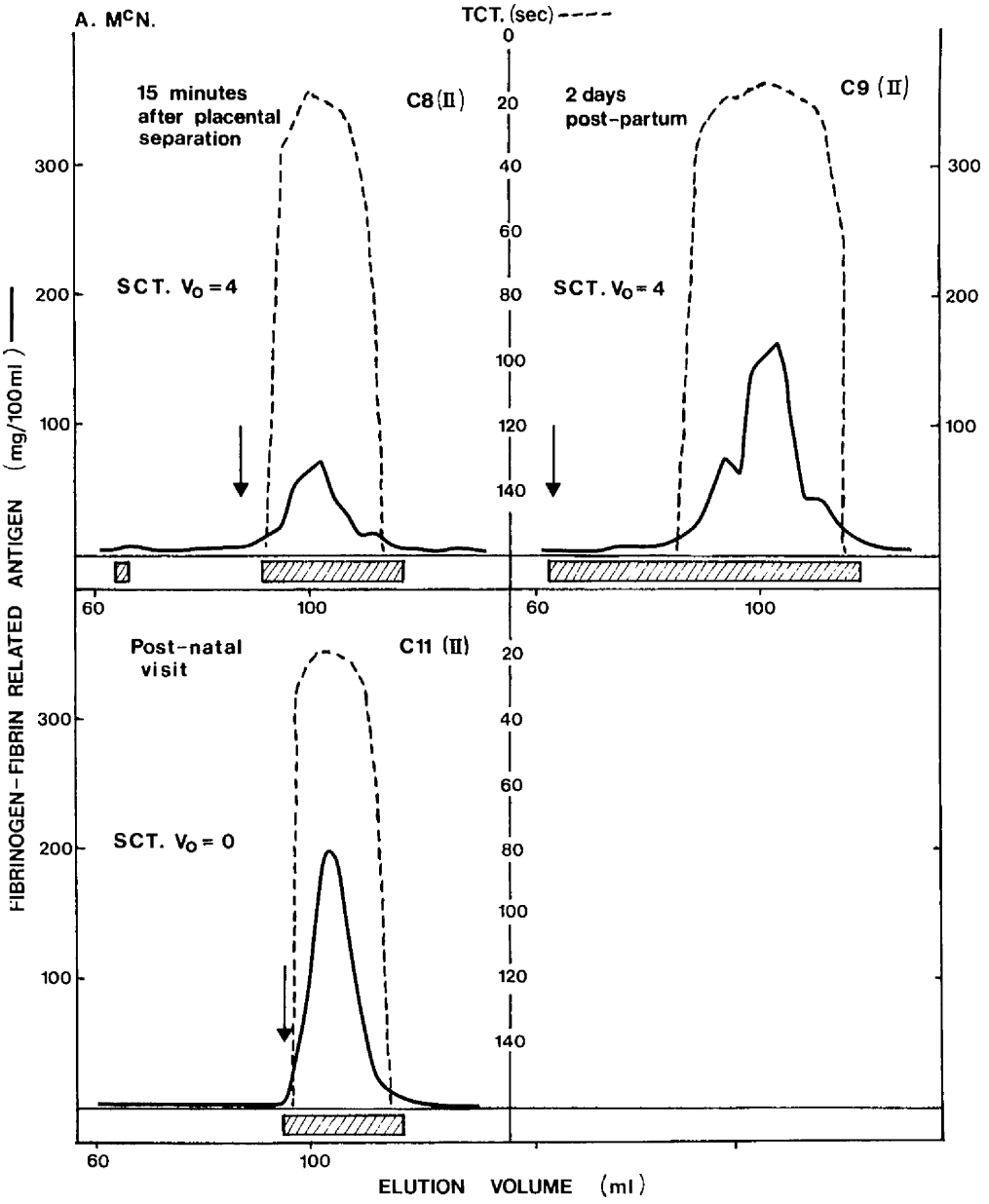


Figure 51c:- Results for Pre-eclamptic Patient 9 (A. McN.).

Figures 52-57

In this set of figures the sequential laboratory results are compared with the clinical findings for six pre-eclamptic patients (Nos. 3, 5, 7, 9, 10 and 11 respectively). Time is shown on the X axis. During the ante-natal period this is expressed as the gestational age (weeks) and post-partum as days, with a break in the scale before the post-natal visit (P.N.V.). The broken vertical line indicates delivery. Each figure is set out in the following way, starting from the top:-

(i) plasma fibrinogen (mg/100 ml)

(ii) serum fibrinogen-fibrin degradation products (FDP/fdp; in μg/ml) measured by the tanned red cell haemagglutination inhibition immuno-assay

(iii) staphylococcal clumping test titre at the void volume (SCT V_o; negative logarithm to base 2)

(iv) area m (percent total area)

(v) area p (percent total area)

(vi) quantitative proteinuria (Q.P.; g/24 hours)

(vii) diastolic blood pressure (D.B.P.; mmHg).

The stippled areas indicate the range of results (mean $\pm$ single standard deviation) found in normal single pregnancies (Tables 51 and 53).

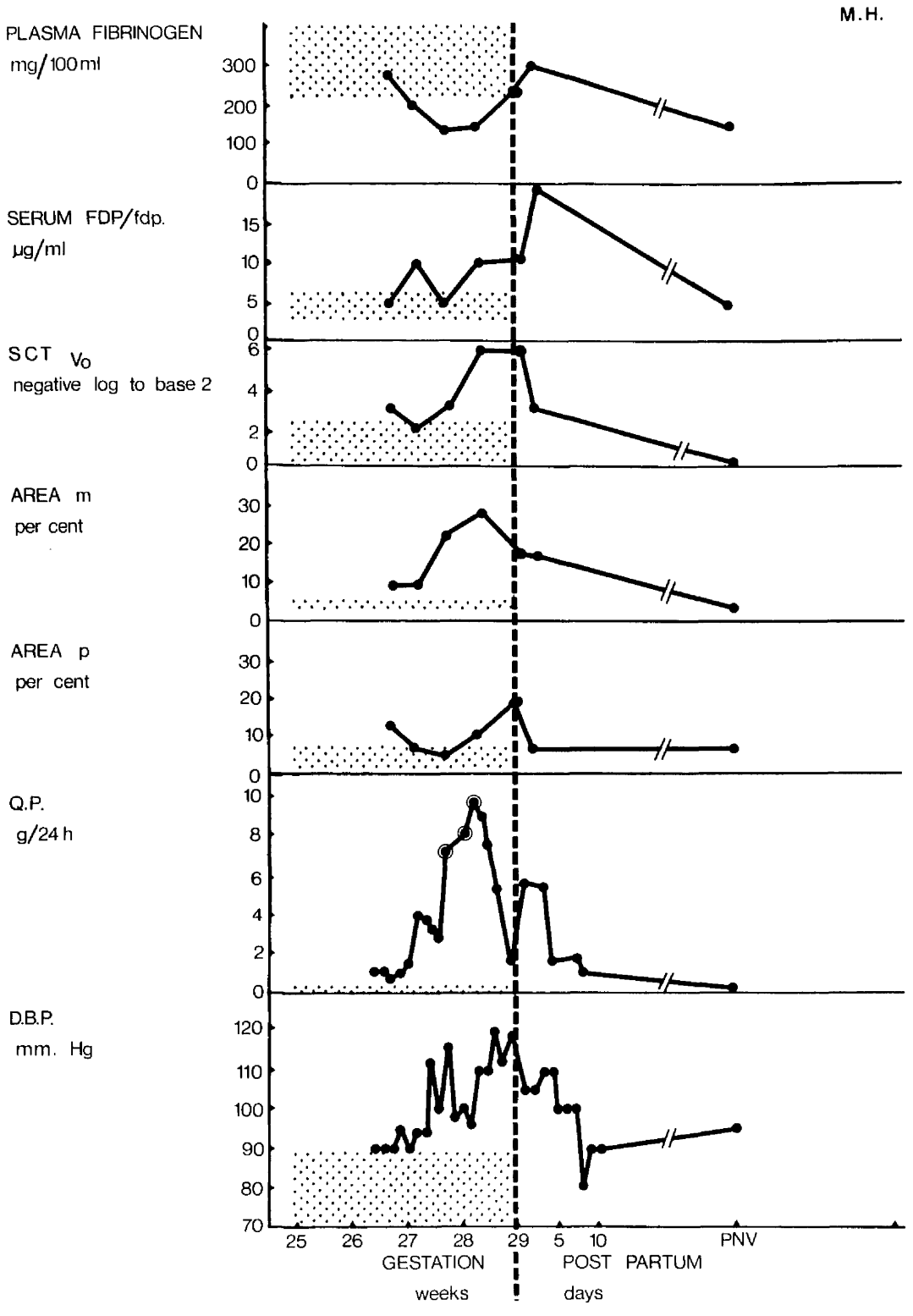


Figure 52:- Results for pre-eclamptic Patient 3 (M.H.).
The blood sample on the day of delivery was taken 10 minutes after placental separation.

◉ "interfering substance" present in urine.

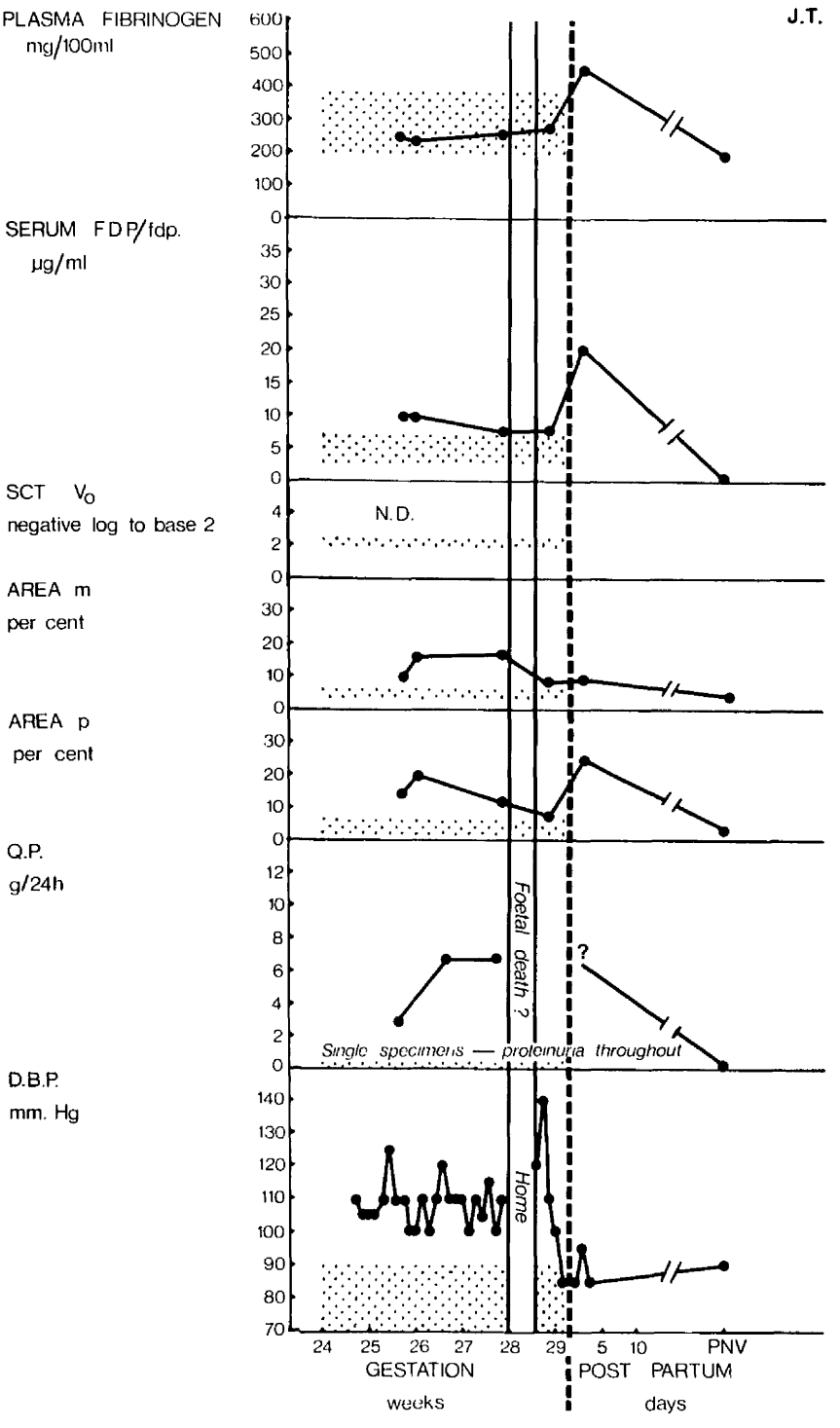


Figure 53:- Results for Pre-eclamptic Patient 5 (J.T.).

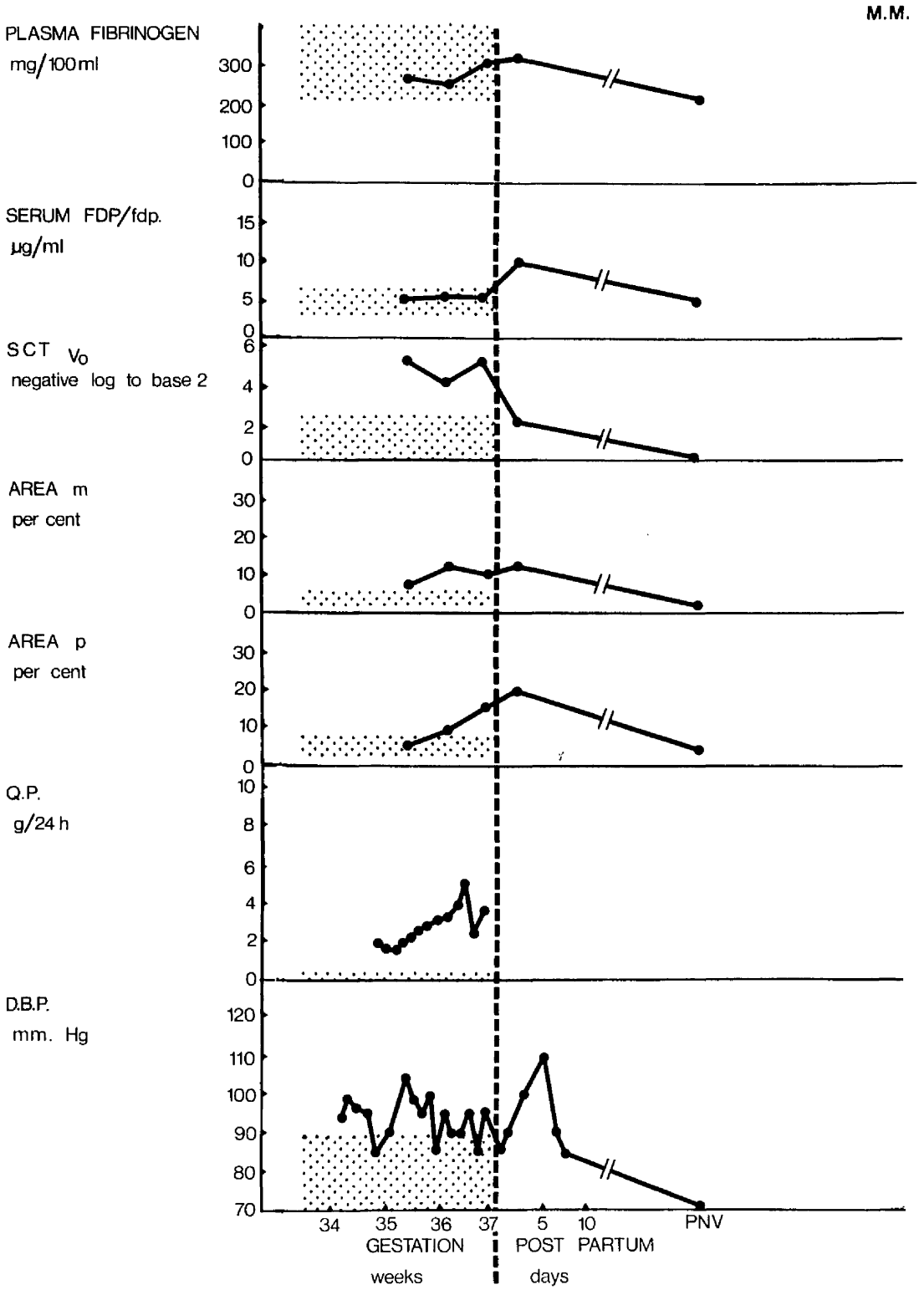


Figure 54:- Results for Pre-eclamptic Patient 7 (M. M.).

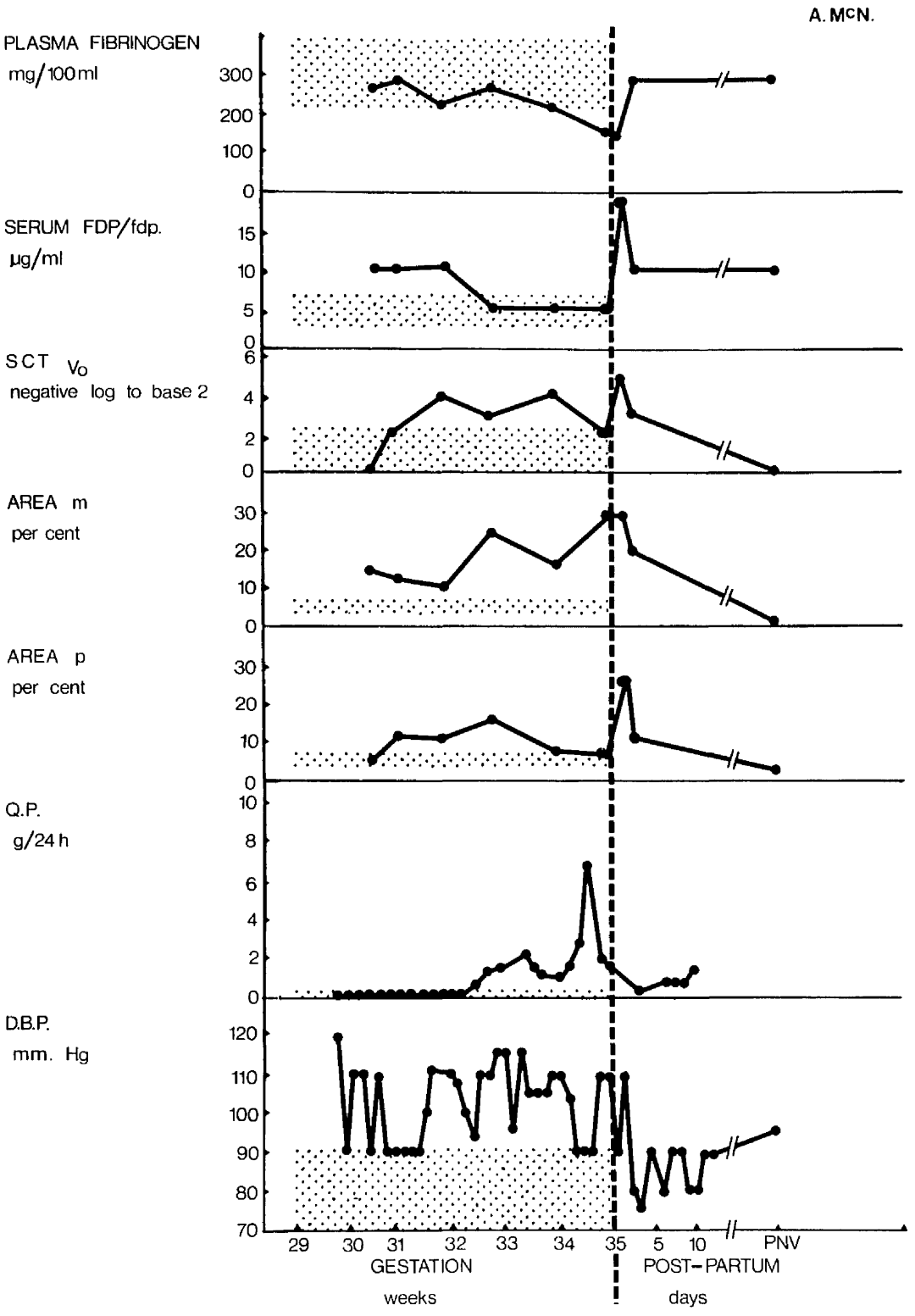


Figure 55:- Results for Pre-eclamptic Patient 9 (A. McN.). A blood sample was taken the day before delivery and also 15 minutes after placental separation.

