

THE STRUCTURE AND PROPERTIES OF HUMAN
FIBRINOGEN FRAGMENT D

Jan S. Lawrie

A Thesis Submitted for the Degree of PhD
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1980

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ABSTRACT

THE STRUCTURE AND PROPERTIES OF HUMAN FIBRINOGEN FRAGMENT D.

Ph.D. Thesis

Jan Sloane Lawrie

(1980)

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(1) Three molecular weight forms of fragment D were isolated from a plasmic digest of human fibrinogen. Further heterogeneities within the preparation were revealed by NH_2 -terminal amino acid analysis and by digestion studies performed in the presence of 2 M-urea; the existence of conformationally different forms of the fragment D molecule is proposed.

(2) Plasmic digestion of fibrinogen in the presence of 2 mM- Ca^{2+} produced a homogeneous high molecular weight preparation of fragment D (designated $\text{D}_{\text{Ca}^{2+}}$). In the absence of Ca^{2+} or in the presence of EDTA, two lower molecular weight forms of fragment D, each containing a more degraded constituent γ chain, were identified. In the presence of 2 M-urea only slight plasmic degradation of fragment $\text{D}_{\text{Ca}^{2+}}$ occurred.

(3) Increased SDS-gel electrophoretic mobilities were demonstrated for the fragments D and γ prepared from fibrinogen in the presence of Ca^{2+} . An anomalous electrophoretic mobility pattern was also described for the constituent γ chain of fragment $\text{D}_{\text{Ca}^{2+}}$ and of fibrinogen exposed to Ca^{2+} . It is suggested that Ca^{2+} bound to the constituent γ chain of fibrinogen and fragment D forms an intra-chain Ca^{2+} -bridge towards the COOH-terminus thereby maintaining a 'hook-like' conformation. This form of fragment D, it is proposed, exhibits a decreased susceptibility to plasmic attack and an anomalously low electrophoretic mobility.

(4) A method was developed employing ion-exchange chromatography and gel filtration for the preparation and isolation of fragment D both in the presence and absence of free Ca^{2+} .

(5) Studies employing purified samples of each type of fragment D confirmed that the two molecules differed in the extent of degradation of the constituent γ chain at the COOH-terminus. Furthermore an influence of Ca^{2+} on the charge

heterogeneity of fragment D preparations was concluded from isoelectric focussing studies.

(6) The proposal of a compact and thereby stable structure for the fragment $D_{Ca^{2+}}$ molecule was strengthened by the results obtained from studies employing chemical crosslinking reagents and the technique of ultracentrifugation.

(7) The possibility that serine, glycine and glutamate amino acid residues are located in the region of the γ chain associated with the binding of Ca^{2+} was suggested from amino acid analysis of fragment D.

THE STRUCTURE AND PROPERTIES OF HUMAN
FIBRINOGEN FRAGMENT D

A THESIS PRESENTED BY
JAN S. LAWRIE B.Sc.(Edinburgh)

to

The University of St. Andrews in application for
the Degree of Doctor of Philosophy

1980



Th 9363

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that it is my own composition and that no part of it has been previously presented for a higher degree.

The research was conducted in the Department of Biochemistry and Microbiology, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr Graham Kemp.

CERTIFICATE

I hereby certify that Jan Lawrie has spent nine terms engaged in research work under my direction and she has fulfilled the conditions of Ordinance General No. 12, and Resolution of the University Court, 1967, No. 1, and that she is qualified to submit the accompanying Thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I first matriculated at the University of Edinburgh in October, 1971 and graduated with the degree of Bachelor of Science, Upper Second Class Honours in Pharmacology in July, 1975.

I matriculated as a research student in the Department of Biochemistry, University of St. Andrews in October, 1976.

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I should also like to thank Dr J. Hinnie for his helpful comments and Mrs. W.R. McIlwraith for typing this thesis.

THE STRUCTURE AND PROPERTIES
OF HUMAN FIBRINOGEN FRAGMENT D

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Nomenclature

The constituent chains of fibrinogen will be referred to as $A\alpha$, $B\beta$ and δ . Numerical suffixes will be used to denote differing electrophoretic forms of the same plasmic fragments of fibrinogen: e.g. fragment D_1 refers to a fragment of lower electrophoretic mobility than fragment D_2 . The constituent chains of fragment D will be denoted α , β and δ .

XVIII

Main abbreviations

SDS	sodium dodecyl sulphate
DNS-Cl	1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride)
DMS	dimethyl suberimidate
DMA	dimethyl adipimidate

XIX

Summary

- (1) Fragment D was prepared by digestion of human fibrinogen with plasmin isolated from human plasma. Three molecular weight forms of fragment D which exhibited differing susceptibilities to plasmin in the presence of 2 M-urea were identified. The two higher molecular weight species were rapidly degraded to fragment d while the third displayed the same electrophoretic mobility even after extensive degradation of the constituent polypeptide chains by plasmin. These observations are evidence of the existence of conformationally different forms of the fragment D molecule.
- (2) Further molecular heterogeneities within the fragment D preparation were revealed by NH_2 -terminal amino acid analysis.
- (3) Plasmic degradation of fibrinogen in the presence of Ca^{2+} produced a homogeneous preparation of fragment D (designated $\text{D}_{\text{Ca}^{2+}}$). In the absence of Ca^{2+} or in the presence of EDTA two lower molecular weight forms of fragment D, each containing a more degraded form of the constituent γ chain, were produced. In the presence of 2 M-urea only slight degradation of fragment $\text{D}_{\text{Ca}^{2+}}$ by plasmin was detected. These results support the theory that Ca^{2+} protect fragment $\text{D}_{\text{Ca}^{2+}}$ from further attack by plasmin and that the well

documented heterogeneity of fragment D preparations may be attributable to the digestion of fibrinogen in a medium containing insufficient levels of Ca^{2+} to saturate completely the ion binding sites of the molecule.

(4) Increased SDS-gel electrophoretic mobilities were demonstrated for the fragments D and Y prepared by digestion of fibrinogen in the presence of Ca^{2+} . Furthermore an anomalous electrophoretic mobility pattern was described for the constituent γ chain of fragment $\text{D}_{\text{Ca}^{2+}}$ and of fibrinogen exposed to Ca^{2+} . It is suggested that Ca^{2+} bound to the constituent γ chain of fibrinogen and fragment D forms an intra-chain Ca^{2+} bridge towards the COOH-terminus. Thus Ca^{2+} may maintain a hook-like conformation of the γ chain.

(5) A method was developed employing ion-exchange chromatography and gel filtration for the isolation of fragment $\text{D}_{\text{Ca}^{2+}}$ under conditions compatible with the presence of free Ca^{2+} . This procedure was also applied to the isolation of a fragment D prepared in the absence of Ca^{2+} .

(6) Studies employing purified samples of each type of fragment D confirmed that the two molecules differed in the extent of degradation of the COOH-terminus of the constituent γ chain. Furthermore an influence

of Ca^{2+} on the charge heterogeneity of fragment D preparations was concluded from isoelectric focussing studies.

(7) Ultracentrifugation experiments confirmed that bound Ca^{2+} confers a greater stability on the fragment D molecule.

(8) The influence of Ca^{2+} on the conformation of each type of fragment D molecule was investigated using chemical crosslinking agents. Predominantly intramolecular crosslinking of fragment $\text{D}_{\text{Ca}^{2+}}$ was induced, while significant intermolecular crosslinking of the fragment D prepared in the absence of Ca^{2+} was detected. A more compact molecular structure is envisaged for fragment $\text{D}_{\text{Ca}^{2+}}$.

(9) The possibility that serine, glycine and glutamate amino acid residues are located in the region of the γ chain associated with the binding of Ca^{2+} was suggested by amino acid analysis of fragment D.

GENERAL INTRODUCTION

The work to be described in this Thesis is an investigation into the structure of the plasmin-derived fibrinogen degradation product, fragment D, and of the influence of Ca^{2+} on both the production and properties of this fragment.

This Introduction summarises the present known facts relating to the structure and shape of the fibrinogen molecule. The fragmentation of fibrinogen by plasmin and the influence of Ca^{2+} on this process and on the properties displayed by the fibrinogen molecule will be discussed. The Introduction concludes with a summary of the aims and layout of the Thesis.

(a) The nature of fibrinogen.

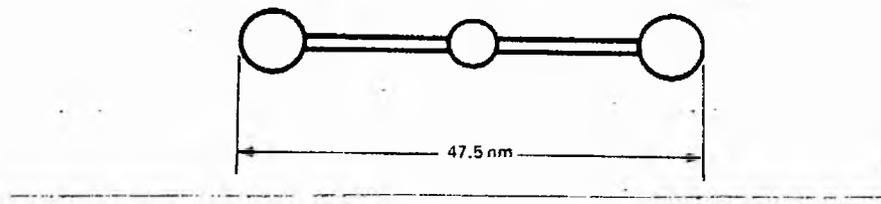
Fibrinogen, the substrate for the final enzymatic event in the mediation of blood coagulation and the formation of fibrin, is a dimeric molecule (Fig. (i)) consisting of three pairs of polypeptide chains (Blombäck & Yamashina, 1958). The $\text{A}\alpha$ chain of fibrinogen has a molecular weight of 67,000, the $\text{B}\beta$ chain 58,000, and the γ chain 47,000 (Gaffney & Dobos, 1971). A molecular weight for the fibrinogen molecule of 340,000 is thereby implied. The conversion of fibrinogen to fibrin is initiated by the thrombin (EC 3.4.215) - induced cleavage of two small peptides from the NH_2 - terminal region of fibrinogen, namely fibrinopeptide A (from the $\text{A}\alpha$ chain) and fibrinopeptide B (from the $\text{B}\beta$ chain) (Bettleheim & Bailey, 1952). The resultant fibrin monomers polymerise spontaneously to produce a

complex fibrillar structure. In the presence of activated factor XIII and Ca^{2+} further stabilisation of the polymer occurs by the rapid formation of specific $\epsilon - (\gamma - \text{glutamyl})$ lysine crosslinks between the γ chains of two adjacent fibrin molecules (Lorand & Ong, 1966). McKee et al. (1970) reported that crosslinking of the α chains also occurs, more slowly to yield α chain polymers. This process extends the linear γ chain polymerisation process into the third dimension to form a reticulum. The resulting stabilized fibrin gel, or clot, is the central event of blood coagulation, its deposition preventing excessive blood loss upon tissue damage. Blood flow is resumed following fibrinolysis. It has been proposed by Astrup (1956) that the processes of in vivo coagulation, constantly laying down fibrin on the vessel walls, and fibrinolysis, removing such deposits after they have served their haemostatic function, are in dynamic equilibrium. Studies on the metabolism and regulation of fibrinogen, the molecular events associated with its conversion to fibrin and of the physiological actions of the fragments of fibrin and fibrinogen are of great clinical, as well as, biological interest.

(b) The shape of fibrinogen.

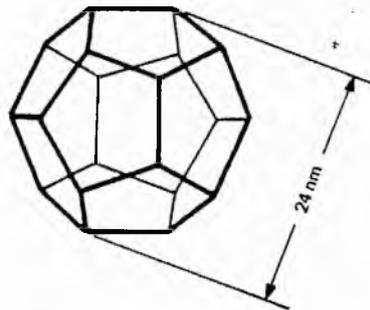
Many different techniques have been employed in the characterisation of the fibrinogen molecule. However, the shape of the molecule remains a matter of controversy.

Fig (ii) Model proposed by Hall & Slayter (1959)
for the fibrinogen molecule



A rod-like trinodular shape is predicted for fibrinogen.

Fig. (iii) Model proposed by Köppel (1966)
for the fibrinogen molecule



A pentagonal dodecahedron shape is suggested for fibrinogen.

(Both figures are from Gaffney (1977).)

Hydrodynamic studies have indicated that the frictional ratio (a parameter characteristic of the size and shape of a molecule) of fibrinogen is higher than would be expected if it were a perfect compact sphere (Socheraga & Laskowski, 1957). Two explanations of this finding are possible; either the molecule has a very elongated shape or it is a very hydrated structure. In the former case a prolate ellipsoid of approximate dimensions 3 x 90 nm would be consistent with the hydrodynamic data while the second proposal suggests a structure involving about 8 grams of water to every gram of protein and a sphere of diameter 20 nm.

Electron microscopy studies have proposed several shapes for the fibrinogen molecule. In 1959 Hall & Slayter proposed from their examination of bovine fibrinogen a trinodular model for the molecule which is still accepted by many investigators (Fig. (ii)). Two terminal spheres of diameter 6.5 nm are each connected by a peptide strand to a central sphere of 5 nm diameter. The overall length of the molecule is 47.5 nm. Hall & Slayter also proposed that the conversion to fibrin was accompanied by end to end association of molecules with a concomitant shrinkage of the fibrinogen molecule to about one half of its original length. This would account for the characteristic cross-banding pattern with an axial repeat distance of about 23 nm observed upon electron microscopic examination of a fibrin polymer. However, Stryer et al. (1963) concluded

from X-ray studies that the pattern suggested by Hall & Slayter represented a staggered arrangement of the molecules rather than shrinkage. Furthermore, the same pattern of results was obtained with fibrinogen gels and fibrin clots which, they claimed, implied that no major internal rearrangement of the monomer occurred in the clotting process. Support for the trinodular model was claimed by Johnson & Mihalyi (1965) from their fluorescence depolarisation studies and they suggested that the nodules might be independently and flexibly linked. However, electron microscopy studies have provided other models for the fibrinogen molecule. Kay & Cuddigan (1967) proposed that the fibrin monomer was a linear array of fifteen nodules while Köppel (1966) proposed a pentagonal dodecahedron shape for bovine fibrinogen (Fig. (iii)). This cage-like structure of polypeptide chains with more than 90% of its volume ascribed to water would explain the unusual hydrodynamic data previously described for the fibrinogen molecule. No unfolding of the molecule upon fibre formation was proposed. Pouit et al. (1972) also reported these polygonal structures but significantly, they envisaged a large conformational change occurring in the fibrinogen molecule during the polymerisation stage. Hitherto models had assumed that the fibrinogen morphology was intact in the monomer.