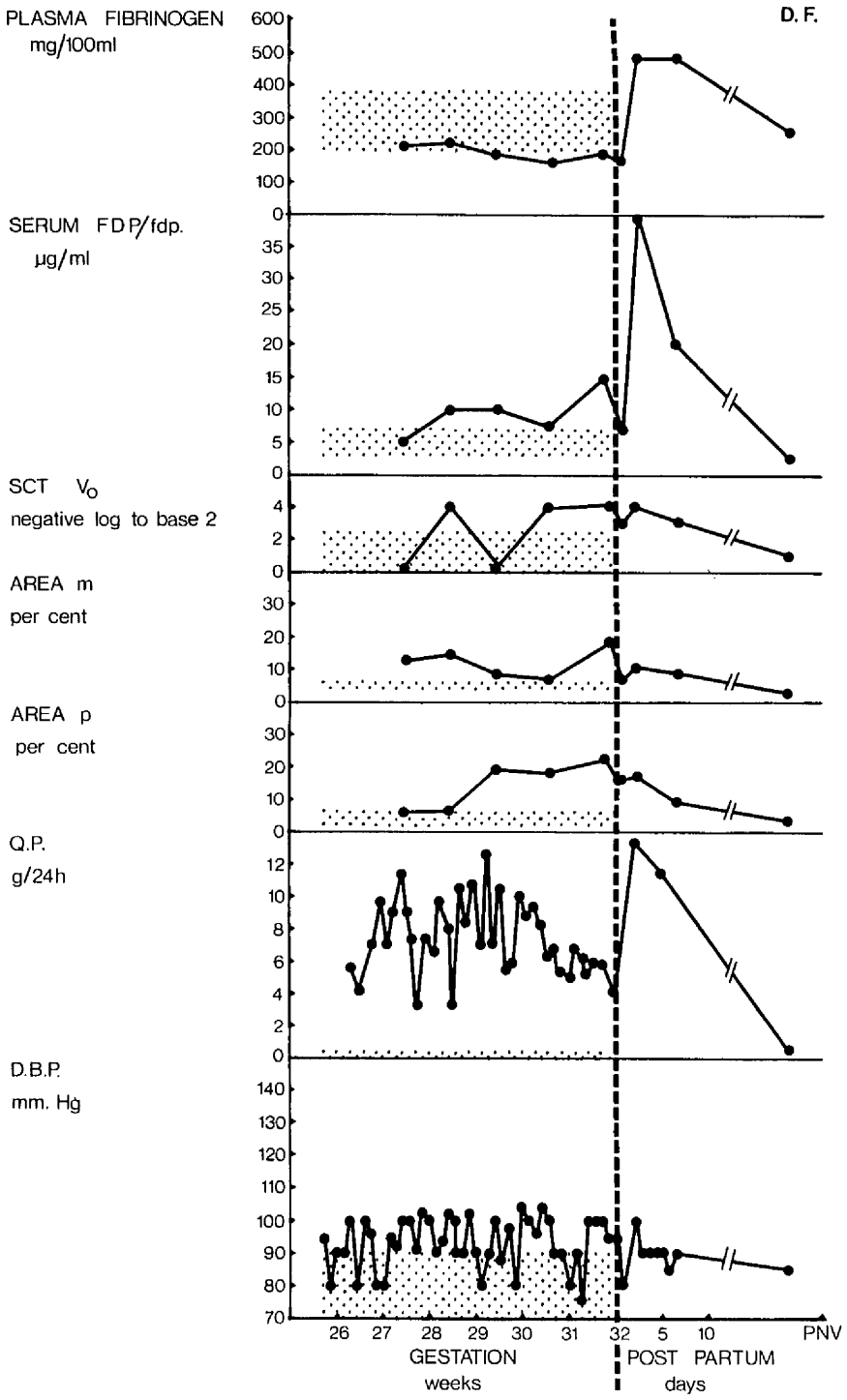


Figure 56:- Results for Pre-eclamptic Patient 10 (D. F.). The blood sample on the day of delivery was taken 5 minutes after placental separation.

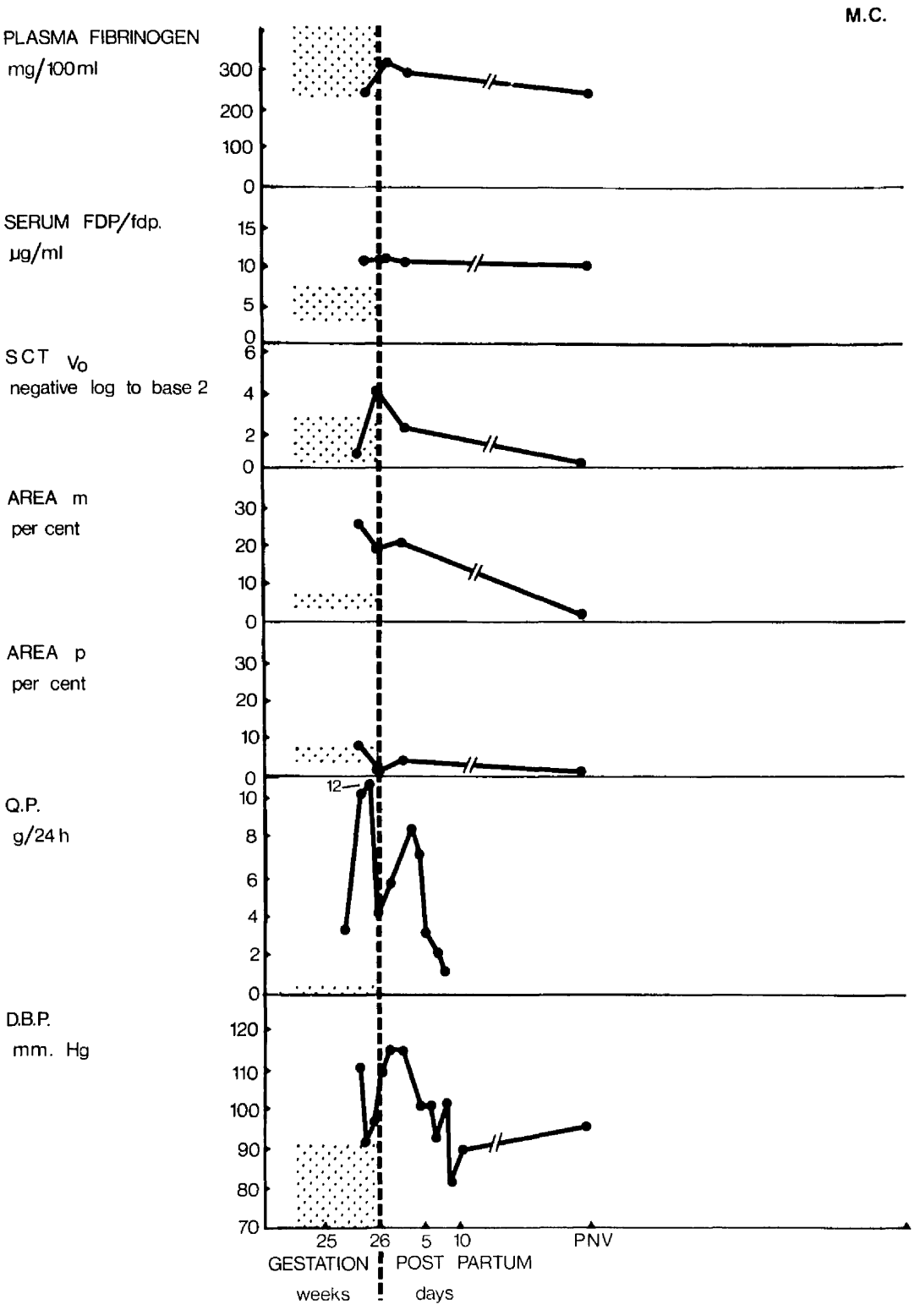


Figure 57:- Results for Pre-eclamptic Patient 1 (M.C.).

The blood sample on the day of delivery was taken following induction but before the onset of eclamptic seizures and delivery.

Figure 58a-d

In this set of figures the sequential results for area m and area p are shown together with the clinical index (Howie, Purdie, Begg et al., 1976) for four pre-eclamptic patients (Nos. 3, 7, 9 and 10 respectively). Time (as gestational age in weeks) is shown on the X axis. The broken vertical line indicates delivery. The area m (percent total area), area p (percent total area) and the clinical index (arbitrary units) results are shown on the Y axis.

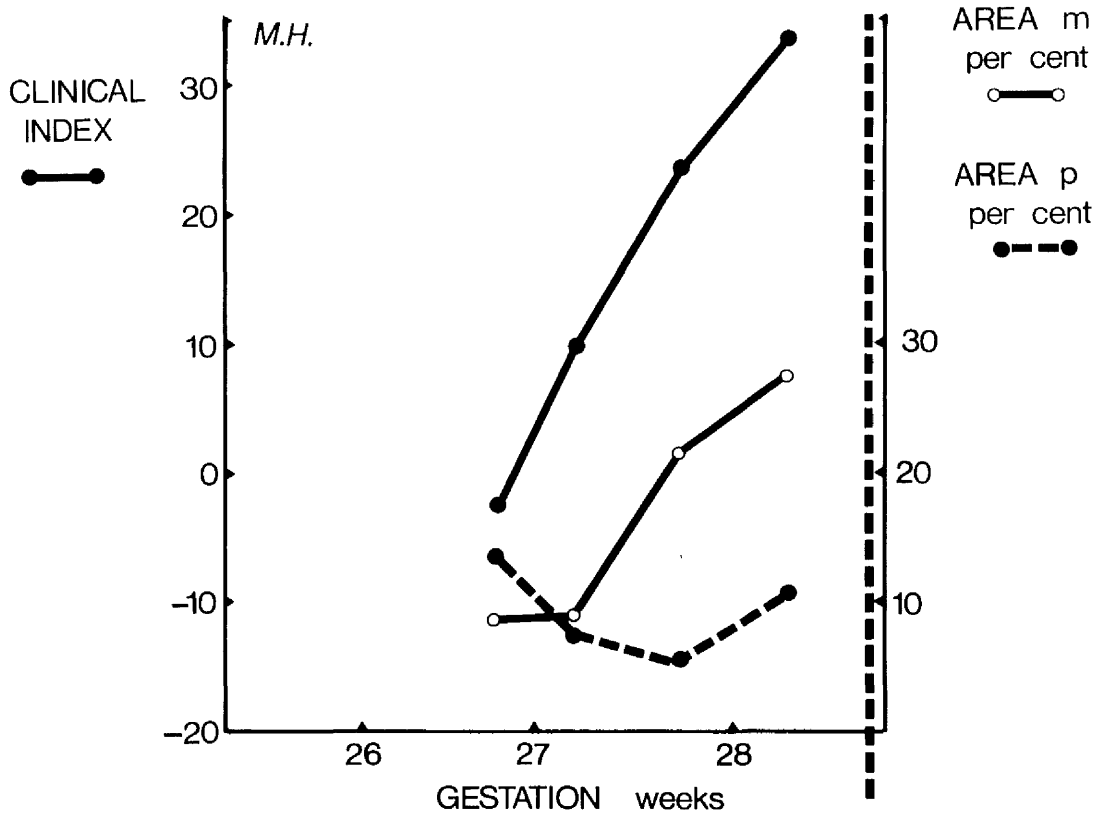


Figure 58a:- Results for Pre-eclamptic Patient 3 (M.H.).

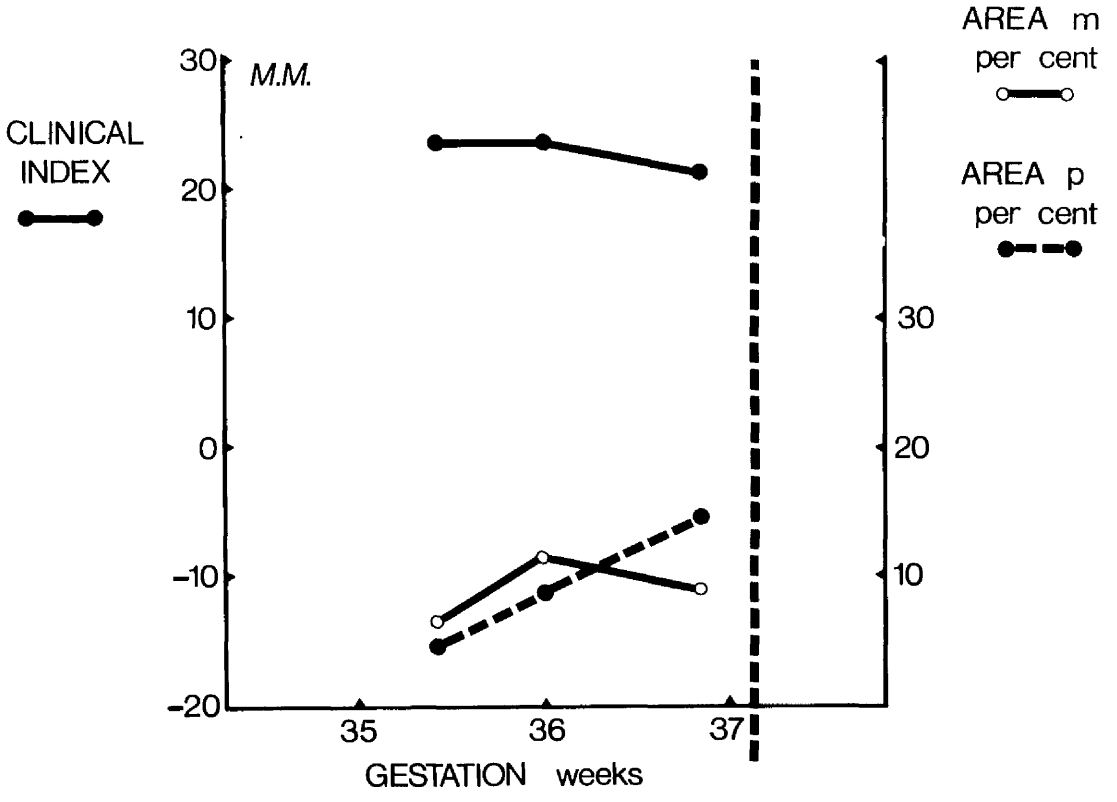


Figure 58b:- Results for Pre-eclamptic Patient 7 (M. M.).

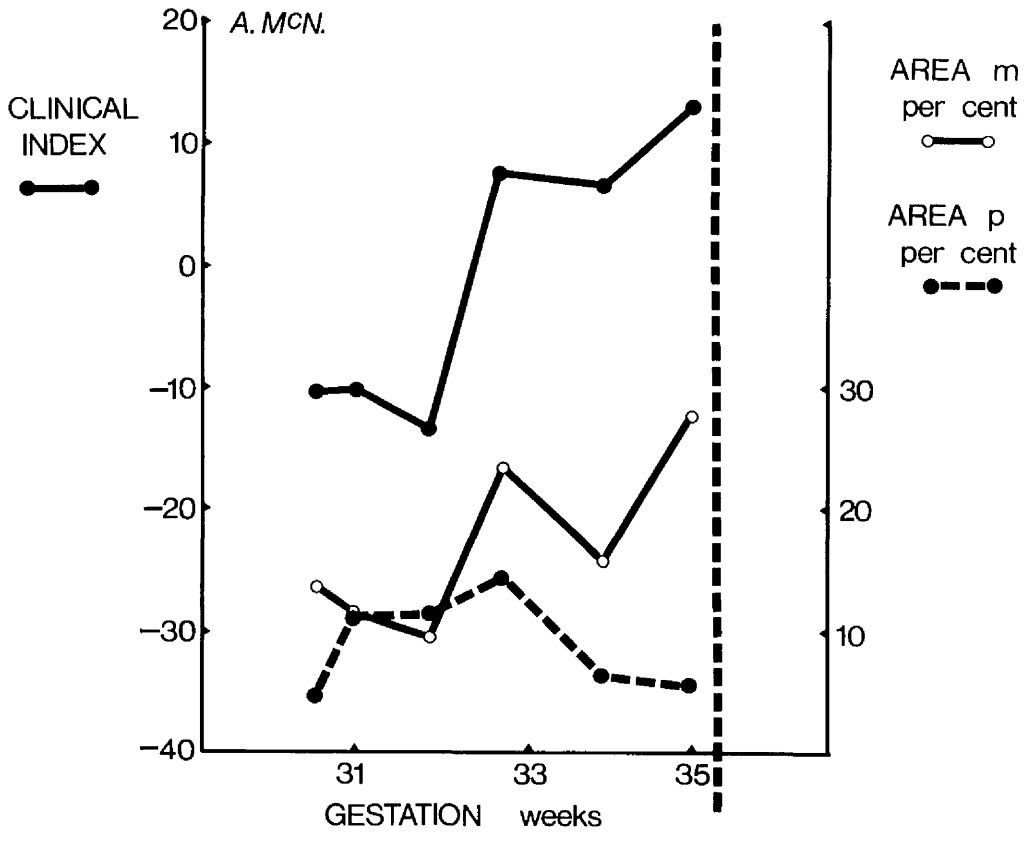


Figure 58c:- Results for Pre-eclamptic Patient 9 (A. McN.).

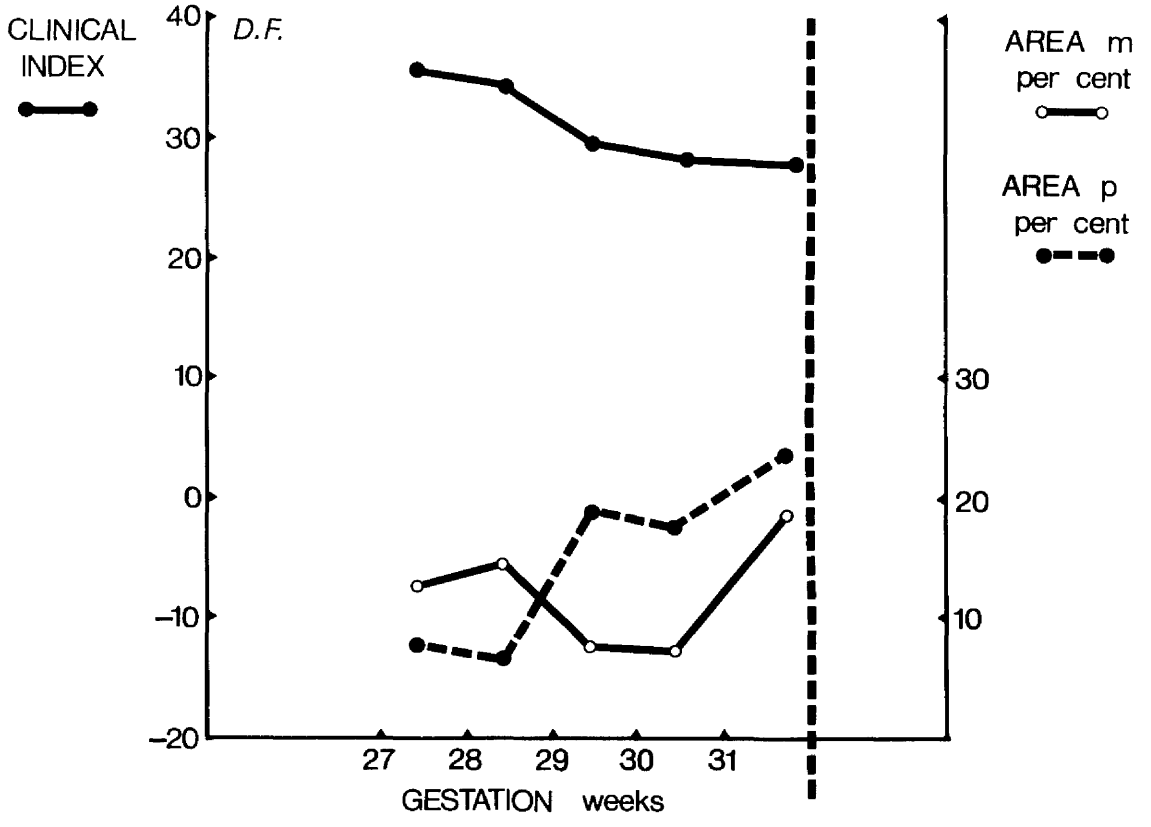


Figure 58d:- Results for Pre-eclamptic Patient 10 (D.F.).

Figure 59

The sequential results for area m, area p and the clinical index (Howie, Purdie, Begg et al., 1970) for a pregnant patient (T.A.) with chronic renal disease. Time (as gestational age in weeks) is shown on the X axis. The broken vertical line indicates the day of delivery. The area m (percent total area), area p (percent total area) and the clinical index (arbitrary units) results are shown on the Y axis.

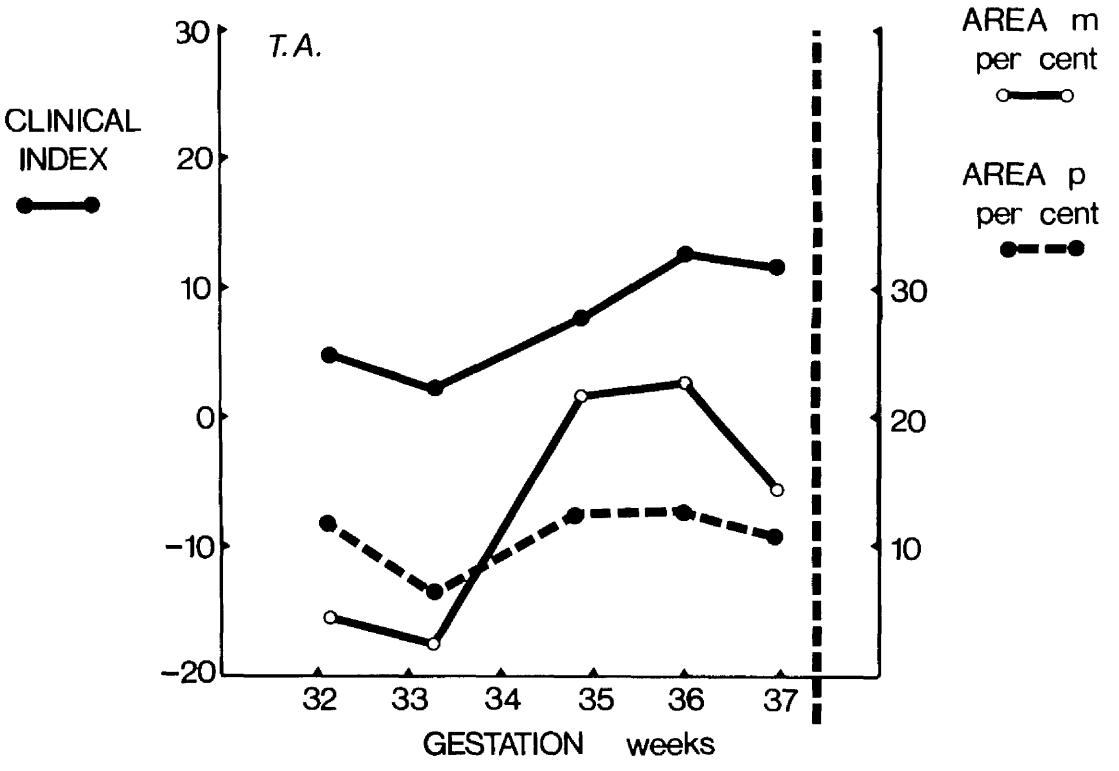

T.A.
CLINICAL INDEX
AREA m per cent
AREA p per cent
30
20
10
0
-10
-20
30
20
10
32
33
34
35
36
37
GESTATION weeks

Figure 59

Figures 60-63

In this set of figures the results for area m (percent total area, Figure 60), SCT titre at V_o (negative logarithm to base 2, Figure 61), area p (percent total area, Figure 62) and serum fibrinogen-fibrin degradation product (serum FDP/fdp; μg/ml) measured by the tanned red cell haem-agglutination inhibition immunoassay (Figure 63) are shown for the main groups of patients studied in this thesis. In each case the scatter of results is shown together with the mean ± single standard deviation. Note that in these figures the zero on the X axis is shown on the right.

I.V. - intravenous.

Pre-eclampsia Total - results in Chapter VI.4.

Pre-eclampsia Group I - results in Chapter V.4.

I.U.G.R. - intrauterine growth retardation.

Normal on "pill" - normotensive women taking oestrogen-containing oral contraceptive drugs.