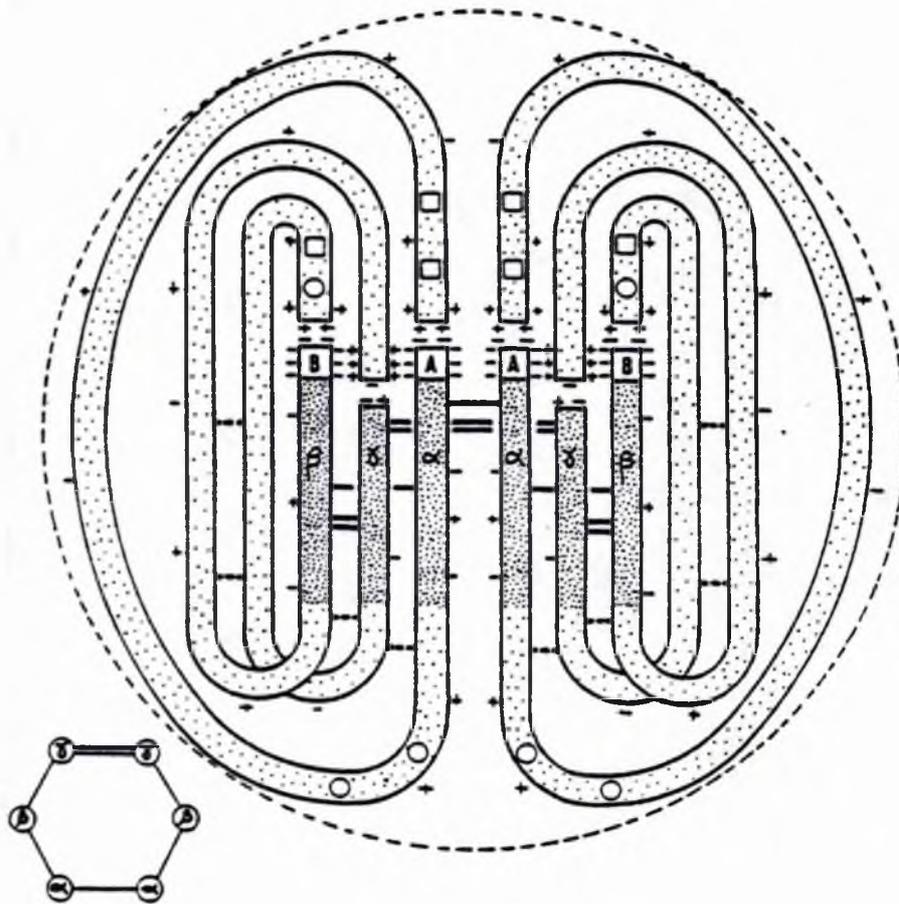
These electron microscopy studies investigated

dried fibrinogen samples prepared by a variety of methods. This may explain, in part, the inconsistency of the results. Recognition of these shortcomings promoted the use of a diversity of techniques to investigate the shape of the fibrinogen molecule.

Native human fibrinogen cannot be crystallised, a property which has been attributed to the free-floating A α chain of the molecule. However Tooney & Cohen (1972) reported the formation of microcrystals of fibrinogen following the removal of a COOH-terminal portion of the A α chain by a bacterial protease. Electron microscopy studies of these crystals revealed a cylindrical shape (9 x 45 nm) for the fibrinogen molecule. In 1974, Cohen & Tooney reported the extension of these studies and concluded from X-ray diffraction work that the molecule had an elongated shape. No major change was envisaged during the fibrinogen to fibrin transition. However, it must be borne in mind that they studied a degraded form of fibrinogen and therefore their findings may not be directly applicable to the intact molecule.

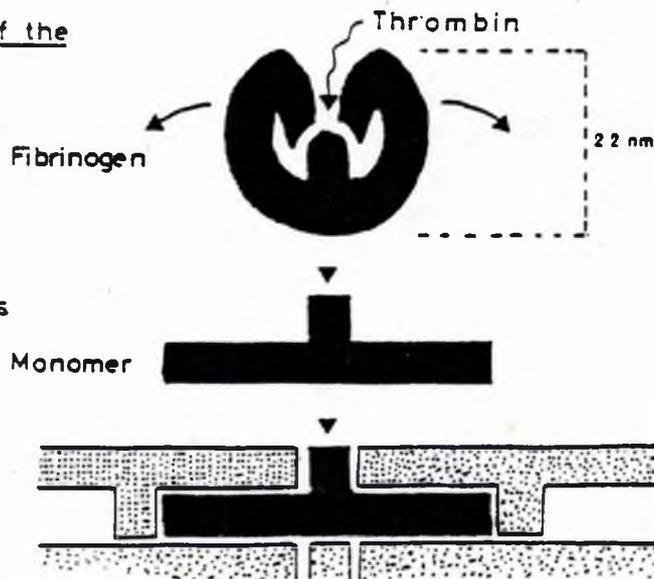
Bachman et al. (1975) presented the results from a modified electron microscopy technique whereby highly hydrated systems could be fixed prior to examination and in which, in addition, both the morphology and the molecular weight of the particles could be determined

Fig.(iv) Schematic representation of the fibrinogen molecule as proposed by Hudry-Clergeon et al. (1975)



The molecule is spherical in shape. The COOH-terminal parts of the constituent chains are folded around the NH₂-terminal region. Disulphide bonds; — and ---, □ ○; crosslinking sites. The morphology is stabilised by electrostatic interactions. The cross section indicates the chain arrangement in the NH₂-terminal region.

Fig.(v)
The initial stages of the enzymatic polymerization of fibrinogen as proposed by Hudry-Clergeon et al. (1975)



The release of the fibrinopeptides alters the intramolecular interactions and structural changes occur.

(Both Fig.(iv) and Fig.(v) are from Hudry-Clergeon et al. (1975).)

independently. Neither the model of Hall & Slayter nor that of Köppel was consistent with the data obtained and instead a flexible, cylindrical molecule (9 x 45nm), possibly in a bent conformation, was proposed. Flow & Edgington (1972) and Chen & Doolittle (1970) suggested that portions of the NH₂ - terminal and COOH-terminal aspects of the molecule must be in close proximity. Furthermore Cierniewski et al. (1977) reported the results of immunochemical studies which suggested that the NH₂ - terminal region of the γ chain of fibrinogen was exposed by plasmic cleavage of the COOH-terminal region of the A α chain. Similar reasoning was applied by Hudry-Clergeon et al. (1975) to develop their model of the fibrinogen molecule shown in Fig. (iv). The shape is similar to that described by Köppel, however, they envisaged a conformational change during the fibrinogen to fibrin transition (Fig. (v)). This scheme, according to Hudry-Clergeon et al., would explain the unmasking of cross-linking sites by the action of thrombin and also the decrease in sensitivity of fibrinogen to factor XIII compared with that of fibrin. They further proposed that since the isoelectric point of the COOH-terminal region of the molecule differs from that of the highly disulphide-linked NH₂ - terminal region, electrostatic interactions occurred between these two domains and contributed to the maintenance of the molecular conformation. Support for these proposals was provided by Tranqui-Pouit et al. (1975) who studied the various

Fig. (vi)

Model proposed by Doolittle et al. (1977)
for the fibrinogen molecule

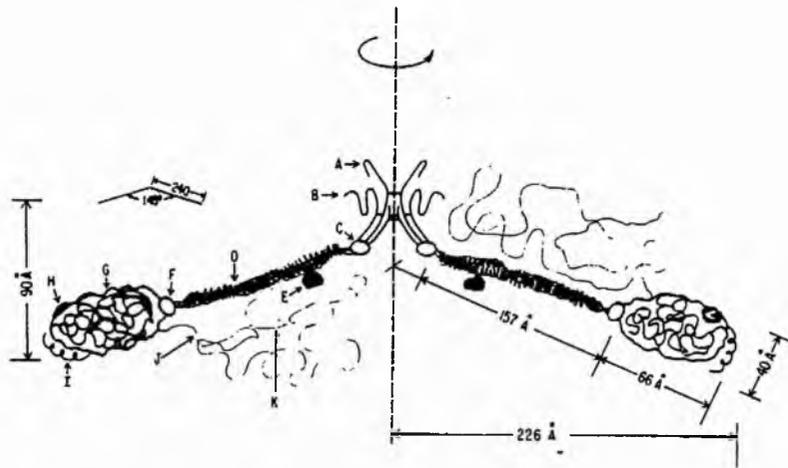


Fig. 6. Detailed model of vertebrate fibrinogen molecule. A, fibrinopeptide A; B, fibrinopeptide B; C, first disulfide ring; D, coiled coil connection between domains; E, carbohydrate attached to γ -chain; F, second disulfide ring; G, terminal domain (cf fragment D); H, carbohydrate on β -chain; I, cross-linking site; J, early plasmin attack point; K, carboxy-terminus of α -chain.

stages of the digestion of fibrinogen using electron microscopy and advocated that the initial structure of fibrinogen was indeed spherical while its degradation precipitated a gradual change to a filamentous structure.

Marguerie et al. (1975) and Marguerie & Stuhmann (1976) applied the technique of neutron small angle scattering to the study of fibrinogen shape. They concluded from their study of bovine fibrinogen solutions that the molecule had a "bent banana" shape with a cleft in the middle. It was proposed that interactions between the constituent polypeptide chains occurred within the cleft thus maintaining the bent structure. The data reported more recently by Marx et al. (1979) from Raman spectroscopy of fibrinogen solutions favour the proposal of Marguerie et al. and of Hudry-Clergeon et al. of a dynamic concept for the conversion of fibrinogen to fibrin.

However controversy still remains. The kinetic data obtained by Schragger et al. (1976) from fibrinogen digestion studies were interpreted as being in accordance with the model originally proposed by Hall and Slayter. Doolittle et al. (1978) and Doolittle et al. (1977) arrived at a similar description of the fibrinogen molecule's shape from their attempt to construct a three dimensional model from the available sequence data (Fig. (vi)). The highly disulphide bonded NH₂ - terminal region is connected by two, three-stranded

"ropes" to each of the separate COOH-terminal regions. Certain regions of the A α , B β and γ fibrinogen constituent chains appeared to be helix permissive, forming interdomainal coiled-coil structures. This spring-like structure would explain the compressibility of fibrin gels. An identical shape for the fibrinogen and fibrin molecules was envisaged. However Parry (1978) from a similar type of study, concluded that the conformation of the interdomainal rope-like regions may be quite complex and that the coiled-coil structures may exist only over relatively short lengths.

A generally accepted description of the shape of the fibrinogen molecule has yet to be achieved.

(c) The structure of fibrinogen.

In sharp contrast to the attempts described above to obtain an unequivocal description of the shape of the fibrinogen molecule, studies aimed at the elucidation of its amino acid sequence are well advanced. The virtually complete amino acid sequences of all three constituent chains have been published culminating in 1979 in the report by Doolittle et al. of the complete sequence of the A α chain (610 residues). In 1972 Blombäck & Blombäck reported the amino acid sequence of the NH₂-terminal disulphide-bonded region of human fibrinogen and in the intervening seven years the sequence of the B β chain (462 amino acid residues) was reported by Henschen &

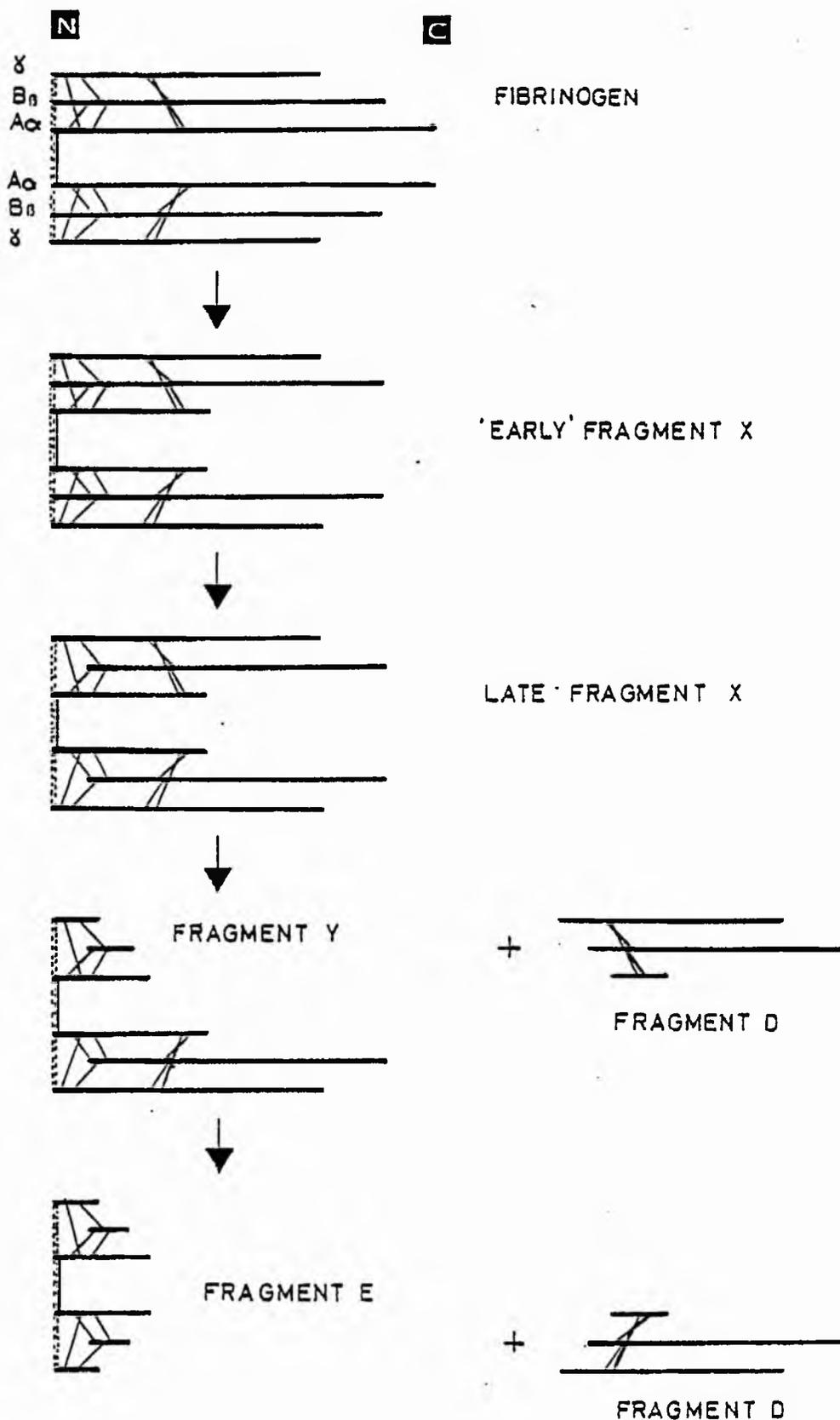
Lottspeich (1977) and by Watt et al. (1978). The δ chain sequence was reported by Lottspeich & Henschen (1977) and comprises 410 amino acid residues. A high degree of homology between the B β and δ chains is apparent, being most striking in the middle and COOH-terminal regions of the chains (Henschen & Lottspeich, 1977a) and supports the view by Doolittle (1976) that all three fibrinogen chains have a common ancestry. However, the degree of homology of the B β and δ chains with the A α chain is less striking.

It has been demonstrated that mammalian fibrinogens contain covalently-linked carbohydrate (Pepper et al., 1974; Mills & Triantaphyllopoulos, 1969) attached to the B β and δ chains. The site of binding on the δ chain was reported to be an asparagine residue in position 52 (Blombäck et al. 1973) while that on the B β chain was identified as an asparagine residue at position 364 by Töpfer-Peterson et al. (1976).

Fibrinogen contains 29 disulphide bridges per molecule and no free sulphydryl groups (Henschen, 1964). When it is cleaved at its methionyl bonds by treatment with cyanogen bromide five fragments containing disulphide bonds or disulphide knots (DSK) are formed (Gårdlund et al., 1977). One such disulphide cluster, the largest, is located in the NH₂ - terminal region of the molecule and is called N-DSK. It is a dimer containing three pairs of polypeptide chains symmetrically

Fig (vii) Schematic diagram of the digestion of fibrinogen by plasmin

Fibrinogen is digested to the intermediates, fragments X and Y, and then to the two terminal core fragments D and E, as proposed by Marder *et al.* (1969). The approximate location of the disulphide bonds (inter-chain) is shown. N and C represent the NH_2 and COOH termini of the molecule.



linked by 11 disulphide bridges (Blombäck et al., 1972; Blombäck et al., 1976). The second largest disulphide knot is monomeric and contains parts from the mid-section of all three chains (Gårdlund et al., 1977). The arrangement of the six disulphide bridges in this DSK were established by Bouma et al. (1978) and Henschen (1978). The three remaining disulphide knots are each located in the COOH-terminal region of one of the three chains of fibrinogen (Gårdlund et al., 1977).

(d) Fibrinogen interactions with plasmin.

Further investigations of fibrinogen have employed the fibrinolytic enzyme plasmin (EC 3.4.21.7) which preferentially catalyses the hydrolysis of peptide bonds between the carboxyl group of lysine or arginine residues and the amino group of another amino acid. The general scheme of in vitro digestion of fibrinogen by plasmin proposed by Marder et al. (1969) is shown in Fig. (vii). Initially plasmin is reported to remove the COOH-terminal two thirds of the A α chain as a single polypeptide of 40,000 to 50,000 molecular weight (Mills & Karpatkin, 1972; Furlan & Beck, 1972). The remaining structure is termed fragment X. Furlan & Beck reported considerable heterogeneity in the molecular weight of this fragment and this may be attributed to the sequential release of these 40,000 molecular weight peptides and also to the presence initially of a partially degraded A α chain in the fibrinogen molecule. (Mosesson et al. (1972) proposed that

plasmin degradation of the A α chain represents a physiologically important catabolic process; a topic which will be returned to later in this section.) Fibrinopeptide B is also rapidly lost from fibrinogen (Budzynski et al., 1974). The approximate molecular weight of fragment X is 240,000 and its conversion to fragment Y (molecular weight 160,000) involves the splitting of three peptide bonds, one on each of the α , β and γ chains. The released fragment is termed fragment D. Likewise three bonds are split in fragment Y to produce fragment E and a second molecule of fragment D. This asymmetric scheme of fibrinogen degradation has been supported by the results of other investigators (Furlan & Beck, 1972; Mills, 1972; Pizzo et al., 1972). However Mosesson et al. (1973) and (1974) postulated the existence of a second region of disulphide bonds linking the two halves of the fibrinogen molecule mid-way between the NH₂ and COOH-terminal ends. Thus one fragment D and one fragment E would be produced from fibrinogen according to this scheme. Such a fragment D molecule would represent a covalently linked dimer of approximate molecular weight 180,000. This value is twice that reported by other investigators. Furthermore this scheme would predict that digestion of crosslinked fibrin would yield a fragment D oligomer. However plasmic degradation of fibrin containing covalently crosslinked γ chains produces only one molecular form of fragment D, a dimer termed D-dimer (Ferguson et al., 1975). Furthermore purified fragment Y contains two distinct sets of

polypeptide chains. One set are almost identical to those of fragment X while the second set are of a lower molecular weight being similar to those of fragment E (Budzynski et al., 1974). Most investigators favour the scheme of fibrinogen digestion proposed by Marder et al.

Fragment D contains sections of all three fibrinogen constituent chains and three of the disulphide knots described above. Fragment D preparations are normally heterogeneous and Furlan et al. (1975) characterised three main molecular weight forms. Each contained identical subunit α (molecular weight 10,000) and β (molecular weight 45,000) chains but three molecular weight forms of the γ chain were isolated. These findings were confirmed by Collen et al. (1975) and by Ferguson et al. (1975). The heterogeneity of fragment D preparations has been attributed to the gradual degradation of the constituent γ chain from its COOH-terminal end by plasmin (Pizzo et al., 1972; Gaffney, 1972; Furlan et al., 1975; Ferguson et al., 1975). With the exception of the highest molecular weight form all other γ chain remnants have lost their ability to react with activated factor XIII; this is in accordance with the proposed location of the γ chain cross-linking acceptor site, 14 residues from the COOH-terminus (Furlan et al., 1975a; Ferguson et al., 1975). However, Takagi &

Doolittle (1975) reported the early cleavage of a 3,000 molecular weight fragment from the NH_2 -terminal end of the γ chain and also the removal of a pentapeptide from the COOH-terminus during the formation of fragment D. Six amino acid residues are also split from the NH_2 -terminus of the α chain in the late forms of fragment D (Furlan et al., 1975.) The β chain component of fragment D appears to be the most resistant towards further plasminic attack and this may be, in part, attributable to its covalently bound carbohydrate.

Fragment E is a dimer, composed of the NH_2 terminal regions of all six fibrinogen constituent chains. Its amino acid sequence overlaps with that of the N-DSK described above (Kowalska - Loth et al., 1973) and is immunologically related to it (Marder et al., 1972). A molecular weight of 52,000 is predicted for fragment E by the available sequence data reported by Kowalska-Loth et al. There is general agreement that the fibrinogen NH_2 -termini of both γ chains are intact in fragment E whilst those of the $\text{B}\beta$ chain are lost. The state of the NH_2 - termini of the $\text{A}\alpha$ chains is uncertain.

Fragments D and E have been termed the terminal digestion products of fibrinogen. However, two investigators reported the production of a low molecular weight (45,000) species with partial immunological identity to

fragment D (Furlan & Beck, 1973; Mills & Triantaphyllopoulos, 1969). This fragment termed fragment d by Furlan and Beck was further investigated by Kemp et al. (1973) who concluded that it was formed by the action of plasmin on a conformationally altered form of fragment D. Appropriate conformational changes were induced in fragment D by its exposure to either a low concentration of urea or a low pH. This fragment d was further characterised by Furlan et al. (1975) who proposed that it contained, like its parent structure, sections of α , β and γ chains of respective molecular weights 9,000, 24,000 and 13,000. The NH_2 - terminal amino acids of the α and β chains were identical to those of fragment D while the γ chain had undergone degradation at its NH_2 - terminus. Fragment d may occupy a similar position in the fibrinogen molecule to that of the second largest DSK described earlier. The presence of a highly disulphide bonded region within the fragment d molecule would explain its resistance to further degradation even in the presence of urea.

It will be recalled that fibrinogen is acted upon by thrombin to produce non-crosslinked fibrin polymers which in turn are crosslinked in the presence of Ca^{2+} by factor XIII. The effect of plasmin on these structures has also been investigated. Gaffney, (1973) reported that the plasminolytic products of non-crosslinked

fibrin were electrophoretically similar to those produced from fibrinogen. Crosslinked fibrin is less susceptible to lysis by plasmin (Gormeson et al., 1967), but dimeric fragment D fragments containing dimeric i.e. crosslinked, δ chains are produced along with a fibrin fragment E, (Kopec et al., 1973 and Gaffney & Brasher, 1973).

Several studies have reported the biological effects of fragment D. Orloff and Michaeli (1977) found that fragment D inhibited the fibrin-induced release of platelet serotonin. Acute pulmonary dysfunction was induced in rabbits by the intravenous infusion of human fragment D (Manwaring et al., 1978) and a pathophysiological role of fibrin degradation products in clinical states associated with disseminated intravascular coagulation has been proposed (Chang & Bang, 1977).

Astrup (1956) proposed that a dynamic equilibrium exists between in vivo coagulation, constantly depositing fibrin in the vessel walls, and fibrinolysis, removing such deposits thereby ensuring a resumption of blood flow. It is widely believed that the fibrinolytic system is devised for the degradation of fibrin, not for circulating fibrinogen. However fibrinogenolysis may occur in man. Mosesson et al. (1972) found that human fibrinogen displayed evidence of degradation of its constituent $A\alpha$ chains at their COOH-terminus. A similar heterogeneity

of fibrinogen samples was demonstrated by Gaffney et al. (1974). However Semeraro et al. (1977) believe that the major portion of fibrinogen A α chain heterogeneity is an artefactual result of storage but concede that a minor part may be ascribed to in vivo fibrinogenolysis and indeed Collen et al. (1977) found an increase in the extent of A α chain degradation following strenuous exercise. Mills & Karpatkin (1970) reported that human fibrinogen contained two A α chain species which differed in molecular weight by 3,000 while Cottrell & Doolittle (1976) isolated a 27-residue polypeptide released during the early stages of plasmin digestion at the COOH-terminus of the A α chain. It displayed a molecular weight of 2859 and since fibrinogen prepared from freshly collected plasma contained both the A α chains described by Mills & Karpatkin, Cottrell & Doolittle ascribed the release of this peptide to the in vivo degradation of fibrinogen.

(e) Calcium and fibrinogen.

The normal total concentration of calcium in human blood plasma is about 10 mg/100 ml (2.5 mM) of which about one half is in the ionized state, the remainder being in combination with protein. Calcium is an essential cofactor in several of the reactions of blood coagulation. It is a requirement in the process of

fibrin polymerisation and Endres & Scheraga (1972) suggested that it may bind to specific sites on the molecule. However, recently, the possibility that this ion has an important perhaps crucial role in the structure and interactions of the fibrinogen molecule has been viewed with increasing interest. Godal (1960) found that EDTA decreased the heat resistance of fibrinogen in plasma while Ca^{2+} had the opposite effect consequently a protective action of Ca^{2+} on the fibrinogen molecule was proposed. Blombäck et al. (1966) reported a dissociation or conformational change induced in fibrinogen by its dialysis against EDTA while Endres & Scheraga (1971) found that EDTA caused a small decrease in the standard sedimentation coefficient of bovine fibrinogen. Both groups however agreed with Bithell (1964) that although such effects were compatible with the loss of bound metal ions essential to the maintenance of the protein's conformation, a direct action of EDTA on fibrinogen by virtue of its electronegativity disrupting hydrogen bonds seemed more likely. However, Kahn et al. (1970) provided evidence for an interaction of calcium with both bovine fibrinogen and fibrin from infrared spectroscopy results and Endres & Scheraga (1972) demonstrated that Ca^{2+} induced proton release from fibrinogen solutions at pH 5.3. Mg^{2+} was without effect. Shen et al. (1975) proposed that three Ca^{2+} were bound

per mole of bovine fibrinogen. One appeared to be removed relatively easily while two were tightly bound. Marguerie et al. (1977) also concluded from equilibrium dialysis experiments that bovine fibrinogen bound three calcium ions. In addition, 14-20 low affinity Ca^{2+} binding sites were demonstrated. The three high affinity sites did not behave identically. At a pH of 6.0 the number of high affinity sites was reduced to two. In a later report (Marguerie, 1977) the finding of Ly & Godal (1973) that Ca^{2+} exerted a protective effect upon the thermal denaturation of fibrinogen was confirmed and was also demonstrated in the case of acid-induced denaturation. Marguerie suggested that the bound Ca^{2+} stabilised a more compact fibrinogen structure. Three high affinity Ca^{2+} binding sites have also been demonstrated recently for rat fibrinogen (Van Ruyven-Vermeer et al., 1978) and human fibrinogen (Lindsey et al., 1978; Nieuwenhuizen et al., 1979). However in the latter case the dissociation constant of calcium binding differed by a factor of 4 from that reported by Marguerie for bovine fibrinogen. In addition Nieuwenhuizen claimed that the three sites did not display identical dissociation constant values. The value of one site was approximately 4 times that of the other two.

Several suggestions have been proposed for the

identity of the amino acids involved in the binding of Ca^{2+} to the fibrinogen molecule. Endres & Scheraga (1972) proposed a role for carboxyl groups and possibly the hydroxyl groups of serine or threonine residues. From studies of fibrin Marguerie et al. (1970) implicated carboxyl groups and Earland et al. (1972) concluded from electron spin resonance spectroscopy results that a tyrosine, a histidine and two aspartate or glutamate residues co-ordinated a Ca^{2+} . Marguerie et al. (1977) demonstrated that two protons were released from fibrinogen by each Ca^{2+} bound and proposed that two ionizable groups of pK 6.5 were involved in the binding of Ca^{2+} . A model was described for the binding site of Ca^{2+} involving two histidine and two carboxyl residues. Histidine residues have been implicated in the process of the polymerisation of fibrin units (Endres & Scheraga, 1966). Data from circular dichroism spectra suggested the involvement of both tyrosine and tryptophan residues close to the site of Ca^{2+} binding (Marguerie, 1977). Furthermore these data suggested that the binding of Ca^{2+} to the fibrinogen molecule did not entail a generalized conformational change, Ca^{2+} being inserted within the areas of fibrinogen which did not involve α -helical or β -form protein structures.

Further information regarding the binding of Ca^{2+} to fibrinogen has been obtained from digestion studies. This procedure is of particular relevance to the

present work. As early as 1963 Khomenko & Belitser reported that Ca^{2+} inhibited the proteolysis of fibrinogen by trypsin and they reasoned that Ca^{2+} maintained a more compact tertiary structure. Marguerie et al. (1977) suggested that since fibrinogen is a dimeric molecule all possible arrangements of three high affinity binding sites within the molecule should involve at least one interchain calcium bridge between at least two identical chains. In a second report Marguerie (1977) monitored the proton release during fibrinogen digestion and found that Ca^{2+} protected at least 1.6 plasmin-susceptible bonds at the beginning of the proteolytic reaction. It has been reported (Furlan & Beck, 1972; Pizzo et al., 1972) that the initial attack of plasmin on fibrinogen occurs at the COOH-terminal region of the $\text{A}\alpha$ chain and Marguerie postulated that the calcium binding centres were located in the COOH-terminal region of the fibrinogen $\text{A}\alpha$ -chains. The influence of Ca^{2+} on the more advanced stages of fibrinogen digestion has also been studied. The considerable heterogeneity of fragment D preparations isolated from plasmic digests of fibrinogen has been described by many investigators (e.g. Jamieson & Pepper, 1970). Thus the reports by Lugovski et al. (1976) and by Haverkate & Timan (1977) that a homogeneous high molecular weight form of fragment D was produced by degradation of fibrinogen in the presence of physiological CaCl_2 concentrations

created considerable interest. This fragment D (designated D_{cate} by Haverkate & Timan but hereafter referred to as $D_{\text{Ca}^{2+}}$) was resistant to attack by plasmin even in the presence of 2M-urea but could be degraded following its exposure to chelating agents. Haverkate & Timan proposed that Ca^{2+} , bound to fragment D, induced a plasmin resistant conformation. Fragment $D_{\text{Ca}^{2+}}$ was apparently identical to a fragment D prepared in the absence of Ca^{2+} except that the latter species contained a shorter constituent δ chain. The in vitro heterogeneity of fragment D preparations has been attributed by various investigators to differing extents of degradation of the constituent δ chain at its COOH-terminal end, (Furlan et al., 1975; Ferguson et al., 1975). Ca^{2+} appears to have protected this region of the molecule in the experiments described by Haverkate & Timan. Furthermore digestion of crosslinked fibrin by plasmin in the presence of CaCl_2 produced a D-dimer whose subunit composition was exactly twice that of fragment $D_{\text{Ca}^{2+}}$. The resistance of D-dimer to further digestion by plasmin was attributed, not to crosslinking, as had been suggested by Ferguson et al. (1975), but to bound Ca^{2+} . This conclusion was ratified by Purves et al. (1978).