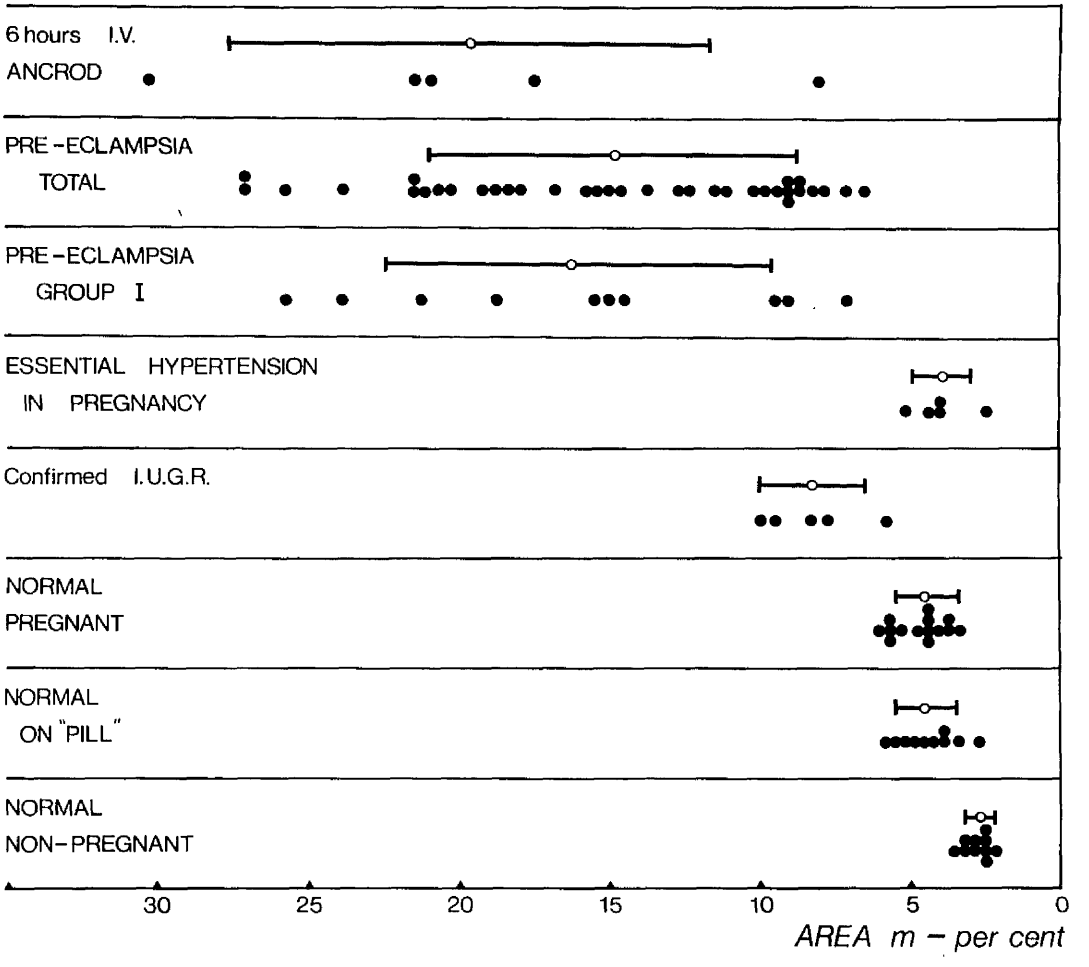


Figure 60:- Area m (percent total area) Results.

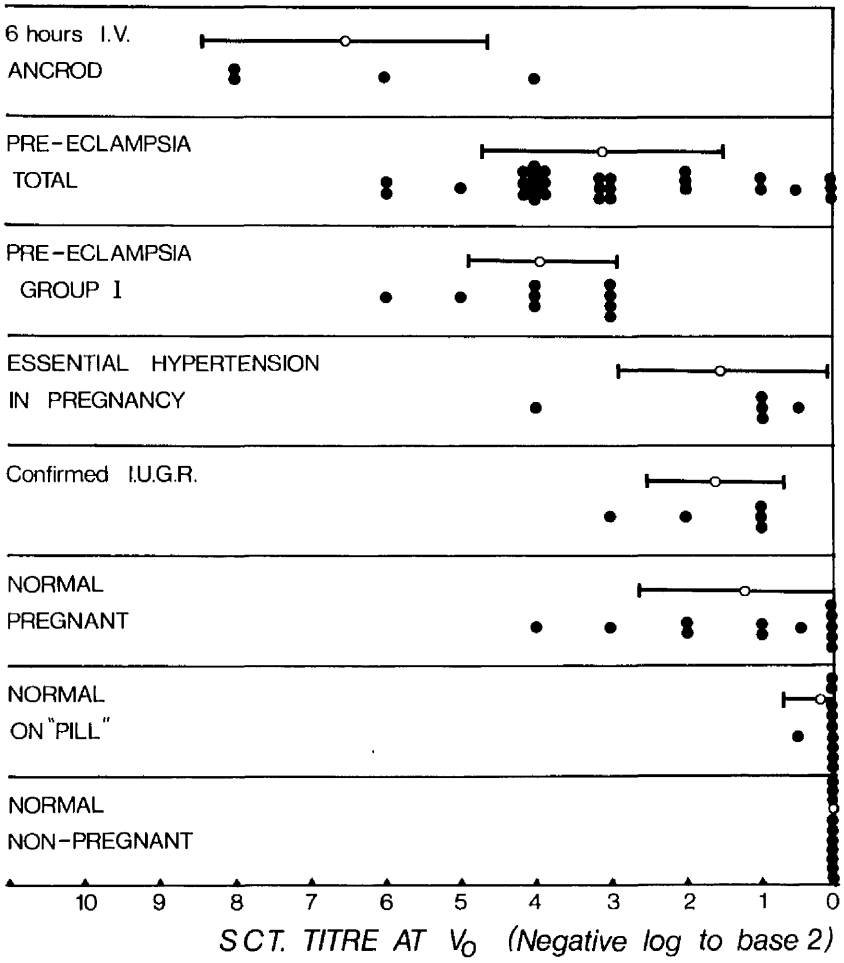


Figure 61:- SCT titre at V_0 (negative logarithm to base 2) Results.

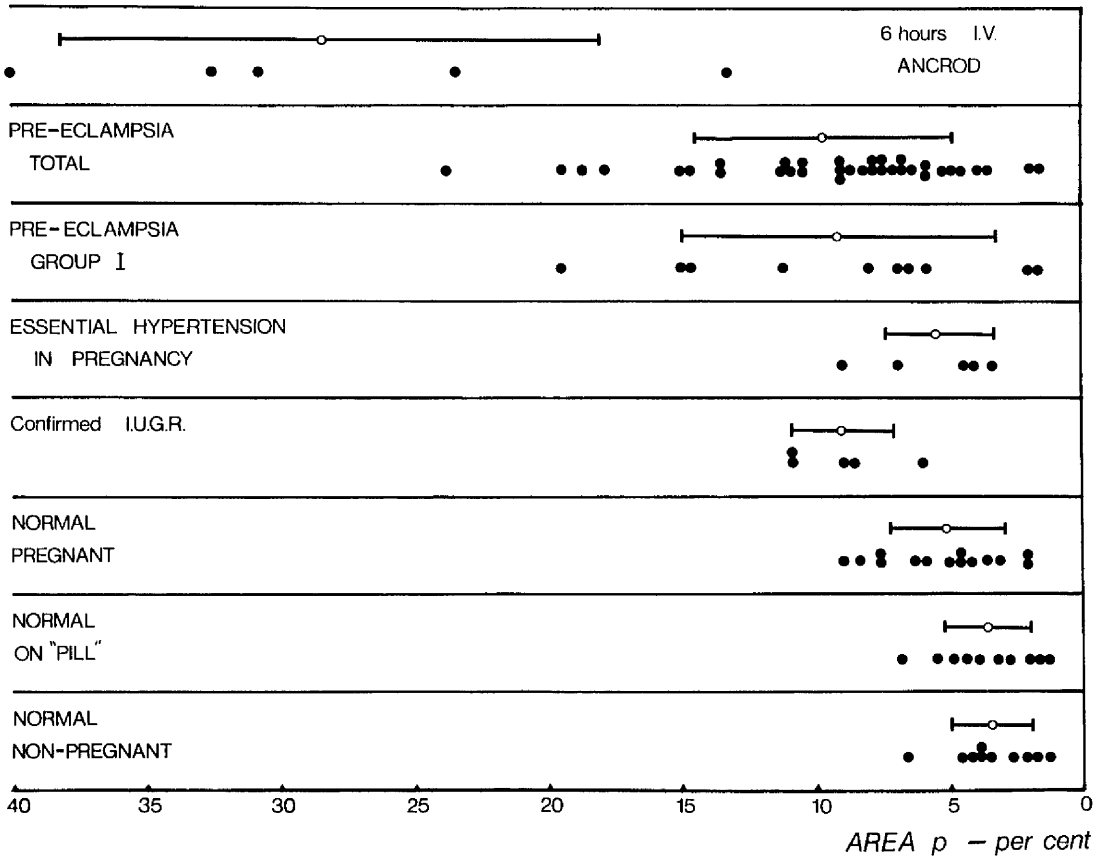


Figure 62:- Area p (percent total area) Results.

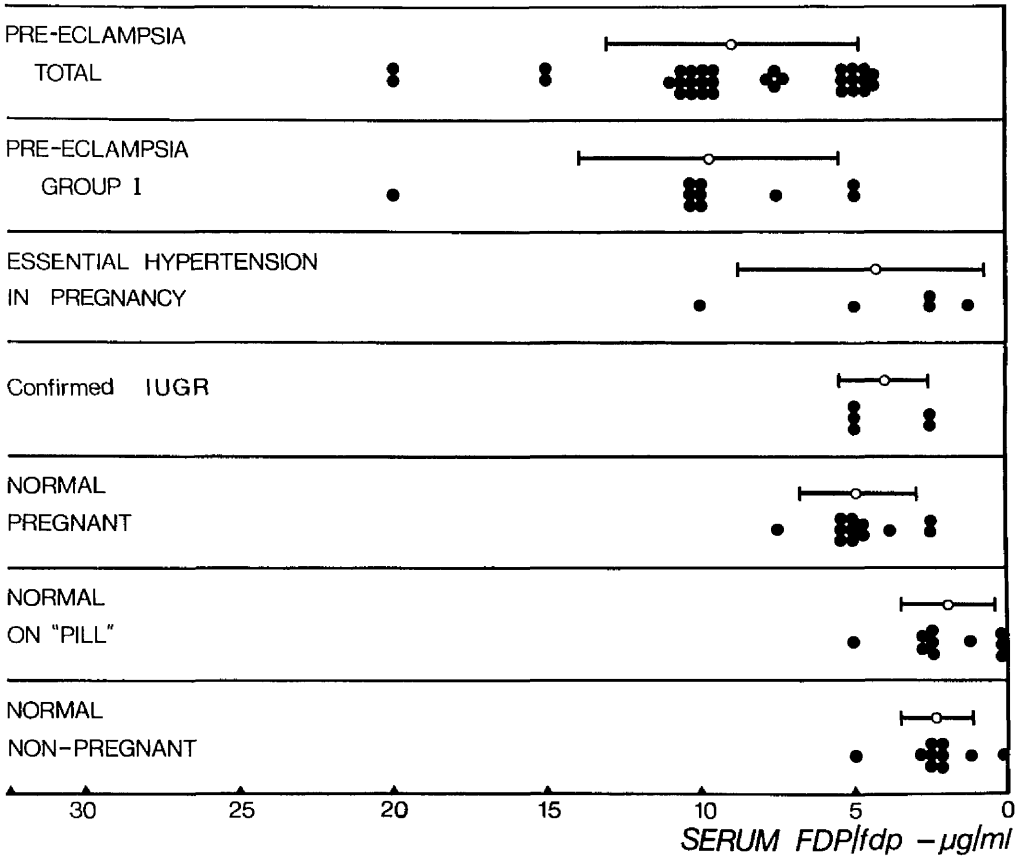


Figure 63:- Serum FDP/fdp (µg/ml) Results.

Figure 64a

In this figure the fibrinogen-fibrin related (FR-) antigen ratio is plotted on the X axis and the area m (percent total area) result for the same samples on the Y axis. The results shown are from the four main groups of subjects studied in the thesis (i. e. non-pregnant control women, normotensive women taking oestrogen-containing oral contraceptive drugs, normal pregnant women and pre-eclamptic patients).

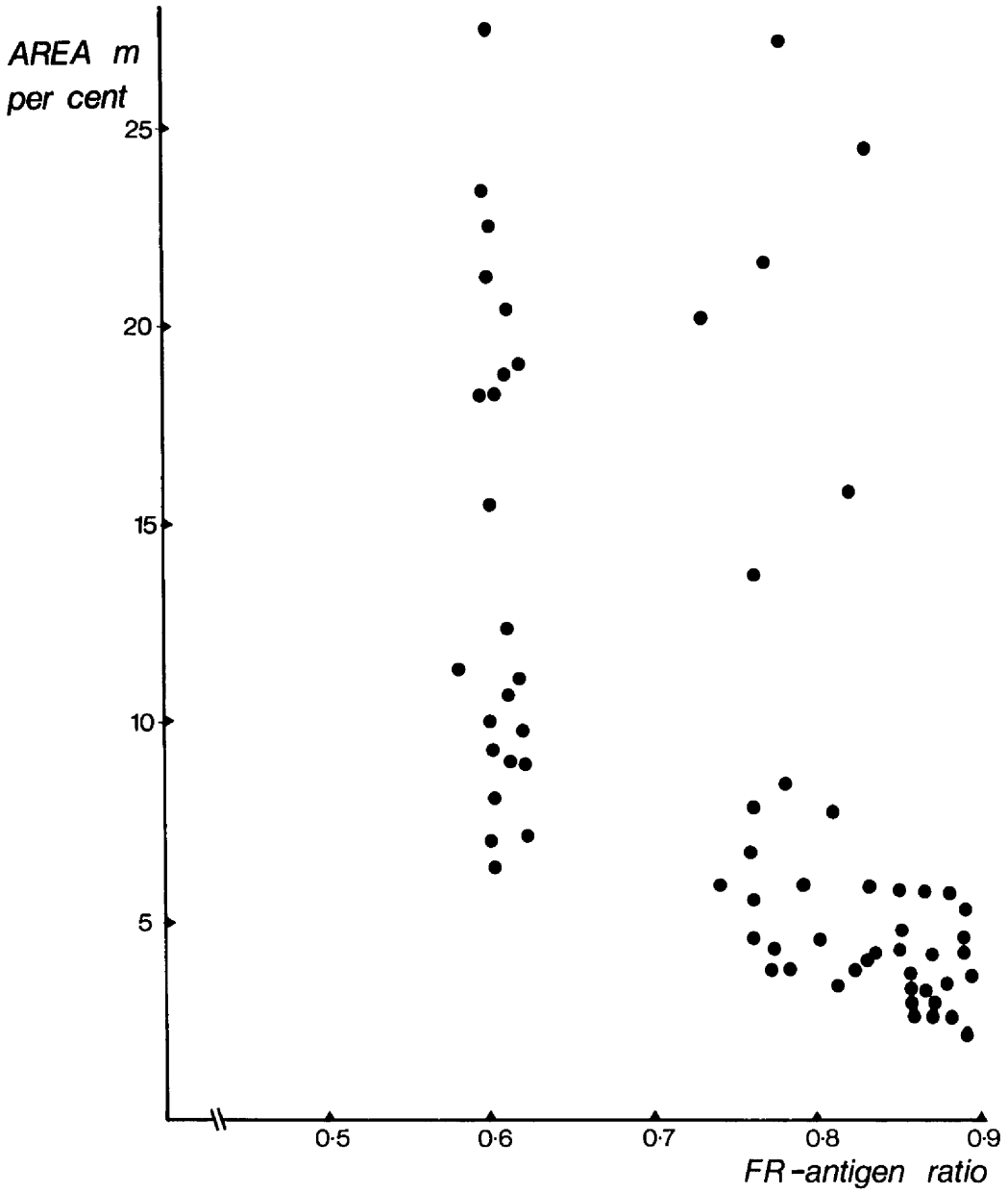


Figure 64a

Figure 64b

This figure shows the same data as Figure 64a, with the addition of the areas occupied by the results from each separate group. It can be seen that the four groups can be separated using these two measurements in combination.

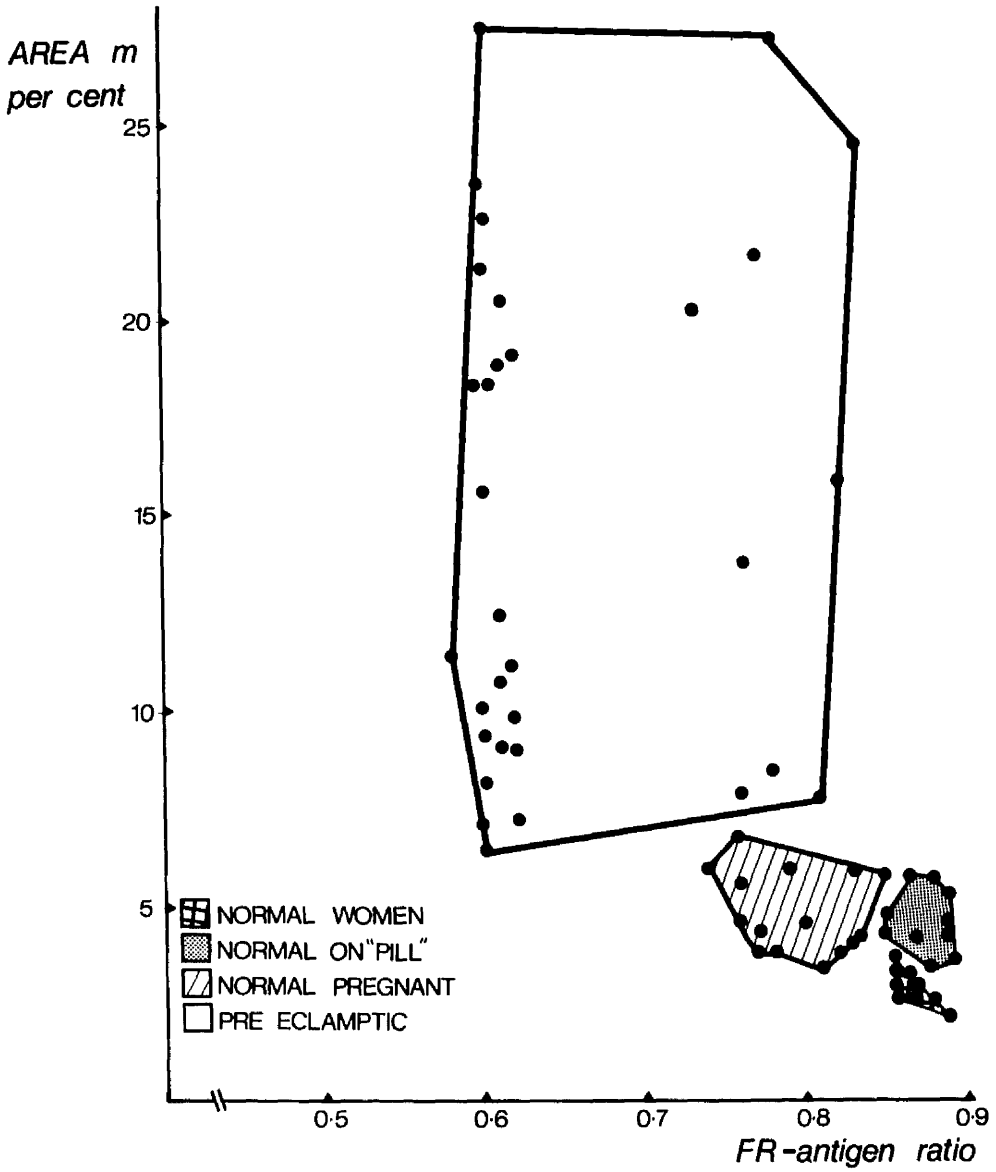

AREA m
per cent
25
20
15
10
5
NORMAL WOMEN
NORMAL ON "PILL"
NORMAL PREGNANT
PRE ECLAMPTIC
0·5
0·6
0·7
0·8
0·9
FR-antigen ratio

Figure 64b

Figure 65

This figure shows the theory behind Figure 66. Area p (percent total area) is plotted on the X axis and area m (percent total area) on the Y axis. The line of complete identity is drawn through all points having the same area m and area p results. When the results from a sample are plotted on this graph those lying on the line will have "balanced coagulation and fibrinolysis/fibrinogenolysis". Any sample found to lie above the dividing line will have a predominant abnormality of "coagulation". Similarly, any sample lying below the dividing line will have a predominant abnormality of fibrinolysis/fibrinogenolysis. The further the sample result lies from zero the greater the degree of abnormality.

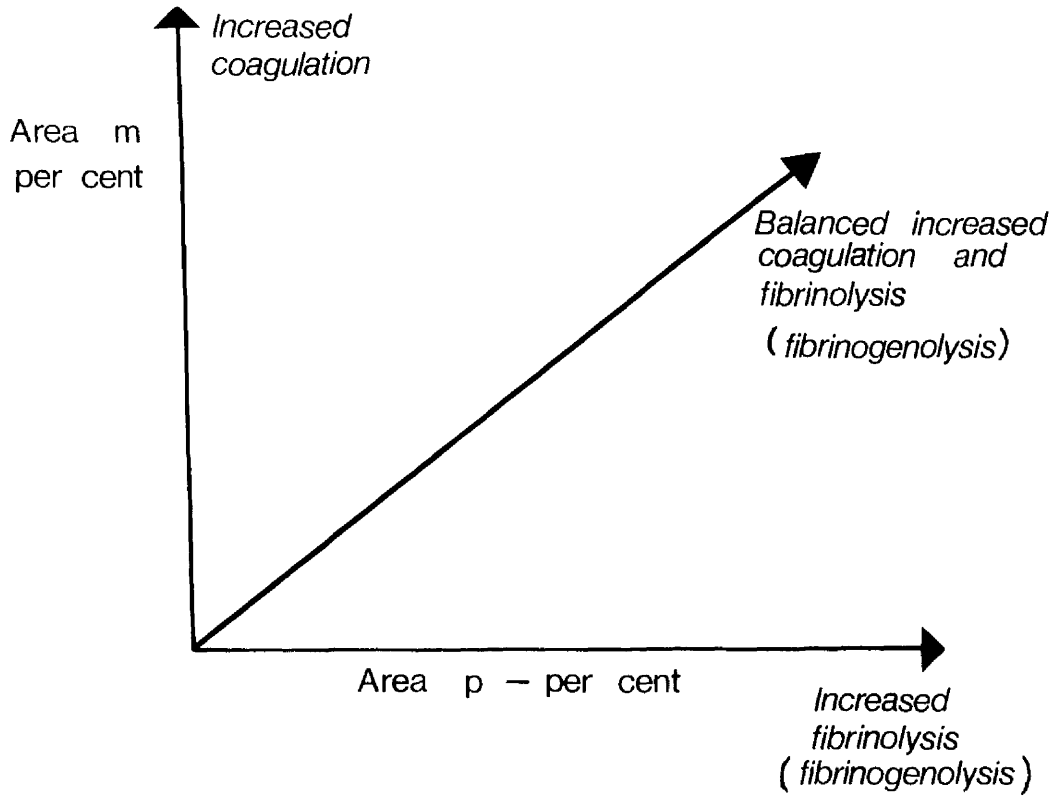

Increased coagulation
Area m per cent
Balanced increased coagulation and fibrinolysis
(fibrinogenolysis)
Area p – per cent
Increased fibrinolysis
(fibrinogenolysis)

Figure 65

Figure 66a

This figure is laid out in the same way as Figure 65, i.e. area p (percent total area) is shown on the X axis and area m (percent total area) on the Y axis. The results shown are from the four main groups of subjects studied in the thesis (i.e. non-pregnant control women, normotensive women taking oestrogen-containing oral contraceptive drugs, normal pregnant women and pre-eclamptic patients).

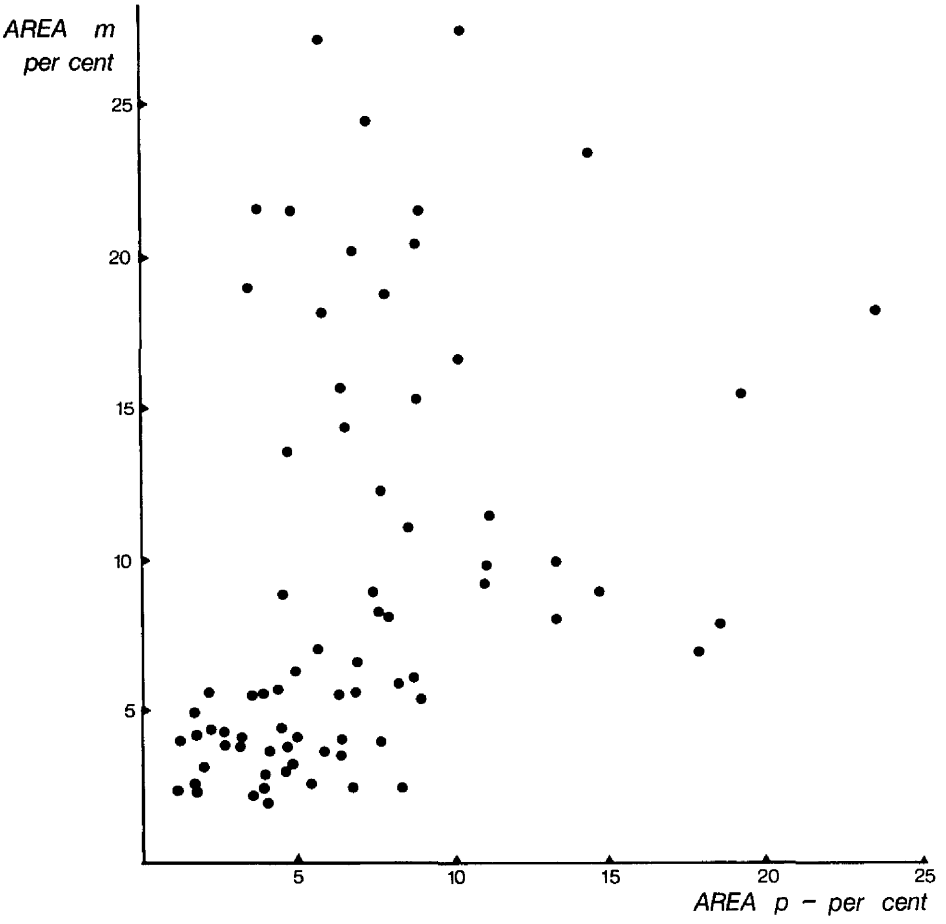


Figure 66a

Figure 66b

This figure shows the same data as Figure 66a, with the addition of the areas occupied by the results from each separate group. The line of complete identity (area m = area p) has also been added (see Figure 65).

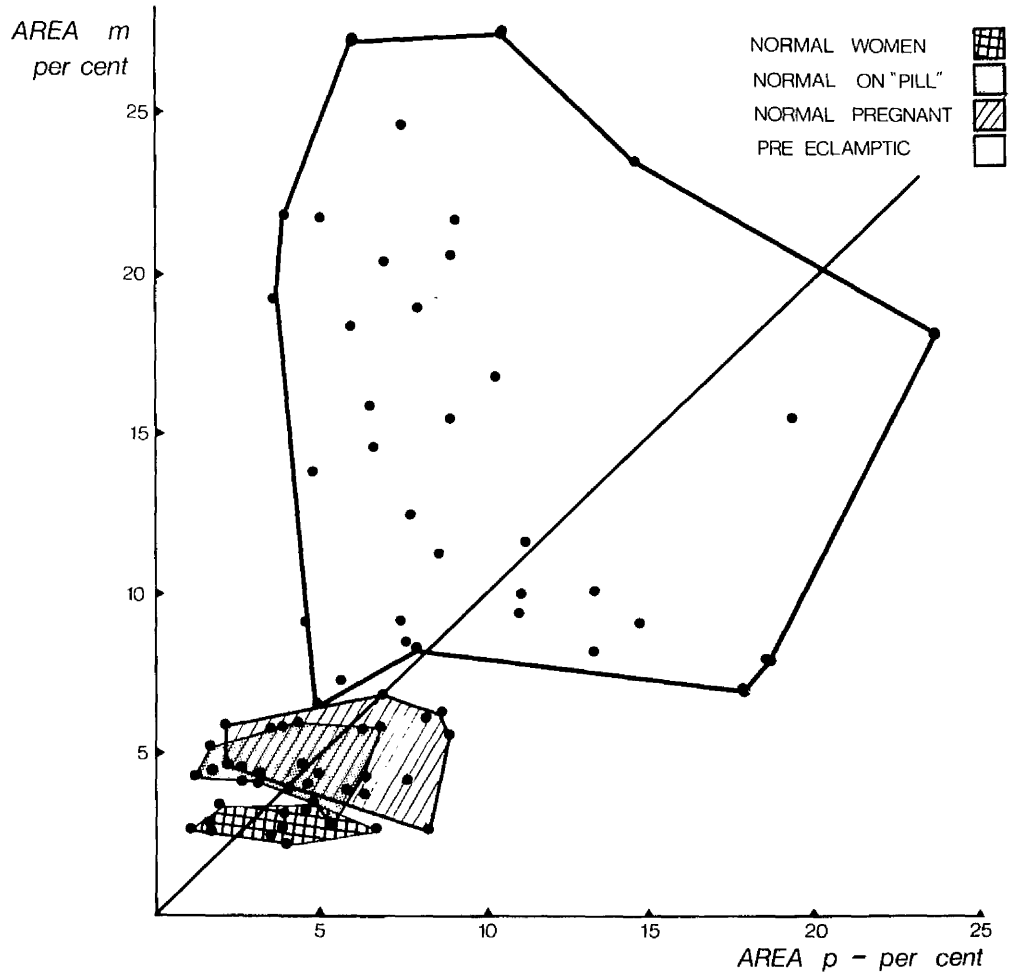

AREA m
per cent
25
20
15
10
5
NORMAL WOMEN
NORMAL ON "PILL"
NORMAL PREGNANT
PRE ECLAMPTIC
5
10
15
20
25
AREA p - per cent

Figure 66b

Figure 67

The plasma fibrinogen chromatography results (Figures 60-62 and Table 80) show a range of abnormality (⟷). This may reflect the underlying type of intravascular coagulation (shown in the boxes). No cases of eclampsia have been studied during the stage of convulsions and coma, but a case report has shown high soluble levels (Graeff and von Hugo, 1972).

D.I.C. - disseminated intravascular coagulation

I.U.G.R. - intrauterine growth retardation

"Pill" - normotensive women taking oestrogen-containing oral contraceptive drugs.

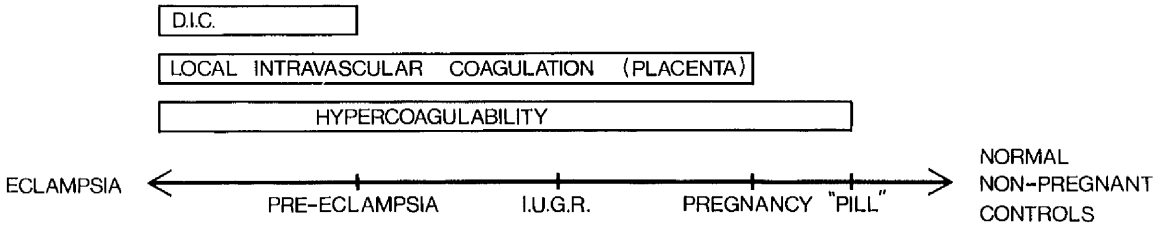

D.I.C.
LOCAL INTRAVASCULAR COAGULATION (PLACENTA)
HYPERCOAGULABILITY
ECLAMPSIA
PRE-ECLAMPSIA
I.U.G.R.
PREGNANCY "PILL"
NORMAL
NON-PREGNANT
CONTROLS

Figure 67

Figure 68

An adaptation of the "inner vicious circle of pre-eclampsia and eclampsia" of Page (1972) is shown incorporating the formation of soluble fibrinogen-fibrin complexes and several of the obstetrical conditions related to pre-eclampsia studied in this thesis.

IUGR - intrauterine growth retardation
DIC - disseminated intravascular coagulation
GFR - glomerular filtration rate

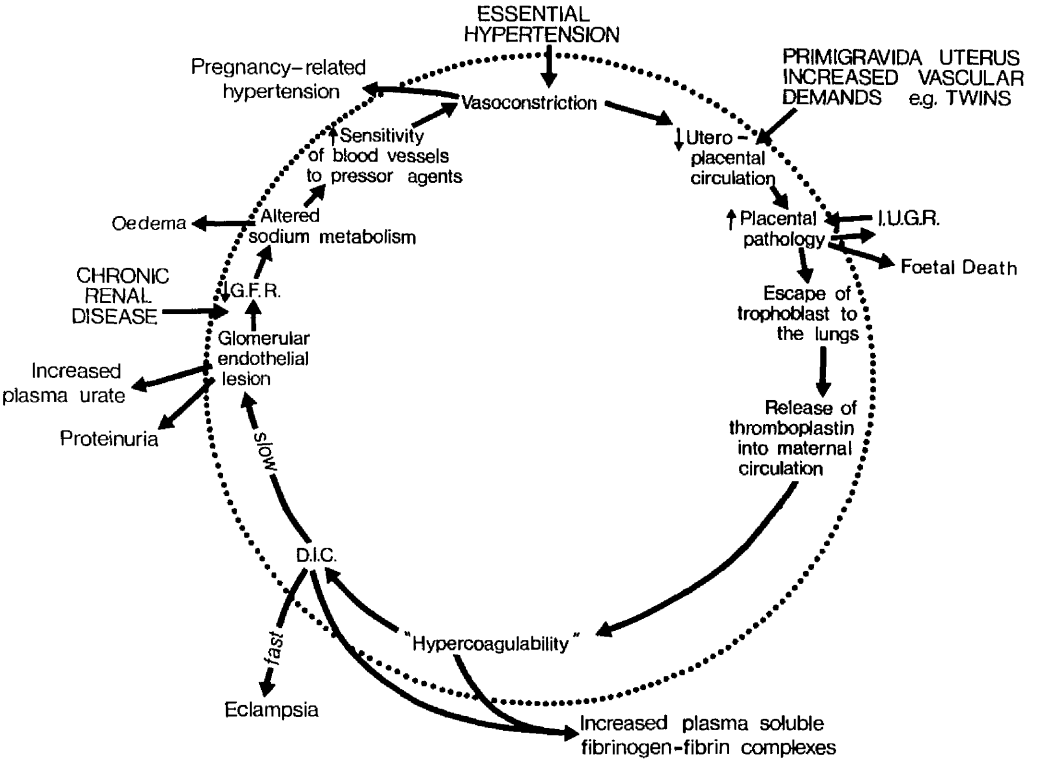

ESSENTIAL HYPERTENSION
Pregnancy-related hypertension
Vasoconstriction
PRIMIGRAVIDA UTERUS INCREASED VASCULAR DEMANDS e.g. TWINS
Sensitivity of blood vessels to pressor agents
Utero-placental circulation
Oedema
Altered sodium metabolism
Placental pathology
I.U.G.R.
Foetal Death
CHRONIC RENAL DISEASE
G.F.R.
Escape of trophoblast to the lungs
Glomerular endothelial lesion
Increased plasma urate
Proteinuria
Release of thromboplastin into maternal circulation
slow
D.I.C.
"Hypercoagulability"
fast
Eclampsia
Increased plasma soluble fibrinogen-fibrin complexes

Figure 68

Figures 69 and 70

In these figures the sequential laboratory results are compared with the clinical findings for two patients (R.S. and J.W.) who were considered to be "at risk" of developing pre-eclampsia and who both received a form of anti-thrombotic therapy. Time is shown on the X axis and is expressed as gestational age (weeks). The broken vertical line indicates delivery. The duration of anti-thrombotic therapy is shown by the solid vertical lines. The following results are shown starting from the top:-

(i) plasma fibrinogen (mg/100 ml)

(ii) serum fibrinogen-fibrin degradation products (FDP/fdp; in μg/ml), measured by the tanned red cell haem-agglutination inhibition immunoassay

(iii) staphylococcal clumping test titre at the void volume (SCT V_o; negative logarithm to base 2)

(iv) area m (percent total area)

(v) area p (percent total area)

(vi) quantitative proteinuria (Q.P.; g/24 hours)

(vii) diastolic blood pressure (D.B.P.; mmHg)

The stippled areas indicate the range of results (mean ± one standard deviation) found in normal single pregnancies (Figure 69) and twin pregnancies (Figure 70) - see Tables 51-54.

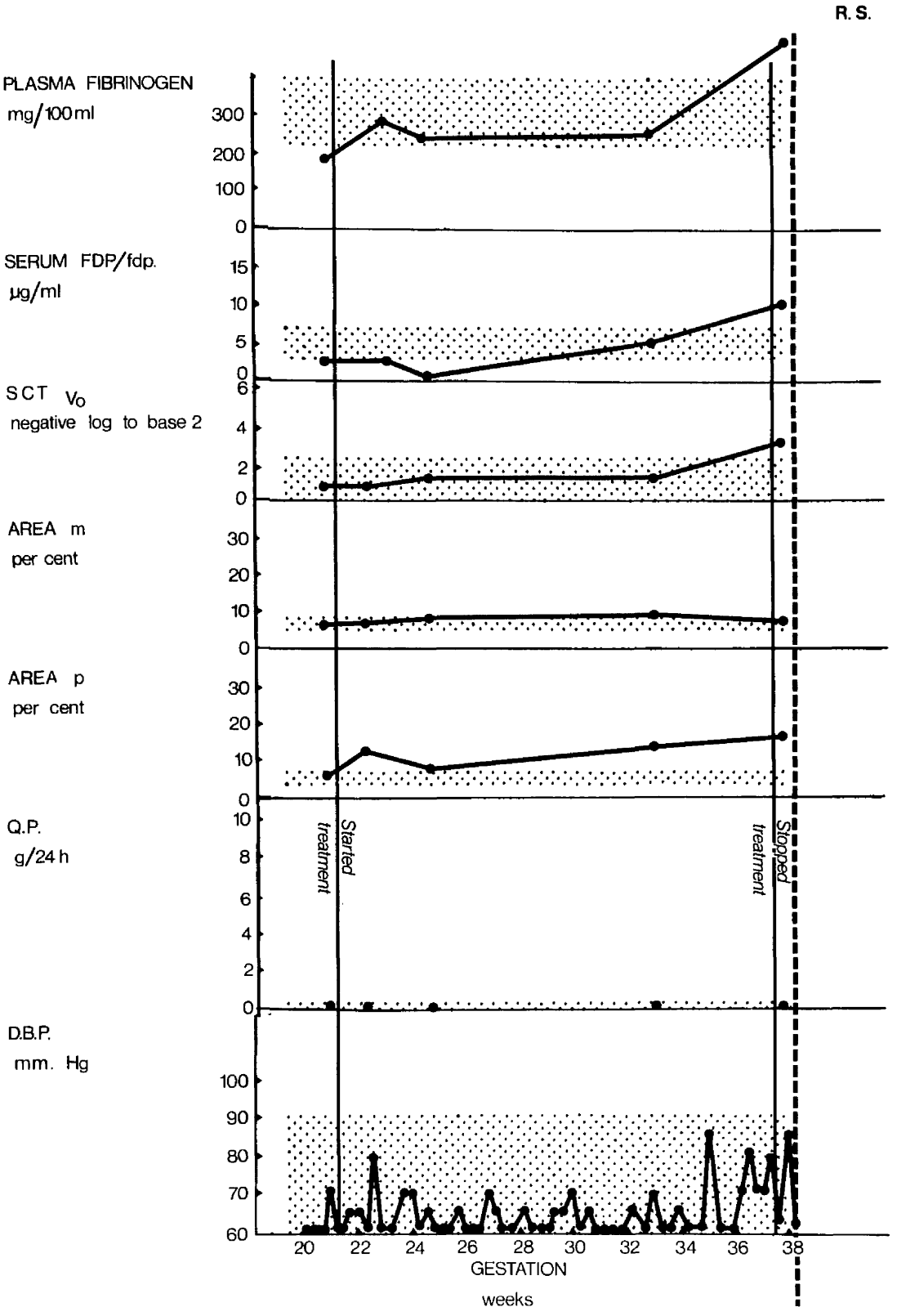


Figure 69:- Results for "High Risk" Patient (R. S.).

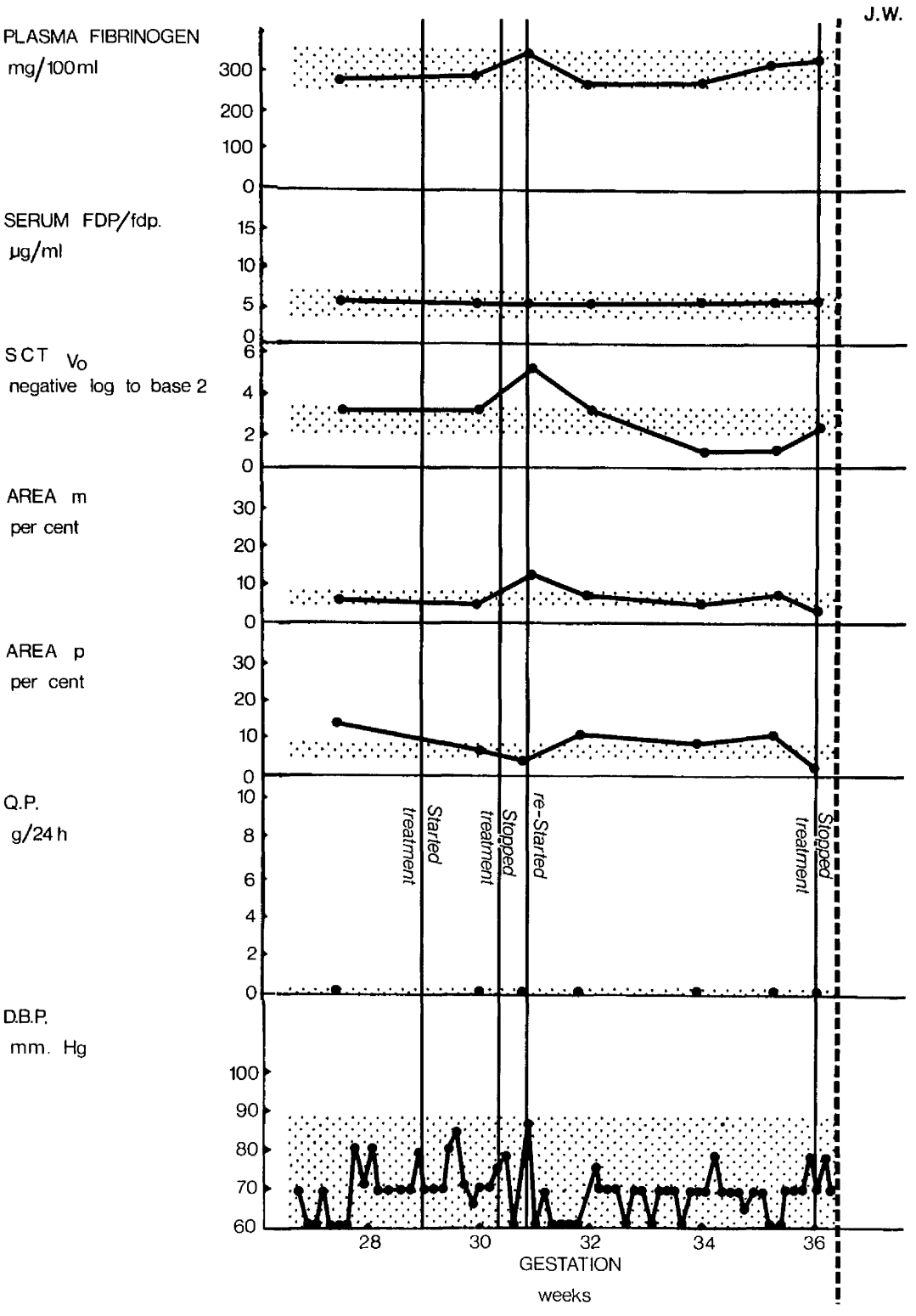


Figure 70:- Results for "High Risk" Patient (J.W.).