To summarise, three high affinity Ca^{2+} binding sites have been demonstrated in human, bovine and rat fibrinogen. Physiological concentrations of Ca^{2+}

affect the course of fibrinogen digestion. This finding has implied the involvement of the COOH-terminal region of the A α chain and of fragment D in the binding of Ca²⁺. Further studies are required to identify the precise location of the three sites of Ca²⁺ binding.

The production of a high molecular weight form of fragment D in the presence of Ca²⁺ has several important implications. Hitherto several publications have described the heterogeneity of fragment D preparations and this may reflect, in part, the use of buffer systems containing varying amounts of Ca²⁺. In a similar fashion the different electrophoretic mobilities displayed by fragments D in the study of abnormal fibrinogens, or dysfibrinogenaemia, may not reflect an abnormality in the fibrinogen molecule but, more simply, the use of varying Ca²⁺ concentrations (Haverkate et al., 1978). Furthermore, a reevaluation of the conclusions from many studies performed to assess the biological activity of fragment D is necessary. This fact was recognized by Belitser et al. (1975) who investigated the anticlotting activity of fragment D_{Ca²⁺}. Several of the fibrinogen degradation products inhibit the clotting of fibrinogen and it is generally accepted that the early digestion products of fibrinogen are more efficient in this respect than fragments D or E (Marder & Schulman, 1969). However Belitser demonstrated an elevated

anticoagulating activity for fragment $D_{Ca^{2+}}$ and concluded that the stabilisation of the crosslinking site within the δ chain permitted fragment $D_{Ca^{2+}}$ to act as a competitive inhibitor of fibrinogen clotting. This finding was confirmed by Haverkate et al. (1979) but it is not yet established whether such an effect occurs in vivo.

To conclude, Ca^{2+} bound to fibrinogen and its degradation product fragment D, may have an important part to play in determining the properties of these molecules.

(f) Aims and layout of the Thesis

This Thesis is concerned with the investigation of the structure of the fibrinogen derivative, fragment D. The experimental results will be presented in two parts, A and B.

Part A will commence with an account of the preparation and isolation of fragment D which will be followed by a report of fragment D digestion studies. At the outset of this work the precise location and extent of the disulphide knot-containing region within fragment D had not been established. However fragment d, produced by plasminic digestion of fragment D, had been shown to contain most of the disulphide bonds of fragment D (Furlan et al., 1975). Accordingly the structure of fragment D was investigated by performing a time-course study of the pathway of digestion of fragment D to fragment d.

The chief experimental problem encountered in Part A was that of the heterogeneity of the isolated fragment D preparation. Thus the report published by Haverkate & Timan (1977) which claimed that a single, high molecular weight form of fragment D was produced by the digestion of fibrinogen in the presence of Ca^{2+} was of considerable significance to the present work. This report prompted the work described in Part B and this will be presented in three Divisions.

In the first Division the experiments performed by

Haverkate & Timan were repeated and extended. The claim made by these authors was confirmed and a desire to compare the properties of fragment D prepared both in the absence and presence of Ca^{2+} prompted the development of a novel method for their isolation. This procedure will be described in Division 2. The results of studies designed to compare the properties of the two fragments D so isolated will be presented in Division 3.

The main points emerging from the practical work will be developed in the General Discussion (Part C).

PART A

1

INTRODUCTION

Part A describes an investigation of the structure of the plasmin derived fibrinogen fragment, fragment D.

As will be recalled two terminal digestion products, fragments D and E, are produced by plasmin digestion of fibrinogen. The structure of fragment E is well characterised (Blombäck et al., 1976a) and this fragment has been shown by Marder et al. (1972) to be immunologically related to one of the five disulphide knot regions attributed to the fibrinogen molecule. Most of the remaining disulphide bonds of fibrinogen are thought to be located within the fragment D structure and evidence from the work of Furlan et al. (1975) suggests that these knots may be mainly concentrated within a relatively small region of the fibrinogen molecule, occupying a similar position to their fragment d. This fragment d is produced from fragment D by plasmin digestion in the presence of 2 M-urea. The rigid disulphide bonded structure of fragment d may explain its resistance to further digestion. However, the position within the fragment D molecule of fragment d proposed by Furlan et al. in 1975 was tentative. Unlike the situation with fragment E, the structure of fragment D, particularly its disulphide bond rich area had yet to be characterised. The analysis of the products of cyanogen bromide treatment of fragment E was invaluable in aiding the elucidation of its primary structure. However this method would not be readily applicable to the study of

fragment D. This molecule is relatively rich in methionine residues, consequently the products arising from cyanogen bromide induced cleavage are small and a theoretical reassembly of the molecule would therefore be complicated. It was proposed in this work therefore, to investigate the structure of the fragment D molecule by degrading it by plasmin according to the method reported by Kemp et al. (1973), who described the degradation of fragment D to fragment d under denaturing conditions. It was intended to monitor the various stages of the production of fragment d and thereby gain insight into the more precise location of the highly disulphide bonded, plasmin resistant fragment d structure within fragment D.

The preliminary sections of Part A will describe the preparation and purification of fragment D from fibrinogen. A report of time-course studies of the plasmic digestion of fragment D to fragment d in the presence of 2 M-urea will follow.

SECTION 2

GENERAL MATERIALS AND METHODS

2.1 Materials

2.1.1 Human plasma

Human plasma was supplied by the Blood Transfusion Service, Ninewells Hospital, Dundee. This gift is gratefully acknowledged.

2.1.2. Chemicals

Sephadex G-200 (Superfine grade), Sephadex G-100, lysine-Sepharose 4B and Sulphopropyl-Sephadex C-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose was supplied by Whatman, Balston Ltd., Kent, England. Human Fibrinogen (Grade L) was obtained from Kabi Pharmaceuticals Ltd., London, U.K., Streptokinase from Hoechst Pharmaceuticals Ltd., Hounslow, U.K., Trasylol from Bayer Pharmaceuticals, Surbiton, Surrey, U.K., and rabbit Antisera (anti-human fibrinogen and anti-human plasminogen) from Boehringer Mannheim (London) Ltd., Ealing, London. Acrylamide, methylenebisacrylamide, N, N, N', N' - tetramethylethylene diamine (TEMED), Coomassie brilliant blue R250, sodium dodecyl sulphate, BDH protomer molecular weight markers, dansyl chloride, polyamide layer sheets and 2-mercaptoethanol were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. Protein standards (phosphorylase a

α -1, 4-glucan: orthophosphate glucosyl transferase; EC 2.3.1.1) from rabbit muscle; bovine serum albumin; ovalbumin; chymotrypsinogen (EC 3.4.4.5) and myoglobin.) and dansyl amino acids were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. α - casein was obtained from the same source. Agarose (ME) was supplied by Miles Labs., Stoke Poges, England. All other chemicals were of AnalaR grade from BDH Chemicals Ltd., Poole, Dorset, U.K. Solutions were prepared in glass-distilled water.

2.1.3 Buffer solution

The following buffer was used routinely throughout Part A. It was prepared as described below and the pH of the solution was measured at 20°C using a Radiometer (Russell) pH meter (Copenhagen).

0.05 M-Phosphate, pH 7.5 was prepared by mixing 420ml of 0.1 M- NaH_2PO_4 , and 80ml of 0.1 M- Na_2HPO_4 monohydrate and distilled water to 1 litre.

Table 2.1 Composition of gels of various porosities

SOLUTION	FINAL CONCENTRATION OF ACRYLAMIDE (% w/v)			
	10	5	4	3
ACRYLAMIDE STOCK (ml)	5.0	2.5	2.0	1.5
WATER (ml)	50	7.5	80	8.5
GEL BUFFER (ml)	100	100	100	100
AMMONIUM PERSULPHATE (g)	0.02	0.02	0.02	0.02

2.2 Methods

2.2.1 SDS - polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out by a modification of the method of Swank & Munkres(1971).

Gels of various acrylamide concentrations were prepared as shown in Table 2.1 and contained 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.075% (v/v) N,N,N',N'-tetra:methylethylene diamine (TEMED), 0.1 % (w/v) ammonium persulphate, 4 M-urea and 0.01 M-sodium phosphate buffer at a pH of 7.2.

The acrylamide stock solution was prepared by dissolving 38.7g of acrylamide and 2.66g of methylene:bisacrylamide in distilled water to a final volume of 100ml. Gel buffer consisted of 0.2% (w/v) SDS, 8 M-urea, 0.15% (v/v) TEMED dissolved in 0.02 M-sodium phosphate buffer, pH 7.2. These two solutions were stored at 4°C but were allowed to reach room temperature prior to use.

Gels were cast in acid-cleaned glass tubes, 6cm long with an internal diameter of 4mm and thereafter overlaid with water. Before sample application the gel top was rinsed twice with reservoir buffer (0.1% (w/v) SDS, 0.01 M-sodium phosphate buffer, pH 7.2).

Samples for electrophoresis were either dissolved directly in, or dialysed against, reservoir buffer. Reduced samples were prepared in an identical fashion but, following the addition of 2-mercaptoethanol (final

concentration 1.5% (v/v)) the sample was incubated at 37°C for 2h. Protein molecular weight standards (phosphorylase a (α -1, 4-glucan: orthophosphate glucosyl transferase; EC 2.3.1.1) from rabbit muscle; bovine serum albumin; ovalbumin and myoglobin) were dissolved in reservoir buffer (0.5mg/ml) containing 1% (v/v) 2-mercaptoethanol and then incubated at 37°C for 2h. Gel loads were prepared from 5-50 μ l of sample mixed with 1 drop of glycerol and 10 μ l of tracking dye (0.05% (w/v) bromophenol blue in distilled water). Reservoir buffer was layered on top of each sample.

BDH protomer molecular weight markers were dissolved, as supplied, in 10ml of 0.01 M-sodium phosphate buffer, pH 7.2, containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. This stock solution was then incubated at 100°C for 2 minutes and stored at 4°C; to 300 μ l of stock solution, 20 μ l of 2-mercaptoethanol, 20 μ l 0.05% (w/v) bromophenol blue, 4 drops of glycerol and 300 μ l of reservoir buffer were added to prepare 100 μ l gel loads.

Electrophoresis was performed at room temperature in a Uniscil electrophoresis tank and a current of 2mA/gel was supplied by a Shandon Southern power pack. Electrophoresis was continued until the bromophenol blue marker band had migrated to the end of the gel (3-4h). After recording the gel length and the

distance moved by the bromophenol blue dye the gels were removed from the tubes.

2.2.2 Staining and destaining of polyacrylamide gels

Gels were stained and destained by a modification of the method of Weber & Osborn (1969). Gels were placed in a tray of staining solution composed of 1.25g Coomassie blue R250, 454ml 50% (v/v) methanol, 46ml glacial acetic acid. After 2 hours the gels were rinsed twice with distilled water and destained in a solution containing 75ml glacial acetic acid, 250ml methanol and 675ml distilled water. Gels were scanned at 570nm using a Vitatron TLD-100 densitometer; the position of protein bands and the length of gel being recorded. The mobility of each protein band was calculated using the following formula,

$$\text{Mobility} = \frac{\text{distance of protein band migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before staining}}{\text{distance of dye migration}}$$

The apparent molecular weight of an unknown protein band was interpolated from a graph of the logarithms of the molecular weights of the protein standards as a function of their electrophoretic mobilities.

2.2.3 Addendum

SDS-polyacrylamide gel electrophoresis has found widespread use as a technique for the estimation of protein molecular weights. It has been employed extensively during

the course of this work. Weber & Osborn (1969) suggested that the molecular weights of protein in the range 15,000 to 100,000 could be estimated with an accuracy of $\pm 10\%$ by this technique. The electrophoretic mobility of a polypeptide chain can, however, be a function solely of its molecular weight only if (a) the charge per unit mass is approximately constant, and (b) the hydrodynamic properties are a function only of the molecular length of the polypeptide chain (Weber et al., 1972). Reynolds & Tanford (1970) concluded from SDS-binding studies of a variety of different proteins that above an SDS concentration of 0.02% (w/v) both of these conditions were met. However, abnormal binding of SDS to proteins has been reported in the case of proteins containing disulphide bonds or carbohydrate (Pitt-Rivers & Impiombato, 1968). These authors also reported an influence of the primary structure of a protein on SDS-binding. A restriction on the binding of SDS may consequently alter the electrophoretic mobility of a protein and thereby yield an inaccurate estimate of its molecular weight. For these reasons the molecular weights calculated from SDS-polyacrylamide gel electrophoresis throughout the course of this work will be referred to as "apparent" molecular weights.

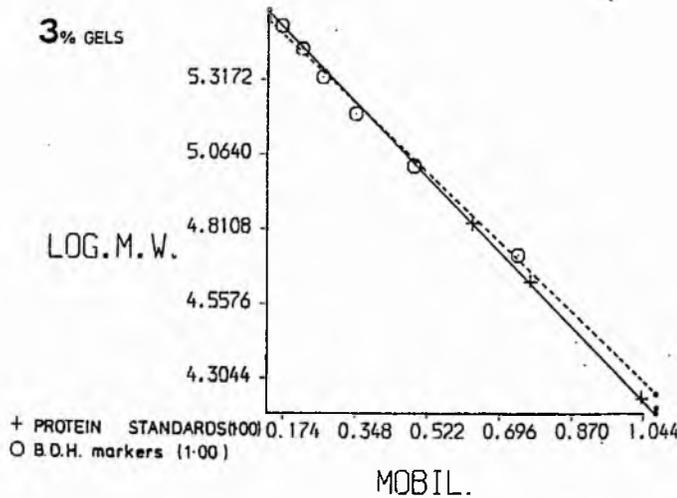
Recently protein molecular weight markers prepared by the crosslinking of a protein monomer to yield a series of oligomers have become commercially available. These preparations were predicted to behave as integral molecular weight multiples of the monomer on SDS-polyacrylamide gel

Fig 2-1 SDS-polyacrylamide gel electrophoresis. Typical calibration plots -1

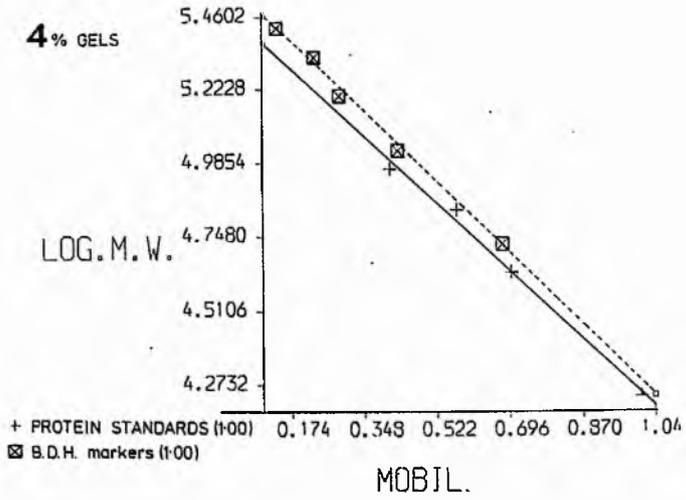
The logarithm of the molecular weights of protein standards (solid line) and of BDH molecular weight markers (broken line) are plotted as a function of electrophoretic mobility. For 3% and 4% gel systems the BDH high molecular weight (range 53,000 to 265,000) markers were employed while for 5% and 10% gel systems the low molecular weight markers (range 14,300 to 71,500) were used.