Table 1

TYPE I Intermediate oligomers	TYPE II Complexes clottable with thrombin	TYPE III Complexes unclottable with thrombin
f-f	f_B-F	x-y
f_B-f_B	f-F	x-Y
x-x	x-F	x-D
	x-X	f_B-y
	f_B-X	f_B-Y
	f-X	f_B-D
		f-y
		f-Y
		f-D

The various types of soluble fibrinogen-fibrin complexes suggested by Węgrzynowicz, Kopeć and Latallo (1971).

F - fibrinogen

f - fibrin monomer produced by thrombin (deprived of fibrinopeptides A and B)

f_B - fibrin monomer produced by ancrod or Reptilase (deprived of fibrinopeptide A)

X, Y, D, E - successive degradation products formed by plasmin digestion of fibrinogen (FDP)

x, y, d, e - successive degradation products formed by plasmin digestion of fibrin (fdp)

Table 2

Solute	M.W.	Log_{10} M.W.	V_e in ml.
Blue Dextran	2,000,000	6.3010	62
Lipopolysaccharide (S. typhosa)	>1,500,000	6.1761	72
Thyroglobulin	670,000	5.8261	90
Fibrinogen	340,000	5.5315	105
Albumin	69,000	4.8388	142
Trypsin	23,800	4.3766	161
Bacitracin	1,411	3.1495	175

Molecular weight (M.W.), logarithm to base 10 of molecular weight (log_{10} M.W.) and elution volume (V_e) of purified solutes analysed by gel filtration technique. Data used to construct graph shown in Figure 23.

Table 3

Sample	No. of analyses	V_o (ml)	V_e fibrinogen (ml)
Blue Dextran	3	69.5 ± 1.3	-
Fibrinogen (Kabi)	3	-	95.7 ± 0.3
Plasma	8	-	95.6 ± 0.5
-alanine precipitate of plasma	10	-	95.4 ± 0.6

Void volume (V_o) and elution volume (V_e) of fibrinogen estimated on Columns A1 and A3 (Batch I gel). Results are shown as the mean ± 1 standard deviation. There is no significant difference (unpaired "t" test) in the V_e of fibrinogen. (Purified fibrinogen/ plasma, $t = 0.149$, $2p < 0.90$; plasma/ β -alanine precipitate, $t = 0.644$, $2p < 0.60$; fibrinogen/ β -alanine precipitate, $t = 0.593$, $2p < 0.60$).

Table 4

	Thrombin (units)	Heparin (units)
a	-	250
b	0.02	250
c	0.04	500
d	0.06	750
e	0.08	1,000
f	0.10	1,250
g	0.12	1,500
h	0.20	2,000

In the set of experiments shown in Figures 27 - 30, 25 mg of fibrinogen was incubated with successively increased concentrations of thrombin at 37°C for 5 minutes. The thrombin was "neutralised" with appropriate concentrations of heparin as shown.

Table 5

	Plasma	Enzyme (1)	Inhibitor (1)	Time (1)	Enzyme (2)	Inhibitor (2)	Time (2)
a	9 ml	-	-	-	-	-	-
b	9 ml	Ancrod 0.315 units	Anti-Ancrod (1:100 dilution) 1 ml	1 min	-	-	-
c	9 ml	-	-	-	Streptokinase 900 units	Aprotinin 9,000 units	20 mins
d	9 ml	Ancrod 0.315 units	Anti-Ancrod (1:100 dilution) 1 ml	1 min	Streptokinase 900 units	Aprotinin 9,000 units	20 mins
e	9 ml	Streptokinase 900 units	Aprotinin 9,000 units	20 mins	Ancrod 0.0315 units	Anti-Ancrod (1:100 dilution) 1 ml	1 min

Details of in vitro experiments, in which plasma was incubated with ancrod and/or streptokinase. Enzyme (1) was added before enzyme (2). The results are shown in Figures 32 - 35.

Table 6

Case No. (Initials)	Sex	Age (Years)	Clinical Details
1 (W.M.)	Male	72	(1) Severe peripheral arterial disease (2) Incipient gangrene of left foot
2 (W.F.)	Male	42	(1) Deep venous thrombosis of right leg (2) Ischaemic heart disease
3 (B.S.)	Male	55	(1) Deep venous thrombosis of right leg (2) Carcinoma of caecum + metastases
4 (J.R.)	Male	26	(1) Deep venous thrombosis of right leg (2) Chronic pyelonephritis
5 (J.McK.)	Female	50	(1) Incipient gangrene of right foot following frost-bite (2) Alcoholism
6 (I.B.)	Female	56	(1) Incipient gangrene of right foot (2) Severe peripheral arterial disease (3) Diabetes
7 (W.McG.)	Male	22	(1) Right sided axillary vein thrombosis

Clinical details of patients included in ancrod study.

Table 7

Plasma Fibrinogen Levels (mg/100 ml)

Patient No.	Pre-treatment	6 hours treatment	24 hours treatment
1	286	130	13
2	364	322	12
3	268	5	0
4	226	60	0
5	421	126	0
6	519	253	0
7	156	0	0
mean ± SD	320 ± 123	128 ± 122	4 ± 6

Plasma fibrinogen levels (mg/100 ml) pre-treatment and after 6 and 24 hours ancrod infusion. Means ± standard deviations are shown for each group of results.

Differences between groups (paired "t" test):-

Pre-treatment/6 hours $t = 5.746$ $2p < 0.005$

6 hours/24 hours $t = 2.767$ $2p < 0.05$

Table 8a

Serum FDP/fdp Levels - T.R.C.H.I.I.
(negative logarithm to base 2)

Patient No.	Pre-treatment	6 hours treatment	24 hours treatment
1	N.D.	N.D.	N.D.
2	2	8	10
3	2	10	10
4	2	9	10
5	1	10	10
6	3	11	10
7	1	10	9
mean ± SD	1.8 ± 0.8	9.7 ± 1.0	9.8 ± 0.4

Serum FDP/fdp levels measured by the Tanned Red Cell Haemagglutination Inhibition Immunoassay (T.R.C.H.I.I.) pre-treatment and 6 and 24 hours after starting ancrod infusion; because of the very high values the results are expressed as the negative logarithm to the base 2 of the last dilution to give a positive reaction (cf. SCT titre). Means ± standard deviations are shown for each group of results. Differences between groups (paired "t" test):-

Pre-treatment/6 hours $t = 16.413$ $2p < 0.001$

6 hours/24 hours $t = 0.349$ $2p < 0.8$ (NS).

Table 8b

Serum FDP/fdp Levels - SCT titre
(negative logarithm to base 2)

Patient No.	Pre-treatment	6 hours treatment	24 hours treatment
1	N.D.	N.D.	N.D.
2	0	4	$\geqslant 10$
3	0	$\geqslant 10$	8
4	0	7	7
5	0	$\geqslant 10$	8
6	0	9	$\geqslant 10$
7	0	8	7
mean $\pm$ SD	0	6.0 $\pm$ 2.2	8.3 $\pm$ 1.4

Serum FDP/fdp levels measured by the Staphylococcal Clumping Test (SCT) titre. Results expressed as the negative logarithm to the base 2. Means $\pm$ standard deviations are shown.
Differences between groups (paired "t" test).
Pre-treatment/6 hours $t = 8.593$, $2p < 0.001$
6 hours/24 hours $t = 0.271$, $2p < 0.8$ (NS)
The correlation coefficient (r) for data in Tables 8a and b calculated by linear regression analysis was 0.964 ($2 \propto < 0.001$).

Table 9a

FR-antigen (mg/100 ml) measured by the Mancini technique at V_o

Patient No.	Pre-treatment	6 hours treatment	24 hours treatment
1	N.D.	N.D.	N.D.
2	N.D.	N.D.	N.D.
3	N.D.	N.D.	N.D.
4	1.0	12.0	4.5
5	0	7.0	13.5
6	7.5	19.0	24.5
7	0	4.5	1.5
mean ± SD	2.1 ± 3.6	10.6 ± 6.4	11.0 ± 10.3

FR-antigen concentrations (mg/100 ml) measured by the radial immunodiffusion (Mancini) technique on the void volume (V_o) fractions from samples obtained pre-treatment and after 6 and 24 hours ancrod infusion. Means ± standard deviations are shown. Differences between groups (paired "t" test):-

Pre-treatment/6 hours $t = 5.087$ $2p < 0.02$

6 hours/24 hours $t = 0.111$ $2p < 0.95$ (NS).

Table 9b

SCT Titre at V_o
(negative logarithm to base 2)

Patient No.	Pre-treatment	6 hours treatment	24 hours treatment
1	N.D.	N.D.	N.D.
2	N.D.	N.D.	N.D.
3	N.D.	N.D.	N.D.
4	0	6	3
5	0	8	7
6	5	8	8
7	0	4	5
mean ± SD	1.3 ± 2.5	6.5 ± 1.9	5.8 ± 2.2

FR-antigen concentrations measured by the SCT titre on the same void volume (V_o) fractions shown in Table 9a. Results are expressed as the negative logarithm to the base 2.
Differences between groups (paired "t" test):-
Pre-treatment/6 hours $t = 4.735$ $2p < 0.02$
6 hours/24 hours $t = 0.878$ $2p < 0.5$ (NS).

The correlation coefficient (r) for the data in Tables 9a and b calculated by linear regression analysis was 0.804 ($2 \propto < 0.01$).

Table 10

Area m (percent total area)

Patient No.	Pre-treatment	6 hours treatment	24 hours treatment
1	N.D.	N.D.	N.D.
2	N.D.	N.D.	N.D.
3	4.2	20.9	26.6
4	7.0	30.2	32.9
5	8.2	21.6	12.7
6	3.6	8.0	18.0
7	4.1	17.5	18.5
mean ± SD	5.4 ± 2.0	19.6 ± 8.0	21.9 ± 7.9

Soluble complex concentrations (area m, percent total area, calculated as described in Chapter II.4(k)) for samples obtained pre-treatment and after 6 and 24 hours anerod infusion. Means ± standard deviations are shown. Differences between groups (paired "t" test):-

Pre-treatment/6 hours $t = 4.681$ $2p < 0.01$

6 hours/24 hours $t = 0.699$ $2p < 0.6$ (NS).

Table 11

Area p (percent total area)

Patient No.	Pre-treatment	6 hours treatment	24 hours treatment
1	N.D.	N.D.	N.D.
2	N.D.	N.D.	N.D.
3	10.7	32.7	29.2
4	7.8	13.3	22.2
5	9.9	23.6	37.3
6	9.5	40.0	55.0
7	7.3	30.9	28.9
mean ± SD	9.0 ± 1.4	28.1 ± 10.1	34.5 ± 12.6

Early degradation product concentration (area p, percent total area, calculated as described in Chapter II. 4(k)) for samples obtained pre-treatment and after 6 and 24 hours ancrod infusion. Means ± standard deviations are shown.
Differences between groups (paired "t" test):-
Pre-treatment/6 hours $t = 4.415$ $2p < 0.02$
6 hours/24 hours $t = 1.652$ $2p < 0.2$ (NS).

Table 12

Area n (percent total area)

Patient No.	Pre-treatment	6 hours treatment	24 hours treatment
1	N.D.	N.D.	N.D.
2	N.D.	N.D.	N.D.
3	85.0	46.4	44.1
4	85.2	56.5	44.9
5	81.8	54.7	59.9
6	87.0	52.0	26
7	86.7	51.6	52.7
mean ± SD	85.1 ± 2.1	52.2 ± 3.8	43.5 ± 10.4

"Non-degraded, uncomplexed fibrinogen" (area n, percent total area, calculated as described in Chapter II. 4(k)) for samples obtained pre-treatment and after 6 and 24 hours ancrod infusion. Means ± standard deviations are shown.
Differences between groups (paired "t" test):-
Pre-treatment/6 hours $t = 15.255$ $2p < 0.001$
6 hours/24 hours $t = 1.818$ $2p < 0.2$ (NS).

Table 13

FR-antigen Ratios

Patient No.	Pre-treatment	6 hours treatment	24 hours treatment
1	0.86	0.66	0.67
2	0.80	0.69	0.69
3	0.66	0.63	0.62
4	0.69	0.65	0.62
5	0.78	0.69	0.60
6	0.66	0.68	0.68
7	0.83	0.67	0.67
mean ± SD	0.75 ± 0.08	0.67 ± 0.02	0.65 ± 0.04

FR-antigen ratios (calculated as described in Chapter II. 4(k)) for samples obtained pre-treatment and after 6 and 24 hours ancrod infusion. Means ± standard deviations are shown.
Differences between groups (paired "t" test):-
Pre-treatment/6 hours $t = 3.000$ $2p < 0.025$
6 hours/24 hours $t = 1.315$ $2p < 0.3$ (NS).

Table 14

TCP Ratios

Patient No.	Pre-treatment	6 hours treatment
1	N.D.	N.D.
2	0.68	0.68
3	0.61	0.66
4	0.74	0.65
5	0.84	0.66
6	0.68	0.72
7	0.77	0.67
mean ± SD	0.72 ± 0.08	0.67 ± 0.03

TCP ratios (calculated as described in Chapter II. 4(i)) for samples obtained pre-treatment and after 6 hours ancrod infusion. (Results could not be calculated for 24 hour samples because no T.C.T. results were recorded ⟨3 minutes). Means ± standard deviations are shown. Differences between groups (paired "t" test):- Pre-treatment/6 hours $t = 1.254$ $2p \langle 0.3$ (NS).

Table 15

T.C.T. Ratios

Patient No.	Pre-treatment	6 hours treatment
1	0.89	0.68
2	0.81	0.68
3	0.84	0.66
4	0.91	0.68
5	0.88	0.66
6	0.86	0.74
7	0.90	0.67
mean ± SD	0.87 ± 0.04	0.68 ± 0.03

T.C.T. ratios (calculated as described in Chapter II. 4(i)) for samples obtained pre-treatment and after 6 hours ancrod infusion. No T.C.T. results (3 minutes were recorded in the 24 hour samples. Means ± standard deviations are shown. Differences between groups (paired "t" test):-
Pre-treatment/6 hours $t = 12.001$ $2p < 0.001$.

Table 16

Pre-Eclampsia Project

Group I	10 pre-eclamptic patients
Group II	10 age, parity and gestation matched pregnant control women
Group III	10 age matched non-pregnant control women

Subjects included in initial pre-eclampsia study.

Table 17

Clinical details used in matching subjects in Group I (pre-eclamptic patients), Group II (normal pregnant controls) and Group III (normal non-pregnant control women). Mean ± standard deviation (SD) of results for age and gestation are shown. There is no significant difference between the groups using the unpaired "t" test:-

	Age	
Group I/Group II	$t = 0.120$	$2p > 0.95$ (NS)
Group II/Group III	$t = 0.288$	$2p > 0.80$ (NS)
Group I/Group III	$t = 0.420$	$2p > 0.70$ (NS)
	Gestation	
Group I/Group II	$t = 0.159$	$2p > 0.90$ (NS)

Table 17

Patient No.	GROUP I Age (years)	GROUP I Parity	GROUP I Gestation (weeks)	GROUP II Age (years)	GROUP II Parity	GROUP II Gestation (weeks)	GROUP III Age (years)
1	16	0 + 0	25	16	0 + 0	24	17
2	18	0 + 0	34	17	0 + 0	34	18
3	35	0 + 0	29	35	0 + 0	30	34
4	23	1 + 0	36	22	1 + 0	38	22
5	29	0 + 1	26	27	0 + 1	27	28
6	21	1 + 0	30	20	1 + 0	28	20
7	27	0 + 0	36	29	0 + 0	36	26
8	23	0 + 0	34	22	0 + 0	35	21
9	24	2 + 2	32	24	2 + 1	33	22
10	22	0 + 0	29	23	0 + 0	29	20
mean ±	24 ± 5		31 ± 4	23 ± 6		31 ± 4	23 ± 5

Table 18a

Systolic Blood Pressure Readings (mm Hg)

	Group I	Group II	Group III
1	160	110	120
2	160	120	100
3	170	140	105
4	120	130	110
5	150	130	110
6	130	110	105
7	160	110	115
8	165	100	120
9	145	90	105
10	130	130	110
mean $\pm$ SD	149 $\pm$ 17	117 $\pm$ 16	110 $\pm$ 7

Systolic blood pressure readings on subjects in Groups I, II and III (see Table 16) at time of blood sampling. Means $\pm$ standard deviations (SD) for each group are shown. Differences between groups (unpaired "t" test):-

Group I/Group II $t = 4.359$ $2p < 0.001$

Group II/Group III $t = 1.300$ $2p < 0.30$ (NS)

Group I/Group III $t = 6.710$ $2p < 0.001$

Table 18b

Diastolic Blood Pressure Readings (mm Hg)

	Group I	Group II	Group III
1	110	75	75
2	110	80	60
3	110	80	65
4	90	75	70
5	100	80	70
6	90	70	70
7	95	70	80
8	95	60	75
9	110	60	60
10	105	75	75
mean $\pm$ SD	101 $\pm$ 9	73 $\pm$ 8	70 $\pm$ 7

Diastolic blood pressure readings of subjects in Groups I, II and III (see Table 16) at time of blood sampling. Means $\pm$ standard deviations (SD) for each group are shown. Differences between groups (unpaired "t" test):-

Group I/Group II $t = 8.060$ $2p < 0.001$

Group II/Group III $t = 0.785$ $2p < 0.50$ (NS)

Group I/Group III $t = 9.211$ $2p < 0.001$

Table 18c

Proteinuria and Oedema

Group I

	Quantitative Proteinuria (g/24 hours)	Oedema (face and hands)
1	12.5	+
2	5.6	++
3	9.7	+
4	1.7	+
5	3.1	+++
6	5.0	+
7	1.8	+++
8	3.1	trace
9	0.7	trace
10	9.8	++
mean $\pm$ SD	5.3 $\pm$ 4.1	

Quantitative proteinuria results and assessment of oedema at time of blood sampling of subjects in Group I (pre-eclamptic patients). Subjects in Group II (normal pregnant controls) did not have significant proteinuria or oedema of face and hands at time of blood sampling. Subjects in Group III (normal non-pregnant controls) did not have significant proteinuria or oedema.

Table 19

Plasma Fibrinogen Levels (mg/100 ml)

	Group I	Group II	Group III
1	300	253	245
2	445	357	172
3	155	380	238
4	340	350	237
5	235	307	207
6	346	297	150
7	303	295	166
8	200	439	167
9	269	150	262
10	220	180	215
mean ± SD	281 ± 84	301 ± 89	207 ± 38

Plasma fibrinogen values for samples from Groups I, II and III (see Table 16). Means ± standard deviations are shown for each group. Differences between the groups (unpaired "t" test):-

Group I/Group II $t = 0.504$ $2p \langle 0.70$ (NS)

Group II/Group III $t = 3.079$ $2p \langle 0.01$

Group I/Group III $t = 2.546$ $2p \langle 0.025$

Table 20

Serum FDP/fdp Levels - T.R.C.H.I.I. (μg/ml)

	Group I	Group II	Group III
1	10	3.75	2.5
2	7.5	5	2.5
3	10	5	2.5
4	10	5	2.5
5	10	5	2.5
6	20	2.5	1.25
7	5	5	2.5
8	10	5	2.5
9	5	10	5
10	10	2.5	0
mean ± SD	9.7 ± 4.2	4.9 ± 2.1	2.4 ± 1.2

Serum FDP/fdp values for samples from Groups I, II and III (see Table 16). Measurements by the tanned red cell haem-agglutination inhibition immunoassay (T.R.C.H.I.I.). Means ± standard deviations are shown for each group. Differences between the groups (unpaired "t" test):-

Group I/Group II $t = 3.316$ $2p < 0.005$

Group II/Group III $t = 3.264$ $2p < 0.005$

Group I/Group III $t = 5.374$ $2p < 0.001$

Table 21

Serum FDP/fdp Levels - SCT titre
(negative logarithm to base 2)

	Group I	Group II	Group III
1	2	0	0
2	0	0	0
3	0	0	0
4	1	0	0
5	0	0	0
6	2	0	0
7	0	0	0
8	0.5	0	0
9	0	0	0
10	2	0	0
mean ± SD	0.75 ± 0.9	0	0

Serum FDP/fdp values for samples from Groups I, II and III (see Table 16). Measurements by Staphylococcal Clumping Test (SCT) titre. Means ± standard deviations are shown for each group. Differences between groups (unpaired "t" test):-
Group I/Group II $t = 2.577$ $2p < 0.02$
Group I/Group III $t = 2.577$ $2p < 0.02$

Table 22

Serum FDP/fdp - Comparison of T.R.C.H.I.I. and SCT titre measurements

	r	2α
Group I alone	+ 0.635	<0.05
Group II alone	0	-
Group III alone	0	-
Group I and II	+ 0.702	<0.001
Group II and III	0	-
Group I, II and III	+ 0.703	<0.001

Comparison of serum FDP/fdp measurements using the tanned red cell haemagglutination inhibition immunoassay (T.R.C.H.I.I., Table 20) and the staphylococcal clumping test (SCT titre, Table 21) by linear regression analysis. Values of correlation coefficient (r), and probability values (2α) are shown.

Table 23

FR-Antigen Ratios

	Group I	Group II	Group III
1	0.62	0.85	0.86
2	0.62	0.81	0.86
3	0.60	0.80	0.88
4	0.60	0.80	0.86
5	0.60	0.80	0.88
6	0.60	0.83	0.89
7	0.61	0.75	0.86
8	0.61	0.77	0.87
9	0.60	0.76	0.85
10	0.61	0.78	0.86
mean ± SD	0.61 ± 0.01	0.80 ± 0.03	0.87 ± 0.01

Fibrinogen-fibrin related (FR) antigen ratios (calculated as described in Chapter II.4(k)) for samples from Groups I, II and III (see Table 16). Means ± standard deviations are shown for each group. Differences between groups (unpaired "t" test):-

Group I/Group II $t = 17.794$ $2p < 0.001$

Group II/Group III $t = 6.194$ $2p < 0.001$

Group I/Group III $t = 54.881$ $2p < 0.001$

Table 24

Plasma Fibrinogen Levels/FR-antigen ratios

	r	2α
Group I alone	+ 0.391	>0.1 (NS)
Group II alone	+ 0.108	>0.1 (NS)
Group III alone	- 0.407	>0.1 (NS)
Group I and II	+ 0.147	>0.1 (NS)
Group II and III	- 0.469	<0.05
Group I, II and III	- 0.259	>0.1 (NS)

Comparison of plasma fibrinogen levels (Table 19) and FR-antigen ratios (Table 23) for the same samples using linear regression analysis. The groups are shown separately and in combination.

Table 25

SCT titres (V_o) -
(negative logarithm to base 2)

	Group I	Group II	Group III
1	4	0	0
2	4	2	0
3	6	0	0
4	3	0	0
5	++	0	0
6	3	2	0
7	5	1	0
8	3	0	0
9	3	4	0
10	4	0.5	0
mean ± SD	3.9 ± 1.0	1.0 ± 1.3	0

Staphylococcal Clumping Test (SCT) titres at the void volume (V_o) expressed as the negative logarithm to the base 2 for samples in Groups I, II and III (see Table 16). Means ± standard deviations are shown for each group. Differences between groups (unpaired "t" test):-

Group I/Group II $t = 5.263$ $2p < 0.001$

Group II/Group III $t = 2.237$ $2p < 0.05$

Group I/Group III $t = 11.705$ $2p < 0.001$

*Patient 5 was studied early in the project, before the SCT titre was measured, however, the undiluted sample gave a strongly positive result.

Table 26

FR-antigen (mg/100 ml) measured by Mancini technique at V_o

	Group I	Group II	Group III
1	1.0	0	0
2	1.0	0	0
3	1.0	0	0
4	5.0	0	0
5	1.0	0	0
6	4.0	0	0
7	1.0	0	0
8	3.0	0	0
9	1.5	0	0
10	2.0	0	0
mean ± SD	2.2 ± 1.4	0	0

FR-antigen (mg/100 ml) measured by the radial immunodiffusion technique (Mancini) at the same void volume fractions as shown in Table 25. Means ± standard deviations are shown for each group. Differences between groups (unpaired "t" test):-

Group I/Group II $t = 4.435$ $2p < 0.001$

Group I/Group III $t = 4.435$ $2p < 0.001$

Table 27

FR-Antigen Concentration at V_o Measured by Two Techniques

	γ	2α
Group I alone	- 0.658	$>$0.1 (NS)
Group II alone	0	-
Group III alone	0	-
Group I and II	+ 0.424	$>$0.1 (NS)
Group II and III	0	-
Group I, II and III	0.542	$<$0.01

Comparison of the results of the fibrinogen-fibrin related (FR)-antigen concentration in the void volume fractions (V_o) measured by two different techniques (Tables 25 and 26) using linear regression analysis. The groups are shown separately and in combination.

Table 28

T.C.P. Ratios

	Group I	Group II	Group III
1	0.64	0.87	0.88
2	0.64	0.83	0.89
3	0.58	0.88	0.90
4	0.62	0.89	0.94
5	0.61	0.85	0.88
6	0.60	0.80	0.93
7	0.60	0.86	0.90
8	0.61	0.84	0.87
9	0.60	0.87	0.88
10	0.60	0.83	0.85
mean ± SD	0.61 ± 0.02	0.85 ± 0.03	0.89 ± 0.03

Thrombin Clottable Protein (T.C.P.) ratios (calculated as described in Chapter II.4(i)) for samples from Groups I, II and III (see Table 16). Means ± standard deviations are shown for each group. Differences between groups (unpaired "t" test):-

Group I/Group II $t = 23.004$ $2p < 0.001$

Group II/Group III $t = 3.288$ $2p < 0.005$

Group I/Group III $t = 27.080$ $2p < 0.001$

Table 29

Plasma Fibrinogen Levels/T.C.P. Ratios

	r	2α
Group I alone	+ 0.680	< 0.05
Group II alone	+ 0.019	> 0.1 (NS)
Group III alone	- 0.104	> 0.1 (NS)
Group I and II	+ 0.166	> 0.1 (NS)
Group II and III	- 0.370	> 0.1 (NS)
Group I, II and III	- 0.187	> 0.1 (NS)

Comparison of plasma fibrinogen levels (Table 19) and T.C.P. ratios (Table 28) for the same samples using linear regression analysis. The groups are shown separately and in combination.

Table 30

FR-Antigen Ratios/T.C.P. Ratios

	r	2α
Group I alone	+ 0.716	<0.05
Group II alone	- 0.155	>0.1 (NS)
Group III alone	+ 0.316	>0.1 (NS)
Group I and II	+ 0.956	<0.001
Group II and III	+ 0.494	<0.05
Group I, II and III	+ 0.961	<0.001

Comparison of FR-antigen (Table 23) and T.C.P. ratios (Table 28) for the same samples using linear regression analysis. The groups are shown separately and in combination.

Table 31

T.C.T. Ratios

	Group I	Group II	Group III
1	0.90	0.92	0.90
2	0.86	0.88	0.92
3	0.90	0.91	0.93
4	0.85	0.91	0.89
5	0.90	0.87	0.93
6	0.89	0.90	0.93
7	0.90	0.90	0.93
8	0.86	0.91	0.92
9	0.89	0.90	0.93
10	0.89	0.86	0.90
mean ± SD	0.88 ± 0.02	0.90 ± 0.02	0.92 ± 0.02

Thrombin clotting time (T.C.T.) ratios (calculated as described in Chapter II.4(i)) for samples in Groups I, II and III (see Table 16). Means ± standard deviations are shown for each group.

Differences between groups (unpaired "t" test):-

Group I/Group II $t = 1.372$ $2p < 0.2$ (NS)

Group II/Group III $t = 2.789$ $2p < 0.02$

Group I/Group III $t = 4.310$ $2p < 0.001$

Table 32

Area m (percent total area)

	Group I	Group II	Group III
1	15.0	5.5	2.5
2	7.1	4.5	3.1
3	25.7	4.2	3.3
4	21.3	5.8	2.6
5	15.5	3.3	2.4
6	9.3	5.7	2.1
7	9.0	3.6	3.3
8	18.8	5.8	2.5
9	23.9	4.3	2.8
10	14.5	4.0	2.7
mean ± SD	16.0 ± 6.4	4.7 ± 0.9	2.7 ± 0.4

Soluble complex concentrations (area m, percent total area, calculated as described in Chapter II.4(k)) for samples in Groups I, II and III (see Table 16). Means ± standard deviations are shown for each group. Differences between groups (unpaired "t" test):-

Group I/Group II $t = 5.532$ $2p < 0.001$

Group II/Group III $t = 6.134$ $2p < 0.001$

Group I/Group III $t = 6.546$ $2p < 0.001$

Table 33

Area m values/Plasma fibrinogen levels

	r	2α
Group I alone	- 0.656	<0.05
Group II alone	+ 0.344	>0.1 (NS)
Group III alone	+ 0.086	>0.1 (NS)
Group I and II	- 0.663	<0.01
Group II and III	+ 0.623	<0.01
Group I, II and III	+ 0.016	>0.1 (NS)

Comparison of plasma fibrinogen (Table 19) and area m (Table 32) for the same samples using linear regression analysis. The groups are shown separately and together.

Table 34

Area m values/serum FDP/fdp levels

	r	2α
Group I alone	- 0.205	>0.1 (NS)
Group II alone	- 0.216	>0.1 (NS)
Group III alone	+ 0.233	>0.1 (NS)
Group I and II	+ 0.395	<0.1 (NS)
Group II and III	+ 0.446	<0.05
Group I, II and III	+ 0.572	<0.001

Comparison of serum FDP/fdp levels (Table 20) measured by the tanned red cell haemagglutination inhibition immunoassay (T.R.C.H.I.I.) and area m (Table 32). The groups are shown separately and together.

Table 35

Area p (percent total area)

	Group I	Group II	Group III
1	1.7	8.9	6.7
2	5.8	2.6	3.9
3	6.9	3.2	2.0
4	1.8	3.6	3.9
5	19.5	5.0	3.6
6	11.1	6.3	4.0
7	14.8	4.1	4.6
8	8.0	2.1	1.2
9	15	7.5	1.7
10	6.7	4.7	2.7
mean ± SD	9.1 ± 5.9	4.8 ± 2.2	3.4 ± 1.6

Early degradation product concentration (area p, percent total area, calculated as described in Chapter II. 4(k)) for samples in Groups I, II and III (Table 16). Means ± standard deviations are shown for each group. Differences between groups (unpaired "t" test):-

Group I/Group II $t = 2.186$ $2p < 0.05$

Group II/Group III $t = 1.595$ $2p < 0.2$ (NS)

Group I/Group III $t = 2.961$ $2p < 0.01$

Table 36

Area p values/plasma fibrinogen levels

	r	2α
Group I alone	- 0.201	>0.1 (NS)
Group II alone	- 0.731	<0.05
Group III alone	- 0.017	>0.1 (NS)
Group I and II	- 0.332	>0.1 (NS)
Group II and III	- 0.205	>0.1 (NS)
Group I, II and III	- 0.059	>0.1 (NS)

Comparison of plasma fibrinogen (Table 19) and area p (Table 35) for the same samples using linear regression analysis. The groups are shown separately and together.

Table 37

Area p values/Serum FDP/fdp levels

	γ	2α
Group I alone	- 0.135	$>$0.1 (NS)
Group II alone	+ 0.119	$>$0.1 (NS)
Group III alone	- 0.178	$>$0.1 (NS)
Group I and II	+ 0.216	$>$0.1 (NS)
Group II and III	+ 0.235	$>$0.1 (NS)
Group I, II and III	+ 0.372	$<$0.05

Comparison of serum FDP/fdp levels (Table 20) measured by the tanned red cell haemagglutination inhibition immunoassay (T.R.C. H.I.I.) and area p (Table 35). Groups are shown separately and together.

Table 38

Area m values/area p values

	r	2α
Group I alone	- 0.102	>0.1 (NS)
Group II alone	+ 0.059	>0.1 (NS)
Group III alone	- 0.137	>0.1 (NS)
Group I and II	+ 0.314	<0.1 (NS)
Group II and III	+ 0.295	>0.1 (NS)
Group I, II and III	+ 0.442	<0.05

Comparison of area m (Table 32) and area p (Table 35) for the same samples using linear regression analysis. The groups are shown separately and together.

Table 39

Area n (percent total area)

	Group I	Group II	Group III
1	83.2	85.6	89.2
2	87.0	92.9	93.0
3	67.3	92.3	94.6
4	77.0	90.3	93.5
5	64.9	91.5	93.9
6	79.5	87.9	93.9
7	76.0	92.0	93.0
8	78.2	91.9	96.3
9	60.8	88.2	95.3
10	75.8	91.3	94.5
mean ± SD	75.0 ± 8.2	90.4 ± 2.4	93.7 ± 1.9

"Non-degraded, uncomplexed fibrinogen" (area n, percent total area, calculated as described in Chapter II.4(k)) for samples in Groups I, II and III. Means ± standard deviations are shown.
Differences between groups (unpaired "t" test):-
Group I/Group II $t = 5.689$ $2p < 0.001$
Group II/Group III $t = 3.469$ $2p < 0.005$
Group I/Group III $t = 7.018$ $2p < 0.001$

Table 40

Area n values/plasma fibrinogen levels

	r	2α
Group I alone	+ 0.617	$<$0.1 (NS)
Group II alone	+ 0.503	$>$0.1 (NS)
Group III alone	- 0.177	$>$0.1 (NS)
Group I and II	0.397	$<$0.1 (NS)
Group II and III	- 0.172	$>$0.1 (NS)
Group I, II and III	- 0.0003	$>$0.1 (NS)

Comparison of plasma fibrinogen (Table 19) and area n (Table 39) for the same samples using linear regression analysis. The groups are shown separately and together.

Table 41

Area n values/serum FDP/fdp levels

	r	2α
Group I alone	+ 0.262	>0.1 (NS)
Group II alone	- 0.023	>0.1 (NS)
Group III alone	+ 0.084	>0.1 (NS)
Group I and II	- 0.389	<0.1 (NS)
Group II and III	- 0.379	<0.1 (NS)
Group I, II and III	- 0.572	<0.001

Comparison of serum FDP/fdp (Table 20) and area n (Table 39) for the same samples using linear regression analysis. The groups are shown separately and together.

Table 42

Summary

	Group I	Group II	Group III
Plasma fibrinogen mg/100 ml	281 ± 84	301* ± 89	207 ± 38
Serum FDP µg/ml (TRCHII)	9.7* ± 4.2	4.9* ± 2.1	2.4 ± 1.2
FR-antigen Ratio	0.61* ± 0.01	0.80* ± 0.03	0.87 ± 0.01
SCT titre at V_o	3.9* ± 1.0	1.0* ± 1.3	0
TCP Ratio	0.61* ± 0.02	0.85* ± 0.03	0.89 ± 0.03
Area m (%)	16.0* ± 6.4	4.7* ± 0.9	2.7 ± 0.4
Area p (%)	9.1* ± 5.9	4.8 ± 2.2	3.4 ± 1.6

A summary of results expressed as a mean ± single standard deviation for Groups I, II and III (see Table 16). Abbreviations as on previous tables and at the beginning of Tables Section. Results marked with asterix (*) differ significantly (with level of significance taken as $2p < 0.05$) from those of appropriate control group shown on column to right.

Table 43

Post-Natal Visit

Patient No.	Area m	Area p	SCT V_o	Serum FDP/fdp	Plasma fibrinogen
1 *	1.2	1.0	0	10	248
2 *	4.2	2.6	0	2.5	245
3 *	3.2	7.9	0	5	154
4	-	-	-	-	-
5 *	4.0	2.8	0	0	190
6	7.4	3.0	4	0	169
7	1.3	4.1	0	5	212
8 *	2.7	7.0	0	5	240
9 *	1.7	2.6	0	10	290
10	3.4	3.1	1	2.5	252
mean ± SD	3.2 ± 1.9	3.8 ± 2.2	0.6 ± 1.3	4.5 ± 3.7	222 ± 44

Laboratory results obtained on blood samples taken at the post-natal visit from the patients in Group I, Chapter V. (No sample was obtained from Patient 4). Patient 6 was the only patient receiving oral contraceptive therapy at this time. Patients marked with asterix (*) were still hypertensive. The results were compared with those from the non-pregnant control group (Group II, Chapter V) using the unpaired "t" test:-

Plasma fibrinogen $t = 0.815$ $2p < 0.5$ (NS)
Area m $t = 0.813$ $2p < 0.5$ (NS)
SCT titre at V_o $t = 1.322$ $2p < 0.3$ (NS)
Serum FDP/fdp $t = 1.670$ $2p < 0.2$ (NS)
Area p $t = 0.405$ $2p < 0.7$ (NS)

Table 44

Patient No.	Sample No.	Clinical Index: Pre-eclampsia	Clinical Index: Normal pregnancy
1	1	26.3	-13.4
	2	39.2	
2	1	27.3	-13.4
	2 (during labour)	28.7	
3	1	-2.7	- 8.0
	2	9.4	
	3	23.4	
	4	33.1	
4	1	2.6	- 8.0
5	1	15.7	- 8.0
	2	N.D.	
	3	29.3	
	4 (after I.U.D.)	N.D.	
6	1	19.5	-13.4
	2 (after I.U.D.)	-5.2	
7	1	23.8	-13.4
	2	23.8	
	3	21.1	
8	1	10.6	- 9.0
9	1	-10.7	-13.4
	2	-10.7	
	3	-13.4	
	4	7.8	
	5	6.3	
	6	13.0	
10	1	35.5	-13.4
	2	34.7	
	3	29.7	
	4	28.4	
	5	28.0	

Table 45a

Correlations Between Clinical Indices (see Table 44) and Laboratory Results

Clinical index and -	r	2α
area m	0.102	>0.1
SCT titre at V_o	0.236	>0.1
area p	0.095	>0.1
serum FDP/fdp	0.063	>0.1

Table 45b

Clinical index and -	r	2α
area m	-0.137	>0.1
SCT titre at V_o	0.636	nearly $= 0.05$
area p	0.224	>0.1
serum FDP/fdp	0.395	>0.1

The correlation coefficients (r) calculated by linear regression analysis using the clinical index and various laboratory measurements from the total ante-natal pre-eclamptic data (a) and the data from the last sample taken before delivery or intra-uterine death from the pre-eclamptic patients (b).

Table 44

Clinical indices (Howie, Purdie, Begg et al., 1976) for the pre-eclamptic patients (total ante-natal pre-eclamptic data on patients in Group I, Chapter V) and normal pregnant women (Group II, Chapter V) at the time of blood sampling. No quantitative proteinuria results were available for days when samples 2 and 4 were taken from Patient 5. The underlined samples were used for analyses when the last sample before delivery or intrauterine death (I.U.D.) was required.

Table 45c

Correlation Coefficient (r)

	Patient 3	Patient 7	Patient 9	Patient 10
Area m	0.953*	-0.048	0.912*	0.156
SCT titre at V_o	0.861	-0.866	0.140	-0.342
Area p	-0.473	-0.925	-0.844*	-0.961**
Serum FDP/fdp	0.401	0	-0.978***	-0.573

The correlation coefficients (r) calculated by linear regression analysis for ante-natal sample data from four individual pre-eclamptic patients.

$2\alpha < 0.05$ *

$2\alpha < 0.01$ **

$2\alpha < 0.001$ ***

Table 45d

Clinical index and -	r	2α
area m	+0.497	<0.01
SCT titre at V_o	+0.453	<0.01
area p	+0.371	<0.05
serum FDP/fdp	+0.392	<0.05

Table 45e

Clinical index and -	r	2α
area m	+0.702	<0.001
SCT titre at V_o	+0.746	<0.001
area p	+0.508	<0.05
serum FDP/fdp	+0.578	<0.01

The correlation coefficients (r) calculated by linear regression analysis using the total ante-natal data (d) and the data from the last sample taken before delivery or intrauterine death (e) from the pre-eclamptic and normal pregnant patients included in Groups I and II, Chapter V.

Table 46a

Clinical Outcome in the Pregnancies Studied in Groups I and II, Chapter V

Pre-Eclampsia

Patient No.	Time Interval between Diagnosis and Delivery (weeks)	Foetal Outcome	Percentile Birth Weight
1	⟨1	Premature S.B.	50 - 75
2	1	Live	75 - 90
3	2	Premature N.N.D.	10 - 25
4	2	Live I.U.G.R.	⟨10
5	5	I.U.D.	-
6	1	I.U.D.	10 - 25
7	2	Live I.U.G.R.	⟨10
8	⟨1	(1) S.B. (2) I.U.D.	(1) ⟨10 (2) ⟨10
9	3	Premature Live I.U.G.R.	25 - 50
10	8	Live Premature	⟨10

Clinical outcome in the pre-eclamptic pregnancies included in Group I, Chapter V. (Patient 1 developed eclampsia during labour).

S.B. - still-birth

N.N.D. - neo-natal death

I.U.G.R. - intrauterine growth retardation

I.U.D. - intrauterine death

Percentile birth weight calculated according to Lubchenco et al., 1963.

Table 46b

Normal Pregnancy

	Foetal Outcome	Percentile Birth Weight
1	Live	25-50
2	Live	25-50
3	Live	50-75
4	Live	50-75
5	Live	25-50
6	Live	25-50
7	Live	50-75
8	Live	75-90
9	Live	50-75
10	Live	10-25

Clinical outcome in the normal pregnancies included in Group II, Chapter V. Percentile birth weight calculated according to Lubchenco et al., 1963.

Table 47a

Comparison of Laboratory Data Grouped on the Basis of Perinatal Deaths or Live Births (see Table 46)

	Perinatal Deaths	Live Babies
area m	15.9 ± 6.6	14.0 ± 6.4
SCT titre at V_o	2.9 ± 1.6	2.7 ± 1.7
area p	9.6 ± 4.2	10.4 ± 5.7
serum FDP/fdp	9.2 ± 3.9	8.1 ± 3.5

Means ± standard deviations of total ante-natal results on the pre-eclamptic patients grouped on the basis of perinatal deaths or live births. Differences between groups (unpaired "t" test):-

area m	t = 0.781	2p ⟨0.5 (NS)
SCT titre at V_o	t = 0.325	2p ⟨0.8 (NS)
area p	t = 0.424	2p ⟨0.7 (NS)
serum FDP/fdp	t = 0.733	2p ⟨0.5 (NS)

Table 47b

	Perinatal Deaths	Live Babies
area m	10.3 ± 6.5	16.6 ± 8.4
SCT titre at V_o	4.0 ± 1.4	3.4 ± 1.5
area p	8.7 ± 3.1	10.8 ± 8.4
serum FDP/fdp	11.5 ± 4.8	8.1 ± 4.7

Means ± standard deviations of results from last samples taken before delivery or intrauterine death from the pre-eclamptic patients grouped on the basis of perinatal deaths or live births. Differences between groups (unpaired "t" test):-

area m	t = 0.357	2p ⟨0.8 (NS)
SCT titre at V_o	t = 0.607	2p ⟨0.6 (NS)
area p	t = 0.526	2p ⟨0.7 (NS)
serum FDP/fdp	t = 1.045	2p ⟨0.4 (NS)

Table 47c

	Perinatal Deaths	Live Babies
area m	15.9 ± 6.6	10.6 ± 6.8
SCT titre at V_o	2.9 ± 1.6	2.1 ± 1.8
area p	9.6 ± 4.2	8.3 ± 5.4
serum FDP/fdp	9.2 ± 3.9	6.9 ± 3.4

Means ± standard deviations of results from all the ante-natal samples taken from the pre-eclamptic and normal pregnant patients (Groups I and II, Chapter V), the results being grouped on the basis of perinatal deaths and live births. Differences between groups (unpaired "t" test):-

area m	t = 2.337	2p ⟨0.025
SCT titre at V_o	t = 1.263	2p ⟨0.3 (NS)
area p	t = 0.725	2p ⟨0.5 (NS)
serum FDP/fdp	t = 1.956	2p ⟨0.1 (NS)

Table 47d

	Perinatal Deaths	Live Babies
area m	18.3 ± 6.5	8.7 ± 7.4
SCT titre at V_o	4.0 ± 1.4	1.8 ± 1.8
area p	8.7 ± 3.1	6.8 ± 5.6
serum FDP/fdp	11.5 ± 4.9	5.8 ± 3.2

Means ± standard deviations of results from the last sample taken before delivery or intrauterine death from the pre-eclamptic patients and normal pregnant control women, the results being grouped on the basis of perinatal deaths and live births.