The figures in brackets are the correlation coefficients.

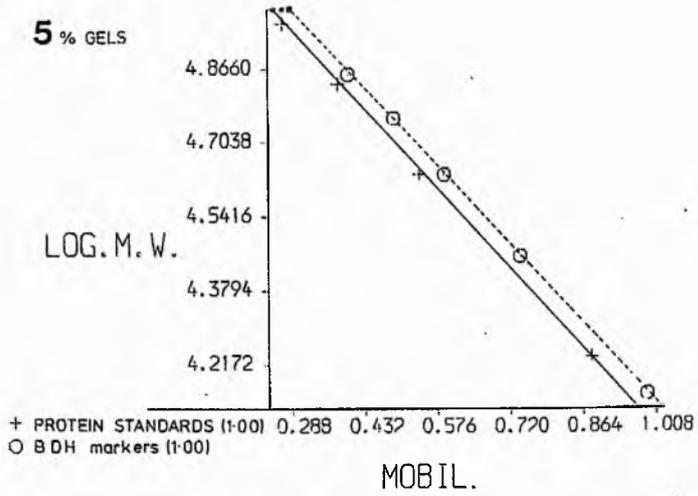
a



b



c



d

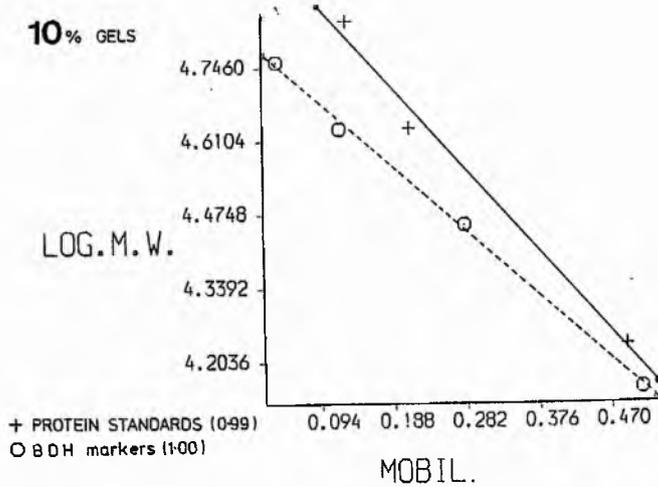
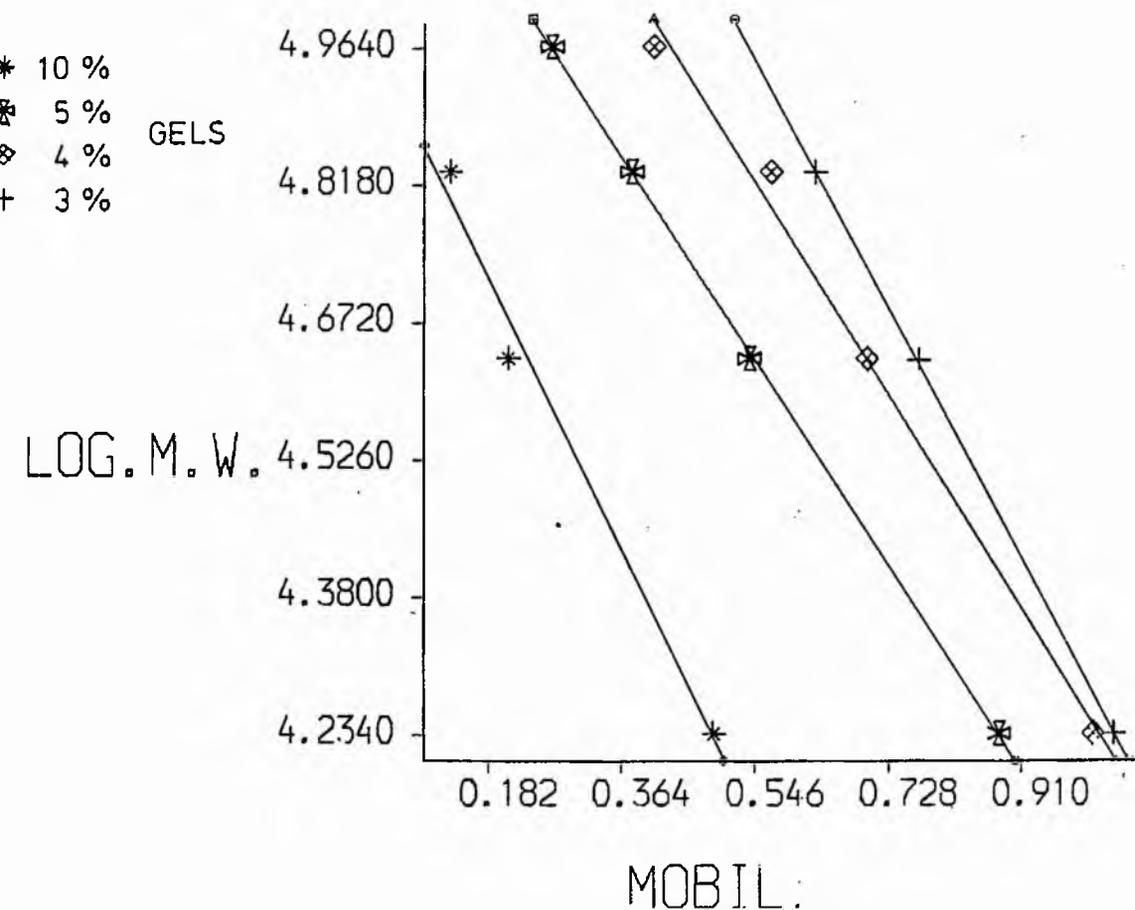


Fig. 2.2 SDS polyacrylamide gel electrophoresis. Typical calibration plots-2

A plot of the logarithm of the molecular weights of the protein standards against electrophoretic mobility for each gel system (3%, 4%, 5% and 10%).



electrophoresis, their uniformity providing a more reliable estimate of the molecular weight of an unknown protein. Fig.2.1 shows the graphs obtained from plotting the logarithm of the molecular weights of protein standards and BDH molecular weight markers as a function of electrophoretic mobility for 3%, 4%, 5%, and 10% polyacrylamide gels. The oligomers displayed electrophoretic mobilities very close to, or slightly greater than those of the single protein standards (3% 4% and 5% gels). However in the 10% gel system, the highest acrylamide concentration studied, the series of oligomers migrated much more slowly. The presence of chemical crosslinks within the oligomer structure may produce two effects. Firstly crosslinks may restrict the ability of the protein to unfold and assume the extended conformation normally favoured in the presence of SDS. Secondly the crosslinked, less extended conformation of the oligomer may bind less SDS per unit mass than a non-crosslinked molecule. Both these effects may alter the apparent mobility of the oligomer. Steele & Nielson (1978) performed a study to evaluate the usefulness of crosslinked oligomers in SDS-polyacrylamide electrophoresis and concluded that the protein oligomers especially trimers and higher multiples, migrated faster than did single standard proteins. As a result an unknown molecular weight was overestimated by 5-15%. They suggested that this result

was attributable to a decreased ability of the crosslinked oligomer to unfold in the presence of SDS. In the present case the results presented for 3%, 4% and 5% gel systems would largely concur with this finding. However, on a 10% gel, decreased mobilities for the oligomeric proteins compared to those of the standard proteins are observed (Fig. 2.1(d)). Thus acrylamide concentration may also influence the observed mobility of crosslinked proteins; a finding suggestive of their anomalous SDS-binding or conformation (Banker & Cotman, 1972).

Routinely a series of single protein standards were employed to estimate protein molecular weights (Fig. 2.2).

2.2.4 Immuno-electrophoresis

Immuno-electrophoresis was performed according to the procedure of Grabar & Williams (1955). Agarose: 1% (w/v) dissolved in 2% (w/v) sodium barbitone buffer, pH 8.6, or 0.04 M-Tris/phosphate buffer, pH 8.6, was layered on glass microscope slides. Samples to be analysed were applied to 1.5mm diameter wells cut in the agarose gel and electrophoresis was performed using the same buffer for 3h. A Shandon Southern power pack supplied a constant voltage of 170 volts. Antiserum was then applied to a central trough cut between the sample wells and diffusion of antiserum and antigen was allowed to proceed for 18h

at room temperature. The slides were washed with 0.9% (w/v) NaCl for 5 days and thereafter with distilled water for 6h. After drying at 40°C (3h) the slides were stained (10min) and destained using solutions identical to those described for polyacrylamide gel electrophoresis (Section 2.2.2).

2.2.5 Estimation of NH₂ - terminal amino acids

The methods of Woods & Wang (1967) and Hartley (1970) were modified to estimate NH₂-terminal amino acids.

5-10mmol of dried sample was redissolved in 10 µl of 0.2 M-NaHCO₃ and, following the addition of 30 µl of 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride, DNS-Cl) the solution was incubated at 37°C for 30min. After drying in vacuo, 100 µl of 6 M-HCl was added, the tube sealed and incubated at 100°C overnight. The sample was again dried and the solid residue extracted twice using 100 µl of the top phase of an ethyl acetate: water mixture (1:1,v/v). The extract was dried and redissolved in 15 µl of 50% (v/v) aqueous pyridine. This solution was spotted in one corner, approximately 0.5cm from each edge of a 5 x 5cm polyamide sheet. A standard DNS-amino acid mixture was similarly applied to the reverse side. After air drying of the spot the chromatogram was developed in the first dimension using 1.5% (v/v) formic acid, dried in a stream of air, and

developed in the second dimension in benzene/glacial acetic acid (9:1, v/v). After air drying the chromatogram was visualized under ultraviolet light.

The standard DNS-amino acid mixture contained each of the following DNS-amino acids (350 μ g/ml in 50% (v/v) aqueous pyridine); DNS-L-Leucine, DNS-L-Serine, DNS-L-Threonine, DNS-L- β -Phenylalanine, DNS-L-Glycine, DNS-L-Glutamic acid, DNS-L-Aspartic acid, DNS-L-Methionine and N-O-Di-DNS-L-Tyrosine.

2.2.6 Estimation of protein concentration

The concentration of fibrinogen, fragment D and fragment E was estimated by measuring their absorbance at 280nm, based upon specific absorption coefficients of 15.1, 20.8 and 10.2, respectively (Marder et al., 1969).

SECTION 3

THE REMOVAL OF CONTAMINATING PLASMINOGEN FROM HUMAN FIBRINOGEN AND THE ISOLATION OF PLASMINOGEN FROM HUMAN PLASMA

3.1 Introduction

The human fibrinogen preparations which are available from commercial sources are contaminated with plasminogen. This readily available supply of proenzyme has frequently been exploited in the study of the plasminic degradation of fibrinogen; fibrinogenolysis being initiated simply by the addition of the plasminogen activator streptokinase to fibrinogen solutions. However the extent of plasminogen contamination may vary. A more reliable and reproducible method of digestion fibrinogen would appear to be that of adding a defined quantity of plasminogen to a plasminogen-free fibrinogen preparation.

The following group of experiments describes the methods employed to (a) achieve the purification of fibrinogen (b) isolate plasminogen from human plasma and (c) standardise the latter preparation. The affinity chromatography technique described by Deutsch & Mertz (1970) offered a relatively quick and simple method of accomplishing both (a) and (b). They demonstrated that plasminogen would bind to lysine coupled to an insoluble gel matrix. The retarded enzyme then being specifically eluted with a 6-amino-n-hexanoic acid solution.

3.2 Methods

3.2.1 The removal of contaminating plasminogen from fibrinogen

Human fibrinogen was freed of contaminating plasminogen

by an affinity chromatography procedure based on the methods of Deutsch & Mertz (1970) and Pharmacia Fine Chemicals Literature (1976). Fibrinogen was dissolved (20mg/ml) in 0.1 M-sodium phosphate buffer, pH 7.5, containing 3 mM-EDTA and applied to a column (1.0 x 12cm) of lysine-Sepharose 4B which had been equilibrated using the same buffer. The column was eluted with starting buffer until absorbance at 280nm of the effluent returned to 0.05. The effluent which had been collected in bulk at 4°C was stored in suitable aliquots at -20°C. Loosely and non-specifically bound protein was then washed from the column by the application of 0.5 M-NaCl dissolved in starting buffer. Finally 0.2 M-6-amino-n-hexanoic acid in distilled water was applied to elute any bound plasminogen from the column. These latter two elutions were each dialysed against starting buffer at 4°C for 24h and then stored at -20°C.

The efficiency of the lysine-Sepharose 4B column in removing contaminating plasminogen from fibrinogen was tested as follows;

(i) Fibrinogen (5mg/ml) both before ("unpurified") and after ("purified") column treatment was incubated (37°C) in the presence of streptokinase (10 units/mg). After 2lh, Trasylol (20 Kallikrein inactivator units (K.I.U.)/mg) was added. Aliquots removed from each incubation were examined, reduced on 5% polyacrylamide gels. In

addition a reduced sample of each type of fibrinogen which had not been exposed to streptokinase or incubated at 37°C was examined.

(ii) Using the technique of immunoelectrophoresis the reactivity of each type of fibrinogen against anti-human fibrinogen and anti-human plasminogen antisera was investigated. Also examined by this technique were the 6-amino-n-hexanoic acid and NaCl-eluted fractions from the lysine-Sepharose 4B column.

3.2.2 The isolation of plasminogen from human plasma

The method followed was that described above for the removal of contaminating plasminogen from fibrinogen.

150ml of human plasma was applied to a column (1.0 x 15cm) of equilibrated lysine-Sepharose 4B. Three elutions were collected as before at 4°C prior to storage at -20°C. After re-equilibration of the column with starting buffer, the cycle was repeated until a total of 600ml of human plasma had been processed. Following every second cycle the column was washed with equilibration buffer containing 0.2 M-6-amino-n-hexanoic acid and 1.0 M-NaCl.

The (plasminogen-containing) fractions eluted by the 6-amino-n-hexanoic acid solution were pooled and then treated following the recommendations of Robbins & Summaria (1970) and Rickli & Otavski (1973). The preparation was

dialysed against 0.05 M-sodium phosphate buffer, pH 7.5 containing 0.15 M-NaCl for 48h at 4°C and then dialysed for a further 24h at 4°C against 0.05 M-Tris/HCl buffer, pH 9.0, containing 0.1 M-NaCl, 1 mM-EDTA, 0.02 M-lysine monohydrochloride. The dialysate was stored at -20°C in suitable volumes.

3.2.3 Assay of Plasminogen

Plasminogen was activated to plasmin and assayed by the caseinolytic procedure described by Johnson et al. (1969) and recommended by the N.H.I. Committee on Thrombolytic Agents.

To a series of plasminogen samples (0-400 µl) were added 200 units of streptokinase and 0.06 M-Tris/HCl buffer, pH 7.5, containing 0.09 M-NaCl, to a final volume of 2.5ml. Following the addition of 2.5ml of an α-casein solution (1.4%, (w/v) in the above buffer) the samples were incubated at 37°C. After periods of 2 and 32min, 2ml aliquots were withdrawn and blown into 3ml 0.5 M-perchloric acid. Following a period of 40min incubation at room temperature the solutions were filtered through Whatman #42 filter paper. The absorbance of each filtrate was read at 275nm in a Fye Unicam SP500 u.v. and visible spectrophotometer (1cm light path) using the 2min sample as the blank.