Differences between groups (unpaired "t" test):-

area m	t = 2.589	2p $<$ 0.02
SCT titre at V_o	t = 2.282	2p $<$ 0.05
area p	t = 0.700	2p $<$ 0.5 (NS)
serum FDP/fdp	t = 2.964	2p $<$ 0.01

Table 48a

Comparison of Laboratory Data Grouped on the Basis of Percentile Birth Weight of the Baby (see Table 46)

	⟨10th Percentile	⟩10th Percentile
area m	12.7 ± 5.3	16.7 ± 7.1
SCT titre at V_o	3 ± 1.7	2.6 ± 1.6
area p	11.5 ± 6.8	8.4 ± 3.3
serum FDP/fdp	8.1 ± 3.5	8.9 ± 4.2

Means ± standard deviations of total ante-natal results on the pre-eclamptic patients grouped on the basis of baby birth weight (below or above the 10th percentile). Differences between groups (unpaired "t" test):-

area m	t = 1.522	2p ⟨0.2 (NS)
SCT titre at V_o	t = 0.542	2p ⟨0.6 (NS)
area p	t = 1.578	2p ⟨0.2 (NS)
serum FDP/fdp	t = 0.517	2p ⟨0.7 (NS)

Table 48b

	⟨10th Percentile	⟩10th Percentile
area m	16.9 ± 5.4	18.0 ± 9.6
SCT titre at V_o	3.8 ± 1.0	3.6 ± 1.8
area p	12.6 ± 8.7	7.4 ± 3.2
serum FDP/fdp	10.0 ± 5.0	10.5 ± 5.7

Means ± standard deviations of the results from the last sample taken before delivery or intrauterine death in the pre-eclamptic patients, the results being grouped on the basis of baby birth weight (below or above the 10th percentile). Differences between groups (unpaired "t" test):-

area m	t = 0.219	2p ⟨0.9 (NS)
SCT titre at V_o	t = 0.148	2p ⟨0.9 (NS)
area p	t = 1.255	2p ⟨0.3 (NS)
serum FDP/fdp	t = 0.125	2p ⟨0.95 (NS)

Table 48c

	⟨10th Percentile	⟩10th Percentile
area m	12.7 ± 5.3	12.1 ± 8.1
SCT titre at V_o	3 ± 1.7	2.0 ± 1.7
area p	11.5 ± 6.8	7.0 ± 3.3
serum FDP/fdp	8.1 ± 3.5	7.4 ± 4.0

Means ± standard deviations of the results from all the ante-natal samples taken from the pre-eclamptic and normal pregnant patients (Groups I and II, Chapter V), the results being grouped on the basis of baby birth weight (below or above the 10th percentile). Differences between groups (unpaired "t" test):-

area m	t = 0.219	2p ⟨0.9 (NS)
SCT titre at V_o	t = 1.626	2p ⟨0.2 (NS)
area p	t = 2.666	2p ⟨0.02
serum FDP/fdp	t = 0.466	2p ⟨0.7 (NS)

Table 48d

	⟨10th Percentile	⟩10th Percentile
area m	16.9 ± 5.4	9.1 ± 8.3
SCT titre at V_o	3.8 ± 1.0	1.8 ± 1.9
area p	12.6 ± 8.7	5.7 ± 2.7
serum FDP/fdp	10.0 ± 5.0	6.8 ± 4.4

Means ± standard deviations of results from the last sample taken before delivery or intrauterine death from the pre-eclamptic patients and normal pregnant control women, the results being grouped on the basis of baby birth weight (below or above the 10th percentile). Differences between groups (unpaired "t" test):-

area m	t = 1.735	2p ⟨0.2 (NS)
SCT titre at V_o	t = 1.884	2p ⟨0.1 (NS)
area p	t = 2.777	2p ⟨0.02
serum FDP/fdp	t = 1.141	2p ⟨0.3 (NS)

Table 49

	Total pre-eclamptic group	Total normal pregnant group	t	2p
Plasma fibrinogen (mg/100 ml)	267 ± 87 (33)	323 ± 68 (14)	2.004	nearly = 0.05
area m percent	14.8 ± 6.2 (35)	4.4 ± 1.0 (14)	6.176	<0.001
SCT titre at V_o (neg. log. to base 2)	3.0 ± 1.6 (28)	1.2 ± 1.4 (11)	3.079	<0.005
FR-antigen ratio	0.66 ± 0.08 (33)	0.80 ± 0.03 (11)	5.493	<0.001
TCP ratio	0.65 ± 0.08 (34)	0.84 ± 0.3 (11)	7.946	<0.001
area p percent	9.7 ± 4.8 (35)	5.1 ± 2.3 (14)	3.347	<0.005
serum FDP/ fdp (μg/ml)	8.9 ± 4.1 (32)	4.9 ± 1.9 (12)	3.272	<0.005

Differences between the total ante-natal data from pre-eclamptic patients (35 samples from 15 patients) and from normal pregnant women (14 samples from 14 patients, Table 53, Chapter VII) analysed using the unpaired "t" test. Numbers in brackets indicate number of observations.

Table 50

	Group I (Chapter V)	Total pre-eclamptic group	t	2p
Plasma fibrinogen (mg/100 ml)	281 ± 84 (10)	267 ± 87 (33)	0.461	<0.7 (NS)
area m percent	16.0 ± 6.4 (10)	14.8 ± 6.2 (35)	0.532	<0.6 (NS)
SCT titre at V_o (neg. log. to base 2)	3.9 ± 1.0 (9)	3.0 ± 1.6 (28)	1.517	<0.2 (NS)
FR-antigen ratio	0.61 ± 0.01 (10)	0.66 ± 0.08 (33)	1.837	<0.1 (NS)
TCP ratio	0.61 ± 0.02 (10)	0.65 ± 0.08 (34)	1.713	<0.1 (NS)
area p percent	9.1 ± 5.9 (10)	9.7 ± 4.8 (35)	0.285	<0.8 (NS)
serum FDP/ fdp (μg/ml)	9.7 ± 4.2 (10)	8.9 ± 4.1 (32)	0.570	<0.6 (NS)

Differences between the selected group of data included in Group I, Chapter V and the total ante-natal pre-eclamptic data (35 samples from 15 patients). Numbers in brackets indicate number of observations.

Table 51

Single Pregnancies

	Age (years)	Parity	Gestation (weeks)	S.B.P. (mmHg)	D.B.P. (mmHg)	Complications
1	16	$0 + {}^{0}$	24	110	75	None
2	27	$0 + {}^{1}$	27	130	80	"
3	20	$1 + {}^{0}$	28	110	70	"
4	23	$0 + {}^{0}$	28	130	75	"
5	35	$0 + {}^{0}$	30	140	80	"
6	35	$4 + {}^{0}$	32	135	80	"
7	27	$1 + {}^{0}$	33	125	75	"
8	22	$0 + {}^{0}$	34	110	70	"
9	24	$2 + {}^{1}$	34	90	60	"
10	17	$0 + {}^{0}$	34	120	80	"
11	29	$0 + {}^{0}$	36	110	70	"
12	22	$0 + {}^{0}$	36	100	60	"
13	25	$0 + {}^{0}$	38	120	80	"
14	22	$1 + {}^{0}$	39	130	75	"
mean ± SD	25 ± 6		32 ± 5	119 ± 14	74 ± 7	
15	27	$0 + {}^{0}$	32	120	75	Hypertension at 36 weeks

Clinical data on the apparently normal single pregnancies studied. Patient 15 developed hypertension later in pregnancy and is therefore placed separately at the foot of the table. Means ± standard deviations of the data from the remaining 14 patients are shown.

Table 52

Twin Pregnancies

	Age (years)	Parity	Gestation (weeks)	S.B.P. (mmHg)	D.B.P. (mmHg)	Complications
16	28	$0 + {}^{1}$	28	110	80	None
17	16	$0 + {}^{0}$	30	120	75	"
18	30	$0 + {}^{0}$	34	150	80	"
19	33	$0 + {}^{0}$	35	110	65	"
mean ± SD	27 ± 7		32 ± 3	123 ± 19	75 ± 7	
20	20	$1 + {}^{0}$	30	110	80	Hypertension at 36 weeks
21	37	$1 + {}^{0}$	34	120	85	Hypertension at 37 weeks

Clinical data on the apparently normal twin pregnancies studied. Patients 20 and 21 developed hypertension later in pregnancy and are therefore placed separately at the foot of the table. Means ± standard deviations of the results from the remaining 4 patients are shown.

Table 53a

Single pregnancies

	Plasma fibrinogen (mg/100 ml)	Serum FDP/fdp (µg/ml)
1	253	3.75
2	307	5.0
3	297	2.5
4	180	2.5
5	380	5.0
6	350	5.0
7	300	5.0
8	460	N.D.
9	150	10
10	357	5
11	295	5
12	439	5
13	400	N.D.
14	350	5
mean ± SD	323 ± 88	4.9 ± 1.9
15	366	5

	Gestational age (weeks)	area m (percent)	area n (percent)	area p (percent)	SCT titre at V_0 (neg. log. to base 2)	FR-antigen ratio	TCP ratio
1	24	5.7	87.9	6.3	0	0.85	0.81
2	27	4.0	91.3	4.7	0	0.83	0.85
3	28	5.8	91.9	2.1	2	0.83	0.80
4	28	3.8	92.0	4.1	0.5	0.78	0.82
5	30	4.5	92.9	2.6	0	0.80	0.87
6	32	4.1	88.3	7.6	N.D.	N.D.	N.D.
7	33	3.8	90.3	5.9	1	0.77	0.88
8	34	4.5	93.6	2.0	N.D.	N.D.	N.D.
9	34	5.5	85.6	8.9	4	0.76	0.86
10	34	3.3	91.5	5.0	2	0.81	0.83
11	36	4.3	88.2	7.5	1	0.75	0.82
12	36	4.2	92.3	3.2	3	0.77	0.84
13	38	2.5	89.3	8.3	N.D.	N.D.	N.D.
14	39	5.8	90.3	3.6	0	0.80	0.88
mean ± SD	32 ± 4	4.4 ± 1.0	90.4 ± 2.3	5.1 ± 2.3	1.2 ± 1.4	0.80 ± 0.03	0.84 ± 0.03
15	32	6.0	90.0	4.1	0	0.82	0.85

Table 54a

Twin pregnancies

	Plasma fibrinogen (mg/100 ml)	Serum FDP/fdp (μg/ml)
16	365	5
17	258	5
18	297	5.0
19	330	2.5
mean ± SD	313 ± 46	4.4 ± 1.3
20	487	10
21	399	5

Laboratory results on the samples from patients in Table 52. Means ± standard deviations are shown for the results from the 4 twin pregnancies uncomplicated by hypertension.

Table 54b

Twin pregnancies

	Gestational age (weeks)	area m (percent)	area n (percent)	area p (percent)	SCT titre at V_o (neg. log. to base 2)	FR-antigen ratio	TCP ratio
16	28	6.7	86.9	6.9	2	0.77	0.76
17	30	6.0	85.6	8.5	3	0.68	0.74
18	34	3.7	90.0	6.4	3	0.82	0.84
19	35	5.9	89.7	4.4	3	0.79	0.78
mean	32	5.6	88.0	6.6	2.8	0.77	0.78
±	±	±	±	±	±	±	±
SD	3	1.3	2.2	1.7	0.5	0.06	0.04
20	30	6.0	85.6	8.5	2	0.75	0.78
21	34	2.7	91.8	5.6	0.5	0.78	0.80

Table 55

Comparison of 14 single and 4 twin pregnancies

	t	2p
Gestation	0.253	<0.9 (NS)
Maternal age	0.633	<0.6 (NS)
S.B.P.	0.456	<0.7 (NS)
D.B.P.	0.363	<0.6 (NS)
Plasma fibrinogen	0.219	<0.9 (NS)
Serum FDP/fdp	0.511	<0.7 (NS)
Area m	1.944	<0.1 (NS)
Area n	1.836	<0.1 (NS)
Area p	1.127	<0.3 (NS)
SCT' titre at V_o	2.133	nearly = 0.05
FR-antigen Ratio	1.286	<0.3 (NS)
TCP Ratio	3.278	<0.01

Differences between single and twin pregnancies (uncomplicated by hypertension) using the data in Tables 51-54 analysed by the unpaired "t" test.

Table 56 a

Suspected intrauterine growth retardation patients

	Age (years)	Parity	Gestation when sample taken (weeks)	S.B.P. (mmHg)	D.B.P. (mmHg)
1	25	2 + 0	36	125	75
2	26	0 + 0	37	125	80
3	31	2 + 0	39	120	70
4	26	4 + 0	37	110	70
5	22	1 + 1	37	100	60
6	19	1 + 0	27	130	80
7	30	3 + 1	37	120	75
8	33	0 + 0	35	110	80
9	32	0 + 0	36	120	85
10	23	1 + 0	37	125	80
mean ± SD	27 ± 5		36 ± 3	119 ± 9	76 ± 7

Clinical data on the 10 patients studied with a diagnosis of suspected intrauterine growth retardation. Where relevant means ± standard deviations are shown.

Table 56b

Suspected intrauterine growth retardation patients

	Approximate gestational age at birth (weeks)	Sex of baby	Birth weight (Kg)	Percentile birth weight	Other complications
1	36	M	2.0	<10	Premature
2	40	M	2.6	<10	None
3	39-40	F	3.2	50-75	None
4	42	F	2.7	10-25	None
5	39-40	F	2.2	<10	None
6	39	M	3.4	50-75	None
7	39	F	2.6	10-25	None
8	35	M	1.6	<10	Premature
9	38	M	2.4	<10	Hypertension at 37 weeks (B.P. 140/90 mmHg). No proteinuria.
10	39	M	3.0	25-50	None

Clinical data on the 10 patients studied with a diagnosis of suspected intrauterine growth retardation.

M = Male

F = Female

Table 57

Suspected Intrauterine Growth Retardation Patients

	Gestation (weeks)	Plasma Fibrinogen (mg/100 ml)	Serum FDP/fdp (μg/ml)
1	36	431	5.0
2	37	322	2.5
3	39	229	2.5
4	37	297	2.5
5	37	322	5.0
6	27	234	3.75
7	37	210	2.5
8	34	322	5.0
9	36	360	2.5
10	37	325	7.5
mean ± SD	36 ± 3	305 ± 67	3.9 ± 1.7

Plasma fibrinogen and serum FDP/fdp results for samples from patients with suspected intrauterine growth retardation (Table 56). Means ± standard deviations are shown.

Table 58

Suspected Intrauterine Growth Retardation Patients

Diagnosis Confirmed

	Gestation (weeks)	Plasma Fibrinogen (mg/100 ml)	Serum FDP/fdp (μg/ml)
1	36	431	5.0
2	37	322	2.5
5	37	322	5.0
8	34	322	5.0
9	36	360	2.5
mean ± SD	36 ± 1.2	351 ± 47	4.0 ± 1.4

Diagnosis Not Confirmed

3	39	229	2.5
4	37	297	2.5
6	27	234	3.7
7	37	210	2.5
10	37	325	7.5
mean ± SD	35.4 ± 4.8	259 ± 49	3.7 ± 2.2

Plasma fibrinogen and serum FDP/fdp results for samples from patients in Table 56, grouped according to whether the diagnosis of intrauterine growth retardation was confirmed on the basis of the baby's percentile birth weight or not. The gestational age of the foetus at the time of blood sampling is also indicated. Means ± standard deviations are shown.

Table 59

Suspected intrauterine growth retardation patients

	m (percent)	n (percent)	p (percent)	SCT titre at V_o (neg. log. to base 2)	FR-antigen ratio	TCP Ratio
1	10.0	79.3	10.7	1	0.79	0.75
2	5.8	83.4	10.7	2	0.77	0.81
3	4.1	87.6	8.6	1	0.78	0.83
4	5.9	89.7	4.5	1	0.80	0.82
5	9.6	84.4	6.0	1	0.77	0.82
6	6.2	86.7	7.1	2	0.77	0.82
7	5.7	89.4	5.0	4	0.79	0.85
8	7.7	83.7	8.6	3	0.86	0.83
9	8.3	82.7	8.9	1	0.77	0.84
10	3.9	91.0	5.0	2	0.75	0.76
mean ± SD	6.7 ± 2.1	85.8 ± 3.7	7.5 ± 2.3	1.8 ± 1.0	0.79 ± 0.03	0.81 ± 0.03

Plasma fibrinogen chromatography results for samples from patients with suspected intrauterine growth retardation (Table 56). Means ± standard deviations are shown.

Table 56a

Suspected Intrauterine Growth Retardation Patients

Diagnosis Confirmed

	m (percent)	n (percent)	p (percent)	SCT titre at V_o (neg. log. to base 2)	FR-antigen ratio	TCP Ratio
1	10	79.3	10.7	1	0.79	0.75
2	5.8	83.4	10.7	2	0.77	0.81
5	9.6	84.4	6.0	1	0.77	0.82
8	7.7	83.7	8.6	3	0.86	0.83
9	8.3	82.7	8.9	1	0.77	0.84
mean ± SD	8.3 ± 1.7	82.7 ± 2.0	9.0 ± 1.9	1.6 ± 0.9	0.79 ± 0.04	0.81 ± 0.04

Plasma fibrinogen chromatography results for samples from patients in Table 55 in whom the diagnosis of intrauterine growth retardation was confirmed. Means ± standard deviations are shown.

Table 60b

Suspected Intrauterine Growth Retardation Patients

Diagnosis Not Confirmed

	m (percent)	n (percent)	p (percent)	SCT titre at V_o (neg. log. to base 2)	FR-antigen ratio	TCP Ratio
3	4.1	87.6	8.6	1	0.78	0.83
4	5.9	89.7	4.5	1	0.80	0.82
6	6.2	86.7	7.1	2	0.77	0.82
7	5.7	89.4	5.0	4	0.79	0.85
10	3.9	91.0	5.0	2	0.75	0.76
mean ± SD	5.2 ± 1.1	88.9 ± 1.7	6.0 ± 1.7	2.0 ± 1.2	0.78 ± 0.02	0.82 ± 0.03

Plasma fibrinogen chromatography results for samples from patients in Table 56 in whom the diagnosis of intrauterine growth retardation was not confirmed. Means ± standard deviations are shown.

Table 61

Comparison of data from the 5 patients in whom the diagnosis of intrauterine growth retardation was confirmed and that from the 5 patients in whom the diagnosis was not confirmed

	t	2p
Gestation	0.272	<0.8 (NS)
Age	0.587	<0.6 (NS)
S.B.P.	0.851	<0.5 (NS)
D.B.P.	0.206	<0.9 (NS)
Plasma fibrinogen	3.019	<0.02
Serum FDP/fdp	0.227	<0.9 (NS)
area m	3.508	<0.01
area n	5.245	<0.001
area p	2.523	<0.05
SCT titre at V_o	0.590	<0.6 (NS)
FR-antigen ratio	0.720	<0.5 (NS)
TCP ratio	0.275	<0.8 (NS)

The data from patients in whom the diagnosis of intrauterine growth retardation was confirmed on the basis of percentile birth weight and those in whom it was not confirmed (Tables 56, 58 and 60) analysed using the unpaired "t" test.

Table 62

Comparison of data from the 5 patients in whom the diagnosis of intrauterine growth retardation was confirmed and that from 14 normal pregnant women

	t	2p
Gestation	1.901	⟨0.1 (NS)
Age	1.059	⟨0.4 (NS)
S.B.P.	0.366	⟨0.8 (NS)
D.B.P.	0.610	⟨0.6 (NS)
Plasma fibrinogen	0.683	⟨0.6 (NS)
Serum FDP/fdp	0.957	⟨0.4 (NS)
area m	6.262	⟨0.001
area n	6.691	⟨0.001
area p	3.295	⟨0.005
SCT titre at V_o	0.553	⟨0.6 (NS)
FR-antigen ratio	0.186	⟨0.9 (NS)
TCP ratio	1.939	⟨0.9 (NS)

The data from 5 patients in whom the diagnosis of intrauterine growth retardation was confirmed (Tables 56, 58 and 60) and the 14 normal single pregnancies (Tables 51 and 53) analysed using the unpaired "t" test.

Table 63

Comparison of data from the 5 patients in whom the diagnosis of intrauterine growth retardation was not confirmed and that from 14 normal pregnant women

	t	2p
Gestation	1.298	<0.3 (NS)
Age	0.425	<0.7 (NS)
S.B.P.	0.361	<0.8 (NS)
D.B.P.	0.421	<0.7 (NS)
Plasma fibrinogen	1.512	<0.2 (NS)
Serum FDP/fdp	1.107	<0.3 (NS)
area m	1.419	<0.2 (NS)
area n	1.345	<0.2 (NS)
area p	0.792	<0.5 (NS)
SCT titre at V_o	1.079	<0.3 (NS)
FR-antigen ratio	1.108	<0.3 (NS)
TCP ratio	1.603	<0.2 (NS)

The data from the 5 patients in whom the diagnosis of intrauterine growth retardation was not confirmed (Tables 56, 58 and 60) and the 14 normal single pregnancies (Tables 51 and 53) analysed using the unpaired "t" test.

Table 64

Comparison of data from the 5 patients in whom the diagnosis of intrauterine growth retardation was confirmed and that from 10 pre-eclamptic patients (Group I)

	t	2p
Gestation	2.820	<0.02
Age	1.321	<0.3 (NS)
S.B.P.	3.895	<0.005
D.B.P.	5.250	<0.001
Plasma fibrinogen	1.709	<0.2 (NS)
Serum FDP/fdp	2.964	<0.02
area m	2.610	<0.025
area n	2.033	<0.1 (NS)
area p	0.055	<0.975 (NS)
SCT titre at V_o	4.089	<0.005
FR-antigen ratio	14.889	<0.001
TCP ratio	14.539	<0.001

The data from the 5 patients in whom the diagnosis of intra-uterine growth retardation was confirmed (Tables 56, 58 and 60) and the 10 pre-eclamptic patients (10 samples) studied in Group I, Chapter V analysed using the unpaired "t" test.

Table 65

	Age (years)	Parity	Gestation (weeks)	S.B.P. (mmHg)	D.B.P. (mmHg)	Diagnosis	Complications
1	28	$0 + {}^{0}$	33	150	120	Essential Hypertension	Borderline intrauterine growth retardation
2	25	$1 + {}^{0}$	20	130	90	Essential Hypertension	Baby had severe congenital heart disease
3	41	$4 + {}^{1}$	18	140	100	Essential Hypertension	None
4	27	$0 + {}^{0}$	36	150	110	Essential Hypertension	None
5	28	$0 + {}^{0}$	22	150	100	Essential Hypertension ?? Renal artery stenosis	None
mean ± SD	30 ± 6		26 ± 8	144 ± 9	104 ± 11		

Clinical data on the 5 patients studied with a diagnosis of essential hypertension in pregnancy. Where relevant means ± standard deviations are shown.