Fig 3.1 Chromatography of human fibrinogen on lysine-Sepharose 4B

Contaminating plasminogen was removed from fibrinogen by lysine-Sepharose 4B chromatography. Column dimensions: 1.0 x 12cm. Flow rate: 30ml/h. Elution procedure: 0.1 M sodium phosphate buffer, pH 7.5 containing 3 mM-EDTA followed by (A) 0.5 M-NaCl in the same buffer and (B) 0.2 M-6-amino-n-hexanoic acid in distilled water.

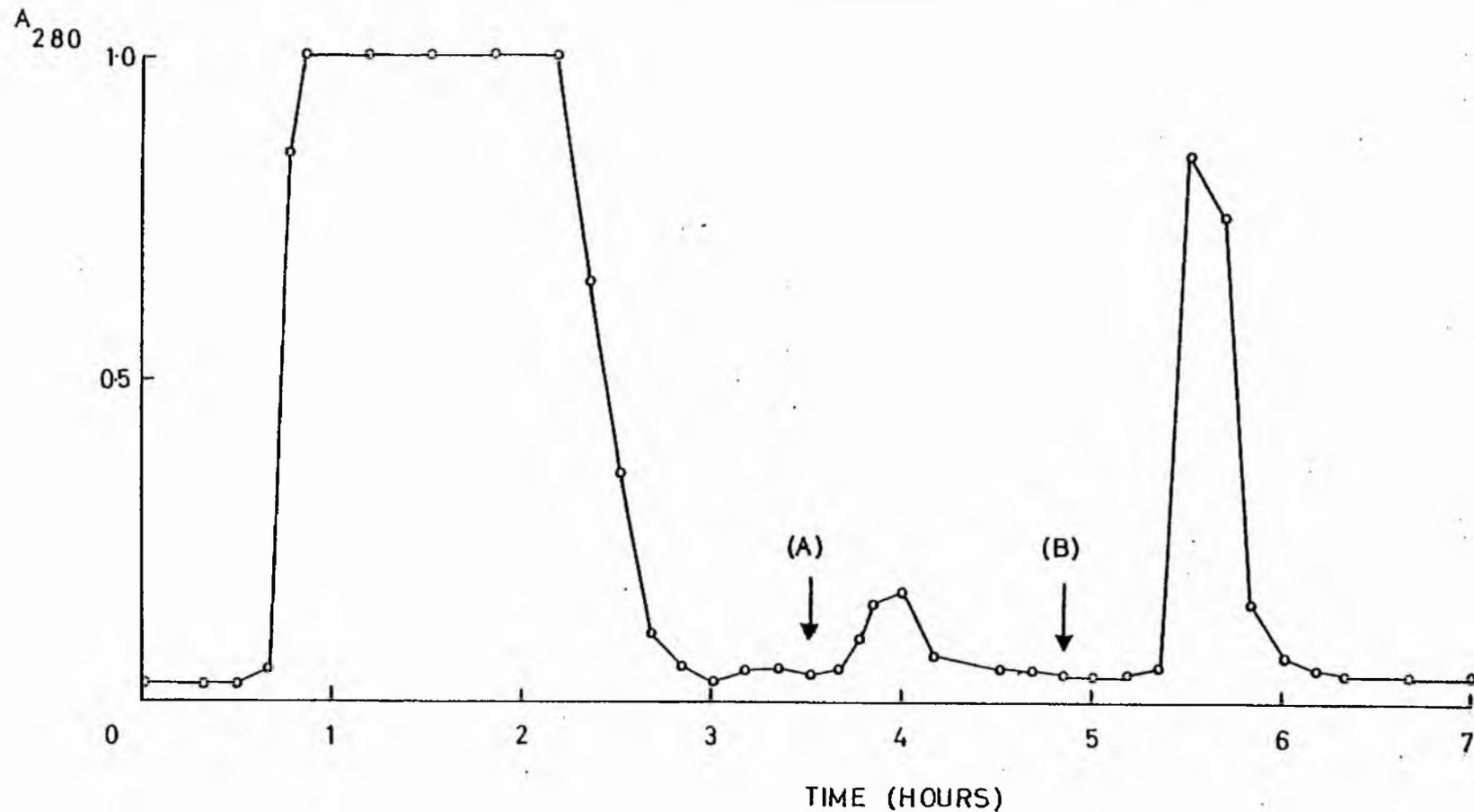
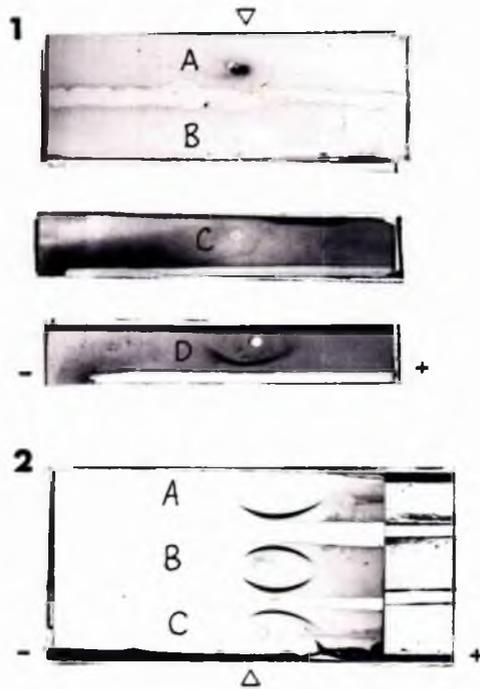


Fig. 3.2 Agar gel immunoelectrophoresis

The various stages of fibrinogen purification by lysine-Sepharose 4B chromatography (Fig. 3.1) were examined by immunoelectrophoresis employing **1** anti human plasminogen and **2** anti human fibrinogen antisera

- Sample A Fibrinogen before application to lys-Sepharose
- B Fibrinogen eluted from lys-Sepharose
- C Material eluted by the NaCl-buffer
- D Material eluted by the 6-amino-n-hexanoic acid solution

The origin is indicated by the arrows and the cathode is on the left.



3.3 Results

3.3.1 The removal of contaminating plasminogen from fibrinogen

The elution pattern obtained following the application of fibrinogen to a column of lysine-Sepharose 4B is illustrated by Fig. 3.1. Three elution peaks are evident. The results obtained from immunoelectrophoretic studies of samples corresponding to each of these elutions (Fig. 3.2) suggest that the first and second contained fibrinogen while the third reacted against anti-human plasminogen antiserum. Thus the lysine-Sepharose 4B treatment had indeed removed a plasminogen-like protein from the fibrinogen preparation. However Fig. 3.2 also illustrates that the fibrinogen preparation failed to react with anti-plasminogen antiserum even before the purification process. Therefore this test alone would not be a reliable indicator of the efficiency of the column procedure.

An alternative method of monitoring the extent of plasminogen contamination of fibrinogen was provided by streptokinase-induced digestion studies (Fig. 3.3). Fibrinogen both before (unpurified) and after (purified) column treatment displays the characteristic $A\alpha$, $B\beta$ and δ reduced chain pattern upon SDS-polyacrylamide gel electrophoresis (Fig. 3.3; a, c). However following their incubation in the presence of streptokinase (a plasminogen

Fig. 3.3 SDS-polyacrylamide gel electrophoresis of fibrinogen

Samples of fibrinogen both before (unpurified) and after (purified) lysine-Sepharose 4B treatment were examined on 5% polyacrylamide gels reduced (gels a and c) and reduced after incubation at 37°C in the presence of streptokinase (gels b and d).

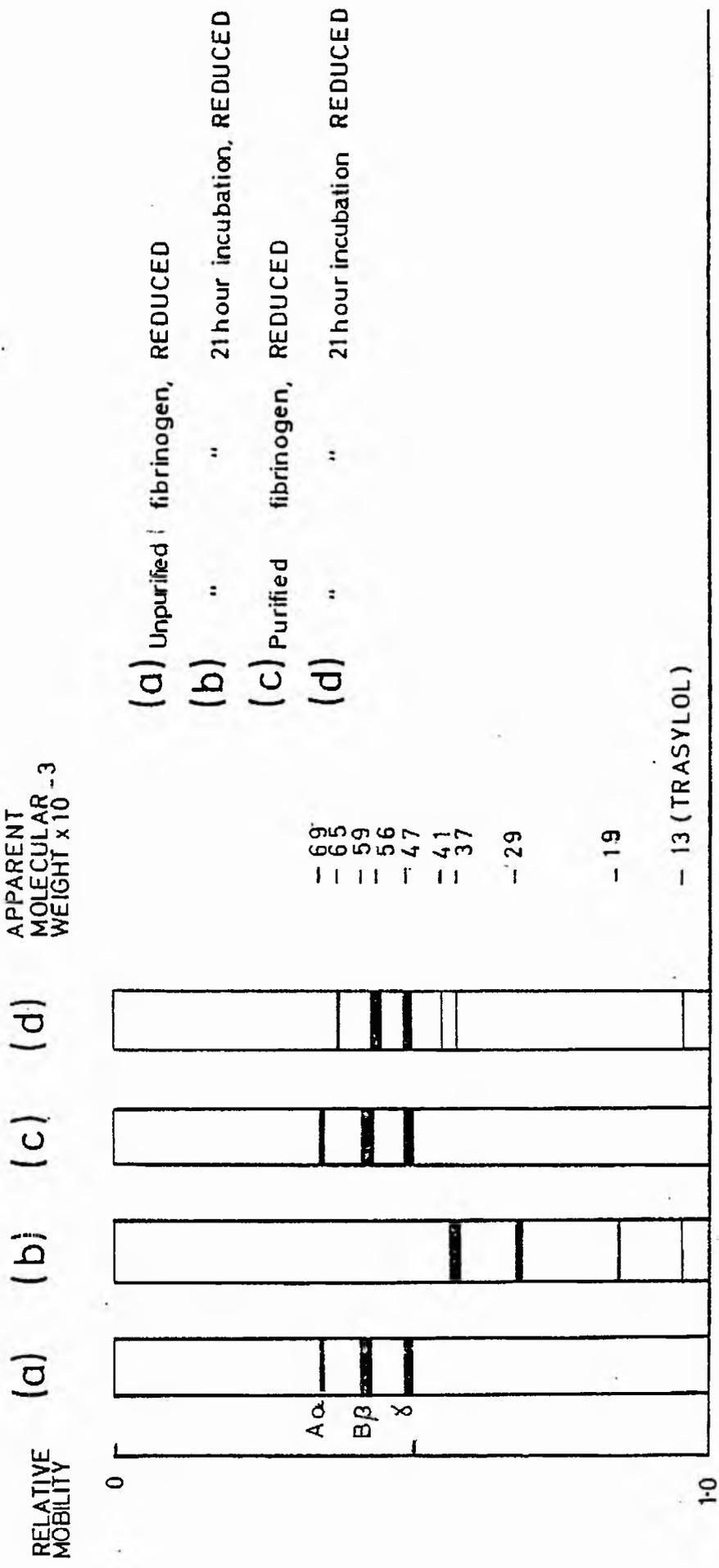
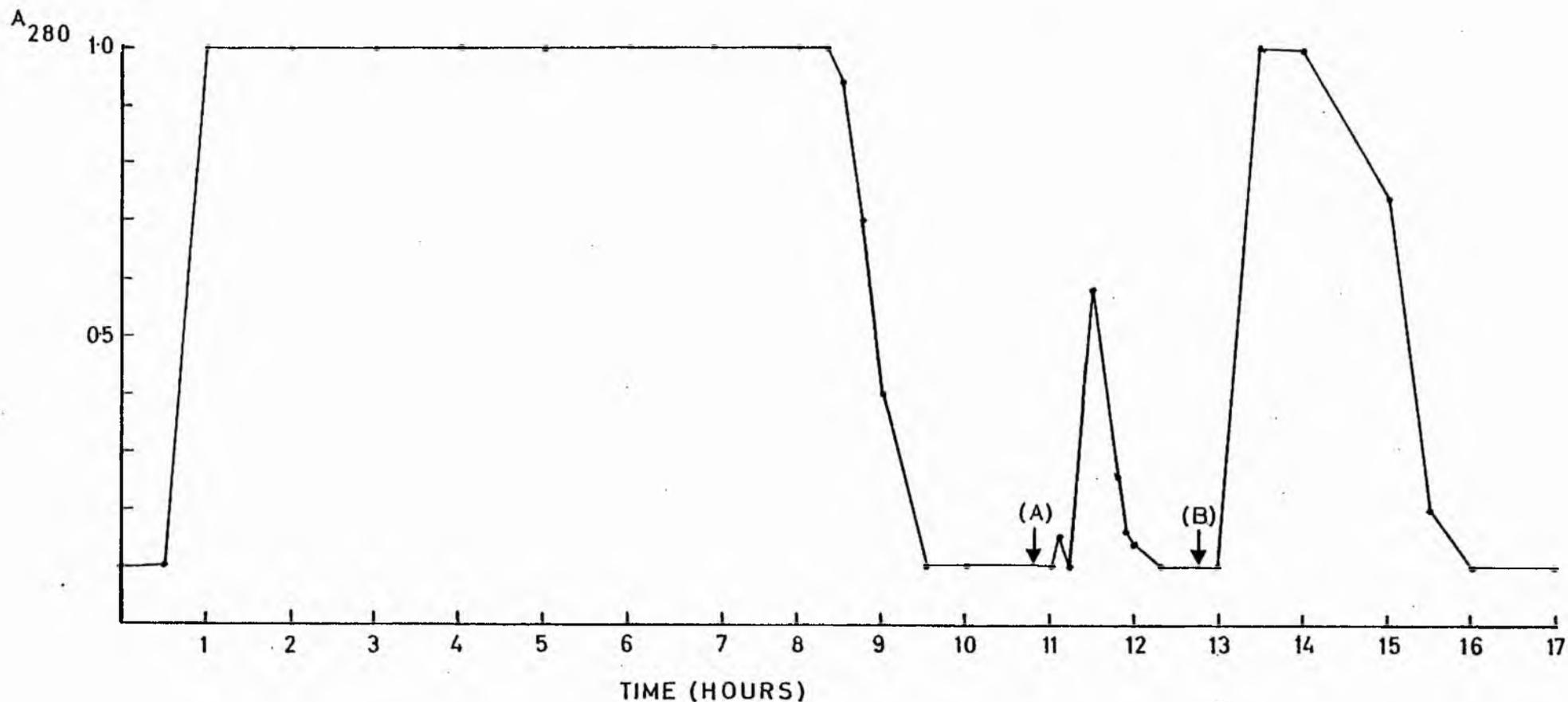


Fig. 3.4 The isolation of plasminogen from human plasma by lysine-Sepharose 4B chromatography

Chromatography of plasma on lysine-Sepharose 4B. Column dimensions 10x15cm. Flow rate: 27ml/h. Sample: 150ml plasma. Elution procedure as for Fig. 3.1.



activator with no inherent proteolytic activity (McClintock & Bell, 1971)) extensive degradation of all three constituent chains of the unpurified fibrinogen is obvious. This is in sharp contrast to the purified fibrinogen; degradation of the latter being restricted to the A α chain (Fig. 3.3; b,d).

3.3.2 The isolation of plasminogen from human plasma

Fig. 3.4 illustrates the elution pattern obtained following the application of human plasma to a column of lysine-Sepharose 4B. Specifically bound plasminogen was eluted from the column at (B) with 0.2 M-6-amino-n-hexanoic acid. This ω -amino acid (an inhibitor of the activation of plasminogen) was subsequently removed by exhaustive dialysis and the activity of the preparation estimated by a standard caseinolytic assay. The results are presented in Table 3.1. The method of Johnson et al. (1969) defines a CTA plasmin unit as follows; one CTA plasmin unit releases 0.1 micro-equivalent of tyrosine/min from Lot GC 1-16 α -casein. In the present case α -casein obtained from a different source (Sigma Chem. Co.) was employed. Since α -casein preparations may vary from batch to batch in their susceptibility to hydrolysis, the plasmin units of the isolated plasminogen preparation will be referred to as CA units and not CTA units. The mean activity of this preparation was 3.46 CA units/ml. The total volume of the preparation was approximately 140ml

Table 3.1 The caseinolytic activity of plasminogen isolated from human plasma

Human plasminogen, isolated by lysine-Sepharose affinity chromatography, was assayed by the caseinolytic procedure of Johnson *et al.* (1969).

VOLUME OF PLASMINOGEN (mls)	NET A ₂₇₅	INITIAL DILUTION FACTOR	CALCULATED ACTIVITY (C. A. units/ml)
0.4	0.439	/	3.46
0.2	0.284	/	4.48
0.4	0.037	1/10	2.92
0.2	0.019	1/10	3.00

MEAN = 3.47 ± 0.62
(±SD.)

and was isolated from 600ml of plasma. Thus a yield of 80.7 GA units/100ml of plasma was obtained.

An aliquot of the preparation was examined by the technique of immunoelectrophoresis. It reacted against anti-human plasminogen antiserum. Further characterization of the preparation by polyacrylamide gel electrophoresis will be described in a later Section (Section 6.2.1).

3.4 Discussion

The purpose of the experiments described in this section was twofold. Firstly to remove contaminating plasminogen from a sample of fibrinogen and secondly to isolate human plasminogen from plasma.

Evidence that the first task had been accomplished was provided by the results of immunoelectrophoretic studies. A protein which reacted against anti-human plasminogen was removed from the fibrinogen sample. Furthermore the results of SDS-polyacrylamide gel analysis of both "pure" and "unpure" fibrinogen following its exposure to streptokinase indicated that the lysine-Sepharose 4B column treatment had markedly reduced the level of contaminating plasminogen in fibrinogen. However, limited degradation of the purified fibrinogen constituent chains was also induced by this treatment which suggests that the removal of

plasminogen had not been complete. Alternatively the degradation may have been induced simply by the conditions of incubation.

All fibrinogen employed throughout the ensuing work was subjected to this purification procedure and then stored at -20°C until required.

(While the bulk of the fibrinogen sample passed unretarded through the lysine-Sepharose 4B gel a small amount of a fibrinogen-type protein was retained and later eluted with the high salt-containing buffer. This may represent a manifestation of the affinity of fibrin for plasmin and plasminogen described by Cederholme-Williams (1977). Alternatively this protein may be a degraded form of fibrinogen with an affinity for the gel matrix.)

The second set of experiments described the isolation of protein assumed to be plasminogen which reacted against anti-plasminogen antiserum and which hydrolysed α -casein. A caseinolytic activity of 3.46 CA units/ml was recorded and this activity value has been assumed in all digestion studies performed with fibrinogen in Part A of this work.

Although sufficient plasminogen was isolated to meet the requirements of the fibrinogen digestion studies of Part A, the yield of plasminogen from plasma does not compare favourably with that obtained by Deutsch & Mertz (1970). Assuming a level of plasminogen in plasma of

2.5 $\mu\text{mol/litre}$ (Mullertz, 1978) and a specific activity of 200 CTA units/mg (Rickli & Cuendet, 1971) a theoretical yield of 400 CTA units/100ml of plasma is predicted. Thus the present results represent 20% of the theoretical yield and 33% of the yield obtained by Deutsch & Mertz. The low figure obtained may indicate either the incomplete removal of the plasminogen activation inhibitor, 6-amino-n-hexanoic acid during the dialysis step or that the α -casein preparation differed in sensitivity to that recommended by Johnson et al. Rickli and Cuendet (1971) also obtained an unsatisfactory yield of plasminogen using this chromatography method and found that lysine coupled to polyacrylamide was a more efficient absorbent for human plasminogen.

SECTION 4

THE ESTABLISHMENT OF THE MOST SUITABLE CONDITIONS
OF FIBRINOGEN DIGESTION.

4.1 Introduction

The aim of Part A of this work was the investigation of the plasmic degradative pathway of fibrinogen fragment D to fragment d, consequently the first task was the isolation of a high molecular weight form of fragment D in a quantity suitable for further study. Preliminary experiments were performed to establish the most suitable conditions of fibrinogen digestion for the production and preservation of a high molecular weight form of fragment D.

4.2 Methods

4.2.1 Determination of the conditions of fibrinogen digestion for the maximum production of fragment D

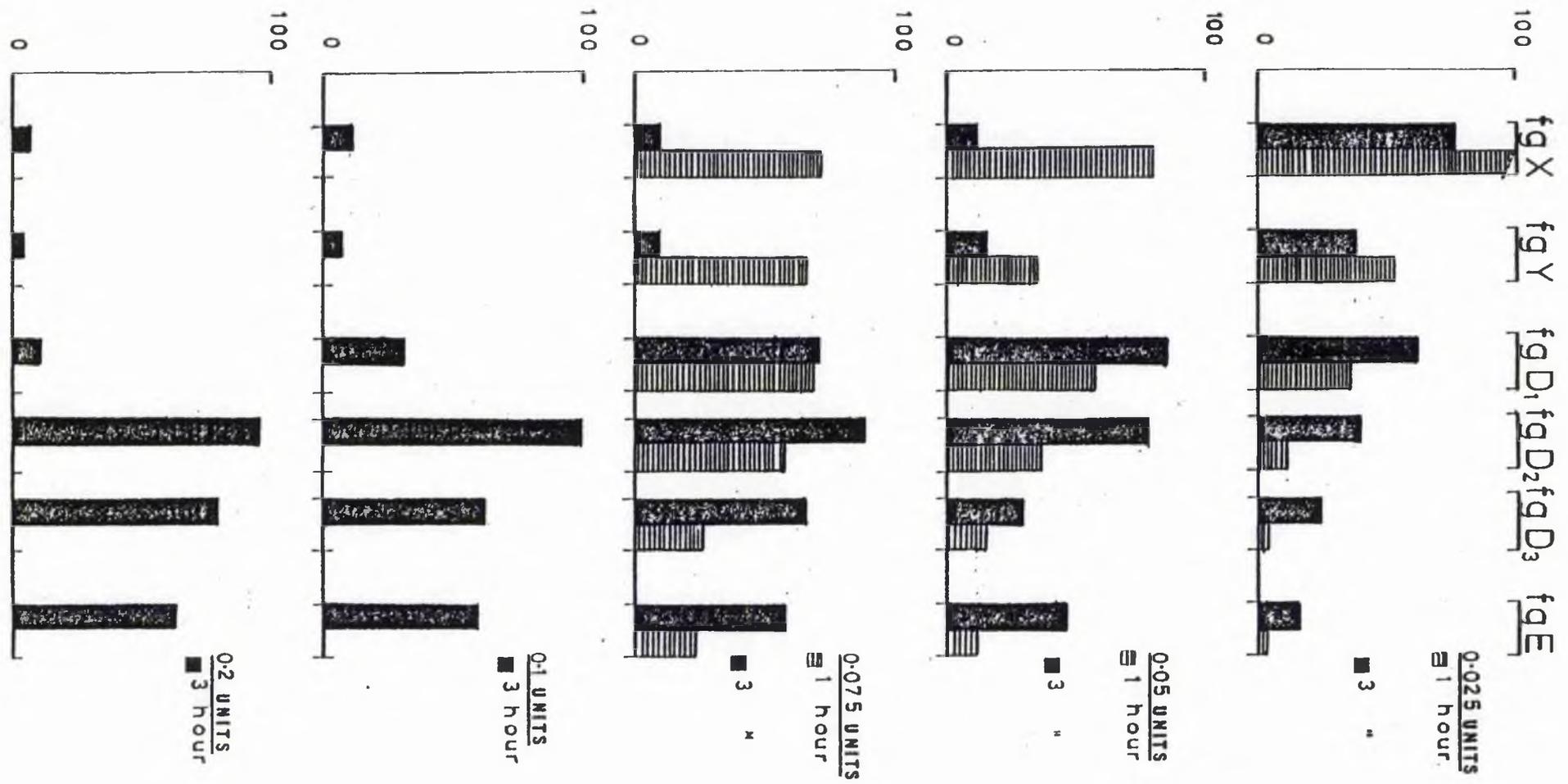
Plasminogen was activated to plasmin by incubation at 37°C for 15min in glycerol (25%, v/v) (Rickli & Otavsky, 1973) and streptokinase (150 units/CA unit plasminogen). Different volumes of this solution were then added to fibrinogen (5mg/ml in 0.05 M-sodium phosphate buffer, pH 7.5). Digestion was allowed to proceed at 37°C for various time intervals and was terminated by the addition of Trasylol (200 K.I.U./ml, final concentration). Samples were examined (unreduced) on 5% polyacrylamide gels.

Fig. 4.1 The digestion of fibrinogen by plasmin
to fragment D

Fibrinogen was digested by plasmin at 37°C employing different levels of plasmin and periods of incubation. Samples from each digest were examined (unreduced) by SDS-polyacrylamide gel electrophoresis (5% gels) and a quantitative estimate of the levels of each of the fibrinogen digestion products, fragments X, Y, D₁, D₂, D₃ and E thereby obtained. The incubation period and the units of enzyme (CA units/5mg of fibrinogen) employed are indicated:

Fig. 4.1

DENSITOMETER SCAN PEAK HEIGHT (ARBITRARY UNITS)



4.2.2 Investigation of alternative methods of plasmin inhibition

Plasmin, prepared as described above, was incubated with fibrinogen (0.001 CA unit/mg) at 37°C for 6h after which time three samples (300 µl each) were removed and treated as follows;

sample (A) was mixed with Trasylol (60 K.I.U.) and then stored at 4°C for 24h.

sample (B) was also mixed with Trasylol (60 K.I.U.) but was incubated at 37°C for 24h.

sample (C) was added to lysine-Sepharose 4B dry powder and after a period of 10min the resulting gel was spun on a bench centrifuge and the resulting supernatant then incubated at 37°C for 24h.

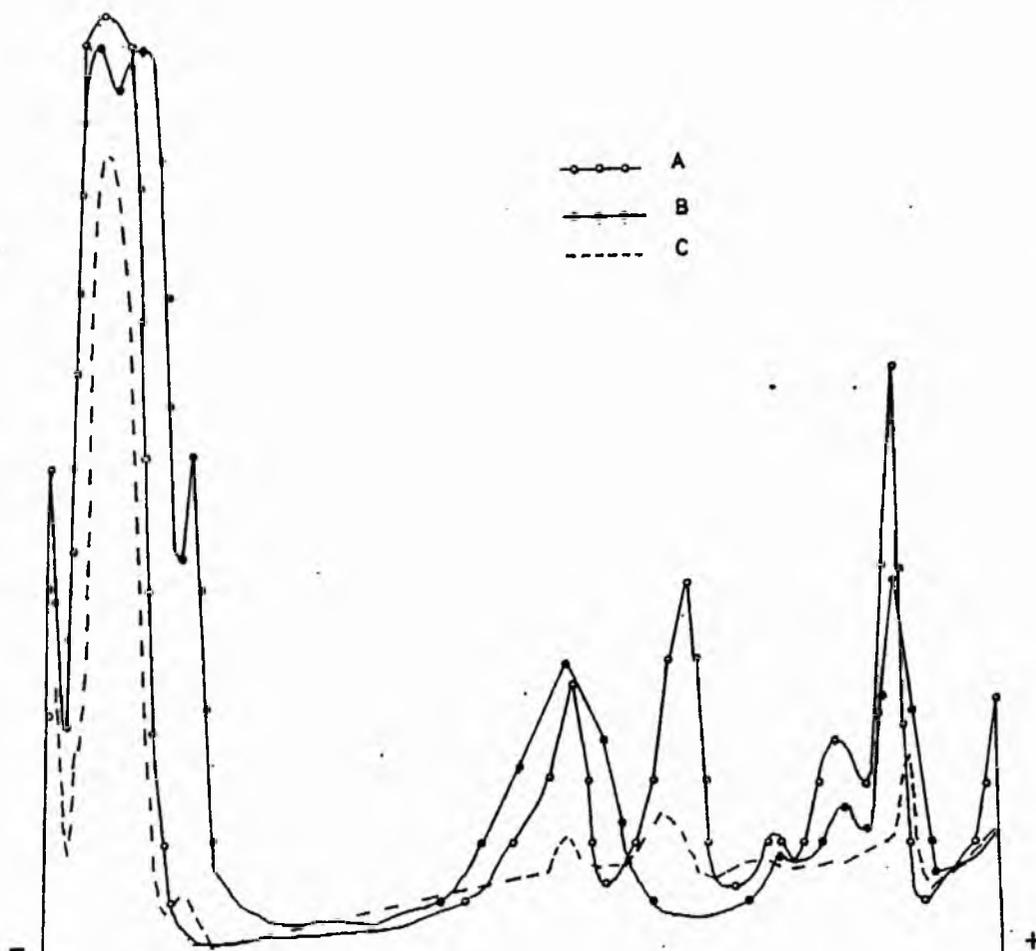
All three samples were examined (unreduced) on 5% polyacrylamide gels.

4.3 Results

4.3.1 Determination of the conditions of fibrinogen digestion for the maximum production of fragment D

Fig. 4.1 illustrates the results of the fibrinogen digestion studies in which both the level of plasmin (expressed as CA units/5mg fibrinogen) and the period of incubation were varied. Following the electrophoretic separation of each digest sample the gels were scanned using a densitometer and a quantitative estimate of the level of each of the digestion intermediates (fragments

Fig. 4.2 SDS-polyacrylamide gel electrophoresis of three samples of a fibrinogen digest



Three samples removed from a plasmic digest of fibrinogen were treated as follows; to A and B was added Trasylol while lysine-Sepharose was added to C. Samples B and C were incubated at 37°C for 24 h. Each sample was then examined (unreduced) on 5% polyacrylamide gels. The corresponding densitometer scans are shown.

X, Y, D₁, D₂, D₃ and E) was thereby obtained.