Table 66

	m (percent)	n (percent)	p (percent)	SCT titre at V_o (neg. log. to base 2)	FR-antigen ratio	TCP ratio	Plasma fibrinogen mg/100 ml	Serum FDP/fdp µg/ml
1	3.3	91.8	4.3	1	0.78	0.84	267	2.5
2	4.0	86.9	9.0	4	0.78	0.84	170	1.25
3	2.6	93.2	4.1	1	0.80	0.78	282	2.5
4	3.9	92.7	3.4	0.5	0.77	0.82	368	10.0
5	5.2	87.9	6.9	1	0.78	0.84	355	5.0
mean ± SD	3.9 ± 0.9	90.5 ± 2.9	5.5 ± 2.3	1.5 ± 1.4	0.78 ± 0.01	0.82 ± 0.03	292 ± 79	4.3 ± 3.5

Laboratory results obtained of samples from patients with essential hypertension in pregnancy (Table 65). Means ± standard deviations are shown.

Table 67

Comparison of data from the 5 pregnant women with essential hypertension and that from 14 normal pregnant women

	t	2p
Gestation	2.280	<0.05
Age	1.709	<0.2 (NS)
S.B.P.	3.711	<0.005
D.B.P.	7.128	<0.001
Plasma fibrinogen	0.675	<0.6 (NS)
Serum FDP/fdp	0.502	<0.7 (NS)
area m	1.015	<0.4 (NS)
area n	0.090	<0.95 (NS)
area p	0.338	<0.8 (NS)
SCT titre at V_o	0.366	<0.8 (NS)
FR-antigen ratio	0.802	<0.4 (NS)
TCP ratio	1.196	<0.3 (NS)

The data from the 5 patients with essential hypertension in pregnancy (Tables 65 and 66) and the 14 normal single pregnancies (Tables 51 and 53) analysed using the unpaired "t" test.

Table 68

Comparison of data from the 5 pregnant women with essential hypertension and that from 10 pre-eclamptic patients (Group I)

	t	2p
Gestation	1.736	⟨0.2 (NS)
Age	1.900	0.1 (NS)
S.B.P.	0.605	⟨0.6 (NS)
D.B.P.	0.481	⟨0.7 (NS)
Plasma fibrinogen	0.245	⟨0.9 (NS)
Serum FDP/fdp	2.533	⟨0.025
area m	4.131	⟨0.005
area n	4.029	⟨0.005
area p	1.297	⟨0.3 (NS)
SCT titre at V_o	3.610	⟨0.005
FR-antigen ratio	34.893	⟨0.001
TCP ratio	18.309	⟨0.001

The data from the 5 patients with essential hypertension in pregnancy (Tables 65 and 66) and the 10 pre-eclamptic patients (10 samples) studied in Group I, Chapter V analysed using the unpaired "t" test.

Table 69

	Age (years)	Parity	Gestation (weeks)	S.B.P. (mmHg)	D.B.P. (mmHg)	Proteinuria (g/24 hrs)	Diagnosis	Complications
1	30	$1 + {}^{0}$	36	170	110	None	Chronic pyelonephritis	Developed proteinuria at 38 weeks
2	30	$0 + {}^{0}$	35	140	85	+++	SLE	Anaemia Severe hypertension at 37 weeks. Acute renal failure post-partum.
3	39	$4 + {}^{1}$	32	170	110	3g/24 hrs	Chronic pyelonephritis	Recurrent urinary tract infection
4	34	$1 + {}^{0}$	32	170	95	+++	Chronic pyelonephritis (nephrectomy 10 yrs previously)	Pre-term and dysmature baby
5	21	$1 + {}^{0}$	32	160	100	3.8g/24 hrs	Chronic pyelonephritis	None
mean ± SD	31 ± 7		33 ± 2	162 ± 13	100 ± 11			

Clinical data on the 5 patients studied with a diagnosis of chronic renal disease in pregnancy. Where relevant means ± standard deviations are shown.

Table 70

	m (percent)	n (percent)	p (percent)	SCT titre at V_o (neg. log. to base 2)	FR-antigen ratio	TCP ratio	Plasma fibrinogen mg/100 ml	Serum FDP/fdp µg/ml
1	4.4	86.0	9.5	-	0.81	0.66	317	-
2	3.8	93.8	2.5	2	0.61	0.78	350	10
3	3.0	94.4	2.7	0.5	0.73	0.81	328	5
4	4.3	81.6	14.2	2	0.79	0.89	300	10
5	4.5	84.1	11.1	0	0.74	0.79	525	20
mean ± SD	4.0 ± 0.6	88.0 ± 5.8	8.0 ± 5.2	1.1 ± 1.0	0.74 ± 0.08	0.77 ± 0.06	364 ± 92	11.2 ± 6.3

Laboratory results obtained on samples from the patients with chronic renal disease in pregnancy (Table 69). Means ± standard deviations are shown.

Table 71

Comparison of data from 5 pregnant patients with chronic renal disease and that from 14 normal pregnant women

	t	2p
Gestation	0.504	<0.7 (NS)
Age	2.015	<0.1 (NS)
S.B.P.	5.982	<0.001
D.B.P.	6.390	<0.001
Plasma fibrinogen	0.888	<0.4 (NS)
Serum FDP/fdp	3.279	<0.01
area m	0.870	<0.4 (NS)
area n	1.341	<0.2 (NS)
area p	1.697	<0.2 (NS)
SCT titre at V_o	0.135	<0.9 (NS)
FR-antigen ratio	2.211	<0.05
TCP ratio	3.373	<0.005

The data from the 5 patients with chronic renal disease in pregnancy (Tables 69 and 70) and the 14 normal single pregnancies (Tables 51 and 53) analysed using the unpaired "t" test.

Table 72

Comparison of 5 pregnant patients with chronic renal disease (? with superimposed pre-eclampsia) and 10 patients with pre-eclampsia

	t	2p
Gestation	1.220	⟨0.3 (NS)
Age	2.186	⟨0.05
S.B.P.	1.486	⟨0.2 (NS)
D.B.P.	0.297	⟨0.8 (NS)
Plasma fibrinogen	1.743	⟨0.2 (NS)
Serum FDP/fdp	0.530	⟨0.7 (NS)
area m	4.107	⟨0.005
area n	3.138	⟨0.01
area p	0.364	⟨0.8 (NS)
SCT titre at V_o	4.390	⟨0.005
FR-antigen ratio	5.378	⟨0.001
TCP ratio	7.693	⟨0.001

The data from the 5 patients with chronic renal disease in pregnancy (Tables 69 and 70) and from the 10 pre-eclamptic patients (10 samples) studied in Group I, Chapter V analysed using the unpaired "t" test.

Table 73

Normotensive women taking oestrogen-containing oral contraceptive drugs

	Age (years)	S.B.P. (mmHg)	D.B.P. (mmHg)	Plasma fibrinogen (mg/100 ml)	Serum FDP/fdp (μg/ml)
1	26	120	80	239	2.5
2	25	115	75	177	0
3	25	115	70	151	0
4	25	110	70	216	1.25
5	27	105	60	203	2.5
6	26	115	80	244	2.5
7	20	110	60	157	0
8	21	105	75	214	5
9	24	115	80	191	2.5
10	21	100	60	228	2.5
mean ± SD	24 ± 2	111 ± 6	71 ± 8	202 ± 33	1.9 ± 1.6

Clinical data on 10 healthy, normotensive women taking oestrogen-containing oral contraceptive drugs (ethinyl-oestradiol or mestranol 50 μg per day). Plasma fibrinogen and serum FDP/fdp results on samples taken from these women are also given. Means ± standard deviations are shown.

Table 74

Normotensive women taking oestrogen-containing oral contraceptive drugs

	m (percent)	n (percent)	p (percent)	SCT titre at V_0 (neg. log. to base 2)	FR-antigen ratio	TCP ratio
1	5.8	90	3.8	0	0.87	0.84
2	5.1	92.9	1.7	0	0.89	0.91
3	4.6	91.0	4.4	0.5	0.85	0.89
4	3.4	91.7	4.9	0	0.89	0.88
5	4.4	93.5	1.9	0	0.89	0.88
6	5.7	87.4	6.8	0	0.86	0.86
7	4.0	93.4	2.7	0	0.87	0.90
8	2.7	91.5	5.4	0	0.85	0.92
9	4.0	92.8	3.1	0	0.89	0.88
10	4.2	94.5	1.3	0	0.85	0.85
mean ± SD	4.4 ± 1.0	91.9 ± 2.1	3.6 ± 1.8	0.1 ± 0.2	0.87 ± 0.02	0.88 ± 0.03

Plasma fibrinogen chromatography data on samples from the 10 normotensive women taking oestrogen-containing oral contraceptive drugs (Table 73). Means ± standard deviations are shown.

Table 75

Comparison of data from 10 normotensive women taking oestrogen-containing oral contraceptive drugs and that from 10 normotensive women not taking these drugs

	t	2p
Age	0.664	⟨0.6 (NS)
S.B.P.	0.349	⟨0.8 (NS)
D.B.P.	0.294	⟨0.8 (NS)
Plasma fibrinogen	0.302	⟨0.8 (NS)
Serum FDP/fdp	0.764	⟨0.5 (NS)
area m	5.018	⟨0.001
area n	2.092	⟨0.1 (NS)
area p	0.223	⟨0.9 (NS)
SCT titre at V_o	1.000	⟨0.4 (NS)
FR-antigen ratio	0.876	⟨0.4 (NS)
TCP ratio	0.935	⟨0.4 (NS)

The data from the 10 normotensive women taking oestrogen-containing oral contraceptive drugs (Tables 73 and 74) and the 10 normotensive women not taking any drug therapy (Group III, Chapter V) analysed using the unpaired "t" test.

Table 76

Comparison of data from 10 women taking oestrogen-containing oral contraceptive drugs and that from 14 normal pregnant women

	t	2p
Age	0.296	<0.8 (NS)
S.B.P.	1.576	<0.2 (NS)
D.B.P.	0.820	<0.5 (NS)
Plasma fibrinogen	4.103	<0.001
Serum FDP/fdp	4.020	<0.001
area m	0.060	<0.975 (NS)
area n	1.641	<0.2 (NS)
area p	1.737	<0.1 (NS)
SCT titre at V_o	2.701	<0.02
FR-antigen ratio	6.712	<0.001
TCP ratio	3.321	<0.005

The data from the 10 normotensive women taking oestrogen-containing oral contraceptive drugs (Tables 73 and 74) and the 14 normal pregnant women (Tables 51 and 52) analysed using the unpaired "t" test.

Table 77

Patients with suspected "pill-related hypertension"

	Age (years)	S.B.P. (mmHg)	D.B.P. (mmHg)	Plasma fibrinogen (mg/100 ml)	Serum FDP/fdp (μg/ml)
1	45	190	120	175	2.5
2	41	140	90	263	7.5
3	44	160	110	278	5.0
4	26	160	110	306	2.5
5	33	170	116	278	5.0
mean ± SD	38 ± 8	168 ± 19	109 ± 12	260 ± 50	4.6 ± 2.1

Clinical data on 5 patients with suspected "pill-related hypertension". Plasma fibrinogen and serum FDP/fdp results on samples taken from these women are also given. Means ± standard deviations are shown.

Table 78

Hypertensive women taking oestrogen-containing preparations

	m (percent)	n (percent)	p (percent)	SCT titre at V_0 log. (neg. to base 2)	FR-antigen ratio	TCP ratio
1	7.3	86.7	6.1	2	0.78	0.85
2	2.7	93.9	3.5	1	0.85	0.87
3	2.0	94.6	3.5	0.5	0.87	0.87
4	7.9	87.5	4.5	3	0.80	0.85
5	4.2	88.5	7.4	3	0.84	0.86
mean ± SD	4.8 ± 2.7	90.2 ± 3.7	5.0 ± 1.7	1.9 ± 1.1	0.83 ± 0.04	0.86 ± 0.01

Plasma fibrinogen chromatography data on the 5 patients with suspected "pill-related hypertension" (Table 77). Means ± standard deviations are shown.

Table 79

Comparison of data from 5 patients with suspected "pill-related hypertension" and that from the 10 normotensive women taking oestrogen-containing oral contraceptive drugs

	t	2p
Age	5.104	<0.001
S.B.P.	8.795	<0.001
D.B.P.	7.343	<0.001
Plasma fibrinogen	2.733	<0.02
Serum FDP/fdp	2.727	<0.02
area m	0.466	<0.7 (NS)
area n	1.106	<0.3 (NS)
area p	1.450	<0.2 (NS)
SCT titre at V_o	5.229	<0.001
FR-antigen ratio	3.254	<0.01
TCP ratio	1.743	<0.2 (NS)

The data from the 5 patients with suspected "pill-related hypertension" (Tables 77 and 78) and the 10 normotensive women taking oestrogen-containing oral contraceptive drugs (Tables 73 and 74) analysed using the unpaired "t" test.

Table 80

A summary of the results (expressed as the mean ± a single standard deviation) for the following clinical conditions studied in this thesis:-

(i) Non-pregnant controls - Chapter V

(ii) Normotensive women taking oestrogen-containing oral contraceptive therapy ("pill") - Chapter VIII

(iii) Normal pregnant controls - Chapter VI

(iv) Intrauterine growth retardation (I.U.G.R.) patients - Chapter VII

(v) Essential hypertension in pregnancy - Chapter VII

(vi) Pre-eclampsia (PET Group I patients) - Chapter V

(vii) Pre-eclampsia (PET total data) - Chapter VI

(viii) 6 hours intravenous (IV) ancrod infusion - Chapter IV

(ix) Local venous thrombosis (local thrombosis) - Appendix IV

Table 80

	Plasma fibrinogen (mg/100 ml)	area m (percent)	SCT titre at V_o (neg. log. to base 2)	FR-antigen ratio	TCP ratio	area p (percent)	Serum FDP/fdp (µg/ml)
Non-pregnant controls	207 ± 38	2.7 ± 0.4	0	0.87 ± 0.01	0.89 ± 0.03	3.4 ± 1.6	2.4 ± 1.2
Normotensive "pill"	202 ± 33	4.4 ± 1.0	0.2 ± 0.6	0.87 ± 0.02	0.88 ± 0.02	3.6 ± 1.8	1.9 ± 1.5
Normal pregnant controls	323 ± 88	4.4 ± 1.0	1.2 ± 1.4	0.80 ± 0.03	0.84 ± 0.03	5.1 ± 2.3	4.9 ± 1.9
I.U.G.R.	351 ± 47	8.3 ± 1.7	1.6 ± 0.9	0.79 ± 0.04	0.81 ± 0.04	9.0 ± 1.9	4.0 ± 1.4
Essential hypertension	292 ± 79	3.9 ± 0.9	1.5 ± 1.4	0.78 ± 0.01	0.82 ± 0.03	5.5 ± 2.3	4.2 ± 3.5
PET - Group I	281 ± 84	16.0 ± 6.4	3.9 ± 1.0	0.61 ± 0.01	0.61 ± 0.02	9.1 ± 5.9	9.7 ± 4.2
PET - Total	267 ± 87	14.8 ± 6.2	3.1 ± 1.6	0.66 ± 0.08	0.65 ± 0.08	9.7 ± 4.8	8.9 ± 4.1
6 hrs. IV Ancrod	101 ± 96	19.6 ± 8.0	6.5 ± 1.9	0.63 ± 0.01	0.64 ± 0.01	28.1 ± 10.1	2,720 ± 2,234
Local thrombosis (Appendix IV)	222 ± 61	5.7 ± 1.9	2.0 ± 2.3	0.73 ± 0.12	0.69 ± 0.10	8.8 ± 3.0	25.8 ± 29.9

Table 81

X	Y	γ	n	2α
area m	Plasma fibrinogen	0.007	123	>0.1 (NS)
area p	Plasma fibrinogen	0.033	123	>0.1 (NS)
area m	Serum FDP/fdp	0.176	119	<0.1 (NS)
area p	Serum FDP/fdp	0.198	119	<0.05
area m	SCT titre at V_o	0.506	109	<0.001
area m	FR-antigen ratio	-0.546	120	<0.001
area m	TCP ratio	-0.604	114	<0.001
FR-antigen ratio	TCP ratio	0.782	114	<0.001
SCT titre at V_o	FR-antigen ratio	-0.646	109	<0.001
SCT titre at V_o	TCP ratio	-0.604	109	<0.001
area m	Area p	+0.312	125	<0.001

The paired laboratory results (X and Y) for the samples (n = number of pairs) described in this thesis. The correlation coefficient ( ) and 2α values are shown.

Table 82a

Batch I

Column	approximate height (cm)	radius (cm)	bed volume (ml)	V_o (ml)	V_e fibrinogen (ml)	$\frac{V_e}{V_o}$
A1 (I)	40.0	1.25	196	69.6 ± 0.6	95.4 ± 0.4	1.37
A3 (I)	40.0	1.25	196	68.1 ± 0.6	95.6 ± 0.6	1.40
B1 (I)	35.0	1.3	186	58.7 ± 0.9	87.0 ± 0.8	1.48
B2 (I)	34.5	1.3	183	59.5 ± 0.8	84.5 ± 1.0	1.42
B3 (I)	35.0	1.3	186	61.2 ± 1.5	88.4 ± 0.6	1.44
B4 (I)	35.0	1.3	186	60.0 ± 0	88.1 ± 0.8	1.47
C1 (I)	35.0	1.3	186	62.2 ± 1.2	89.4 ± 1.4	1.43
C2 (I)	34.5	1.3	183	56.7 ± 0.5	84.7 ± 0.5	1.49

Details of packed agarose gel column systems used in this thesis.

V_o = void volume

V_e = elution volume

Table 82b

Batch II

Column	approximate height (cm)	radius (cm)	bed volume (ml)	V_o (ml)	V_e fibrinogen (ml)	$\frac{V_e}{V_o}$
B5 (II)	34.5	1.3	183	56.0 ± 0.9	95.4 ± 0.5	1.70
B7 (II)	34.5	1.3	183	57.4 ± 1.6	97.6 ± 0.4	1.70
B8 (II)	35.0	1.3	186	61.1 ± 1.2	101.2 ± 1.0	1.66
B9 (II)	34.5	1.3	183	58.6 ± 1.6	96.9 ± 1.4	1.65
B10 (II)	34.5	1.3	183	58.4 ± 1.4	96.5 ± 1.3	1.65
C3 (II)	35.0	1.3	186	61.2 ± 1.2	100.3 ± 0.5	1.64
C5 (II)	34.5	1.3	183	59.0 ± 0.7	98.3 ± 0.6	1.67
C6 (II)	34.0	1.3	181	56.2 ± 0.7	97.9 ± 0.5	1.74
C7 (II)	35.0	1.3	186	64.6 ± 0.7	104.8 ± 0.3	1.62
C8 (II)	34.5	1.3	183	60.9 ± 1.0	97.7 ± 1.4	1.60
C9 (II)	34.5	1.3	183	56.3 ± 0.9	97.9 ± 1.5	1.74
C10 (II)	35.0	1.3	186	60.0 ± 1.6	101.2 ± 0.8	1.69

Details of packed agarose gel column systems used in this thesis.

V_o = void volume. V_e = elution volume.

Table 82c

Batch III

Column	approximate height (cm)	radius (cm)	bed volume	V_o (ml)	V_e fibrinogen (ml)	$\frac{V_e}{V_o}$
C12 (III)	35.0	1.3	186	60.4 ± 0.7	105.5 ± 0.5	1.75
C13 (III)	35.0	1.3	186	61.0 ± 0.5	105.0 ± 1.3	1.72
D1 (III)	35.0	1.3	186	63.3 ± 1.4	107.0 ± 1.1	1.69
Batch IV						
B11 (IV)	34.0	1.3	181	54.3 ± 1.5	89.8 ± 1.5	1.65

Details of packed agarose gel column systems used in this thesis.

V_o = void volume

V_e = elution volume

Table 83

Local Venous Thrombosis

	Age	Sex	Diagnoses
1	54	Male	(1) Recurrent ileo-femoral vein thrombosis with pulmonary embolism. (2) Metastatic carcinoma - primary never discovered, several hepatic secondaries.
2	40	Male	(1) Recurrent ileo-femoral vein thrombosis.
3	22*	Male	(1) Axillary vein thrombosis.
4	50	Male	(1) Subclavian vein thrombosis. (2) Gangrene of hand. (3) Raynaud's syndrome.
5	55*	Male	(1) Ileo-femoral vein thrombosis. (2) Primary carcinoma of colon with hepatic secondaries.
6	25*	Male	(1) Recurrent ileo-femoral vein thrombosis (post-traumatic). (2) Chronic pyelonephritis.

Clinical details of the 6 patients with established local venous thrombosis studied. * included in the ancrod study (Chapter IV).

Table 84

Local venous thrombosis patients

	m (percent)	n (percent)	p (percent)	SCT titre at V_0	FR-antigen ratio	TCP ratio	Plasma fibrinogen mg/100 ml	Serum FDP/fdp µg/ml
1	8.4	83.7	8.0	4	0.84	0.56	293	80.0
2	3.7	91.2	5.1	5	0.61	0.58	N.D.	40.0
3	4.1	86.7	7.3	0	0.83	0.78	156	5.0
4	6.5	79.7	13.8	0	0.85	0.81	166	20.0
5	4.2	85.0	10.7	N.D.	0.63	0.66	268	5.0
6	7.0	85.2	7.8	1	0.62	0.71	226	5.0
mean ± SD	5.7 ± 1.9	85.3 ± 3.8	8.8 ± 3.0	2.0 ± 2.3	0.73 ± 0.12	0.69 ± 0.10	222 ± 61	25.8 ± 29.9

Laboratory results of samples from the 6 patients with venous thrombosis (Table 83). Means ± standard deviations are shown.

Table 85

Comparison of data from the 6 patients with venous thrombosis and that from 10 healthy controls

	t	2p
Plasma fibrinogen	0.592	<0.6 (NS)
Serum FDP/fdp	2.536	<0.025
area m	4.751	<0.001
area n	6.050	<0.001
area p	4.650	<0.001
SCT titre at V_o	2.807	<0.02
FR-antigen ratio	3.639	<0.005
TCP ratio	6.373	<0.001

The data from the 6 patients with deep venous thrombosis (Tables 83 and 84) and the 10 healthy, non-pregnant women (Group III, Chapter V) analysed using the unpaired "t" test.