In all of the digestion samples where the quantity of plasmin had exceeded 0.05 CA units (Fig. 4.1; b, c, d.) the higher electrophoretic mobility forms of fragment D (D₂ and D₃) are prevalent. Digestions employing less than 0.05 CA units of plasmin (Fig. 4.1; a) display considerable amounts of the high molecular weight intermediates, fragments X and Y. Fragment D₁ was produced maximally when 0.05 CA units of plasmin were incubated with 5mg of fibrinogen for 3h.

It is noteworthy that in none of the conditions studied was a single fragment D band produced. The apparent molecular weights of fragments D₁, D₂ and D₃ were 89,000; 80,000 and 76,000 respectively.

4.3.2 Investigation of alternative methods of plasmin inhibition

Densitometer scans of the polyacrylamide gels corresponding to each of the three fibrinogen digest samples A, B and C are presented in Fig. 4.2.

Sample (A) (Trasylol, 4°C) contains a major, low mobility peak and several small high mobility (low molecular weight) peaks. Sample (B) (Trasylol, 37°C) also contains a major, low mobility, peak but it is split into two components and in addition a third peak of slightly increased mobility is obvious. These changes induced in the electrophoretic pattern of the fibrinogen

digest sample by its incubation at 37°C suggest that further degradation has occurred. Sample (C) (lysine-Sepharose, 37°C) has produced an electrophoretic pattern very similar to that described for sample (A). The incubation of sample (C) at 37°C does not appear to have induced further degradation of its constituents. However the peaks displayed by this sample are noticeably less intense than the corresponding peaks of sample (A). Thus the use of lysine-Sepharose to inhibit plasmin may consequently decrease the yield of fibrinogen digest intermediates.

4.4 Discussion

This section of work was concerned with establishing the most suitable conditions for the plasmin digestion of fibrinogen which would give the greatest yield of, and preserve a high molecular weight form of fragment D.

In the first instance fibrinogen was digested for various time intervals by different levels of plasmin. Each digest sample was analysed by SDS-polyacrylamide gel electrophoresis and although problems are associated with the reliability of results obtained by the direct quantification of peaks obtained by densitometric scans, this method does serve to illustrate the rationale behind the selection of fibrinogen digestion conditions which were applied in the following section (Section 5) for the large scale production of fragment D.

The second concern was to ensure that the high molecular weight form of fragment D so produced would not be further degraded by plasmin. Two plasmin-inhibitor agents were investigated. It is assumed that Trasylol binds reversibly to the active site of plasmin, (Bayer Pharmaceuticals Literature). This reversibility of Trasylol inhibition was demonstrated in the present results by the incubation of a fibrinogen digest solution containing Trasylol at 37°C. However no such property was displayed by the second plasmin inhibitor examined, lysine-Sepharose 4B. The characteristics of this gel have already been discussed in relation to the isolation of plasminogen from fibrinogen and from plasma (Section 3). Both plasminogen and plasmin bind to a lysine-Sepharose 4B gel (Siefiring & Castellino, 1975) and this method of plasmin inhibition offers an attractive alternative to the use of Trasylol, for not only will plasmin be inhibited, it will also be removed, complexed to the gel, by the centrifugation step and therefore from the vicinity of fragment D. However, the foregoing results predict that a slightly decreased yield of fragment D must be expected from the use of the lysine-Sepharose method of plasmin inhibition. This may be the result of the binding of fibrinogen degradation products either to the lysine-Sepharose 4B gel matrix or to plasmin (ogen) bound in turn to the lysine groups of the gel.

SECTION 5

THE PREPARATION AND ISOLATION OF FRAGMENT D

5.1 Introduction

The following section of work describes the production and isolation of fragment D by plasmic digestion of fibrinogen according to the recommendations of the foregoing experiments (Section 4).

5.2. Methods

5.2.1 Digestion of fibrinogen

Fragment D was prepared by the plasmic degradation of fibrinogen as follows. Plasminogen (2.5 CA units) was activated to plasmin by incubation at 37°C for 15min in the presence of streptokinase (150 units/CA unit) and glycerol (25%, v/v). This solution was added to 250mg of fibrinogen (5mg/ml in 0.05 M-sodium phosphate buffer, pH 7.5) and digestion was allowed to proceed at 37°C for a period of 3h. At this point digestion was terminated as follows; to a 300 µl sample (designated fraction A) removed from the digestion mixture, Trasylol (60 K.I.U.) was added and to the remainder (approximately 57ml) was added 0.5g of lysine-Sepharose 4B which had been washed with 0.1 M-sodium phosphate buffer, pH 7.5 containing 1.0 M-NaCl and then by 0.05 M-sodium phosphate buffer, pH 7.5. The combined swollen gel and fibrinogen digest were mixed and left at 20°C for 5min

prior to centrifugation on a bench centrifuge (10min). The supernatant (designated fraction B) was removed and the pellet resuspended in 0.05 M-sodium phosphate buffer, pH 7.5, containing 0.5 M-NaCl. Following a second centrifugation step the resulting supernatant (fraction C) was stored at 4°C along with fractions A and B. Aliquots removed from each of these fractions were examined (unreduced) on 5% polyacrylamide gels.

5.2.2 Isolation of Fragments D and E from the fibrinogen digest by gel filtration

The bulk of the fibrinogen digest mixture (designated fraction B, above) was freeze dried, reconstituted in water (8ml) and dialysed against 0.1 M-sodium phosphate buffer, pH 7.5, for 24h at 4°C. Further dialysis for a similar period against 0.01 M-sodium phosphate buffer, pH 7.5, was followed by freeze drying. This digest concentrate, redissolved in 5ml water, was then fractionated by gel filtration at room temperature employing the procedure of Marder et al. (1969) and Furlan & Beck (1972). The sample was applied to a (2.5 x 35cm) column filled with Sephadex G-200 (Superfine grade) and eluted with 0.025 M-Tris/HCl buffer, pH 7.5, containing 1.0 M-NaCl, 0.2 M-6-amino-n-hexanoic acid and 0.025 M-sodium citrate at a flow rate of 8ml/h. The absorbance of the eluant at 280nm was measured in a Pye Unicam SP500 u.v. and visible spectrophotometer

and fractions (30min) were collected. Eluted fractions were characterised by immunoelectrophoresis (employing anti-human fibrinogen antiserum).

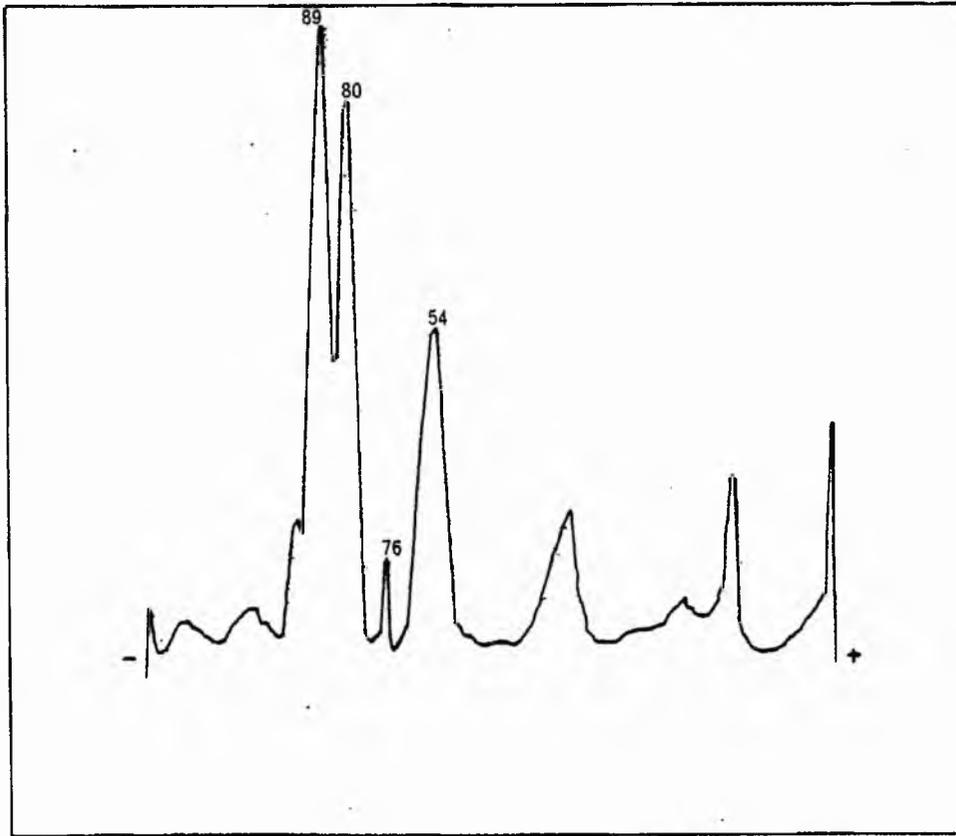
5.2.3 Separation of fragments D and E

Fragments D and E were fractionated by ion-exchange chromatography on sulphopropyl-Sephadex C-25 using the buffer system described by Hagenmaier & Foster (1971).

Those fractions containing fragments D and E (as judged by immunoelectrophoresis) eluted from the Sephadex gel were pooled and dialysed for 18h at 4°C against 0.02 M-sodium acetate buffer, pH 4.3, containing 0.18 M-NaCl. Sulphopropyl-Sephadex C-25 ion exchanger was packed into a column (2.5 x 30cm) and equilibrated using the above buffer. A flow rate of 20ml/h was maintained by a peristaltic pump and the absorbance of the eluant was monitored at 280nm as above. Following the application of the dialysed sample to the ion-exchanger bed surface the column was eluted with equilibration buffer and then by a linear pH gradient (pH 4.3 to 5.5) in 0.02 M-sodium acetate buffer containing 0.27 M-NaCl. Finally the column was eluted with 100ml of the latter buffer adjusted to pH 6.1. Aliquots removed from the eluted fractions were examined (unreduced) on 5% polyacrylamide gels.

5.3 Results

Fig. 5.1 Polyacrylamide gel electrophoresis of the fibrinogen digest applied to the Sephadex G-200 column



The digest sample was examined (unreduced) on a 5% gel. Apparent molecular weights, $\times 10^{-3}$, are shown.

5.3.1 Digestion of fibrinogen

The first stage in the isolation of fragment D was its production from fibrinogen by the action of plasmin. Three fractions removed from the fibrinogen digest solution (designated fractions A, B and C) were examined on 5% polyacrylamide gels. The densitometric scan produced by analysis of fraction B is shown in Fig. 5.1. This fraction was removed following the treatment of the bulk of the digest with lysine-Sepharose 4B gel and therefore illustrates the composition of the digest subsequently applied to the Sephadex G-200 gel. There is very little evidence of the high molecular weight fragments X and Y. Three peaks are evident in the mobility range predicted for fragment D and the following apparent molecular weights were calculated 89,000; 80,000 and 76,000, the latter fragment D being the least obvious. A peak of apparent molecular weight 54,000, attributed to fragment E, is obvious below the fragment D peaks. Thus digestion of fibrinogen has progressed almost exclusively to fragments D and E. The predominant fragment D species is the highest molecular weight form while the lowest molecular weight form is the least evident.

The electrophoretic pattern displayed by digest fraction A (an aliquot of the fibrinogen digest solution to which Trasylol had been substituted for lysine-Sepharose 4B) was identical to that shown in Fig. 5.1. No difference in the ability of each of these plasmin-inhibitor agents to prevent the further breakdown of fragment D was therefore detected.

Fig. 5.2 Sephadex G-200 fractionation of a plasmic digest of fibrinogen

A digest of fibrinogen was fractionated on a column (2.5 x 35cm) of Sephadex G-200 (Superfine). The column was eluted with 0.025 M-Tris/HCl buffer, pH 7.4, containing 1.0 M-NaCl, 0.025-M sodium citrate and 0.02 M-6-amino-n-hexanoic acid at a flow rate of 8ml/h. Fractions, 30min, were collected. Fractions were pooled as indicated by the horizontal bar for further chromatography.

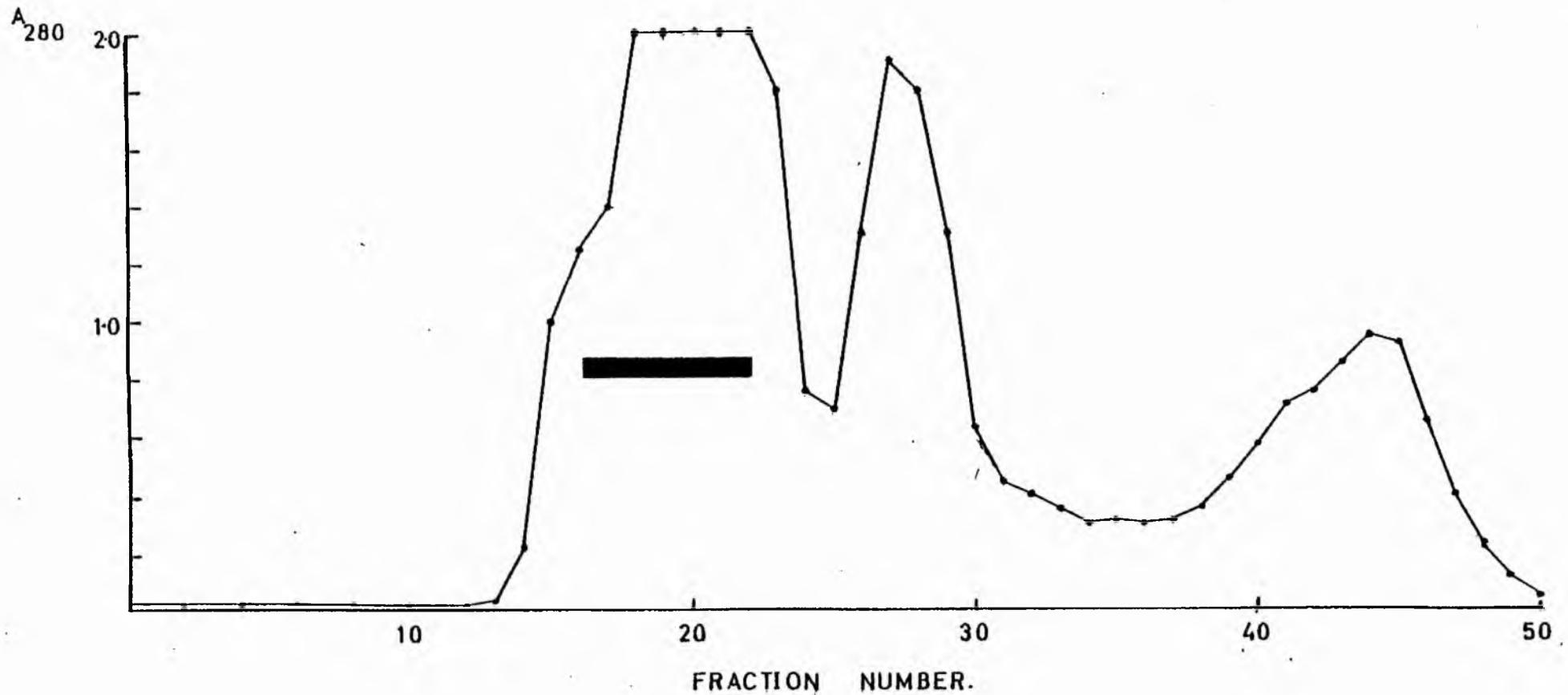
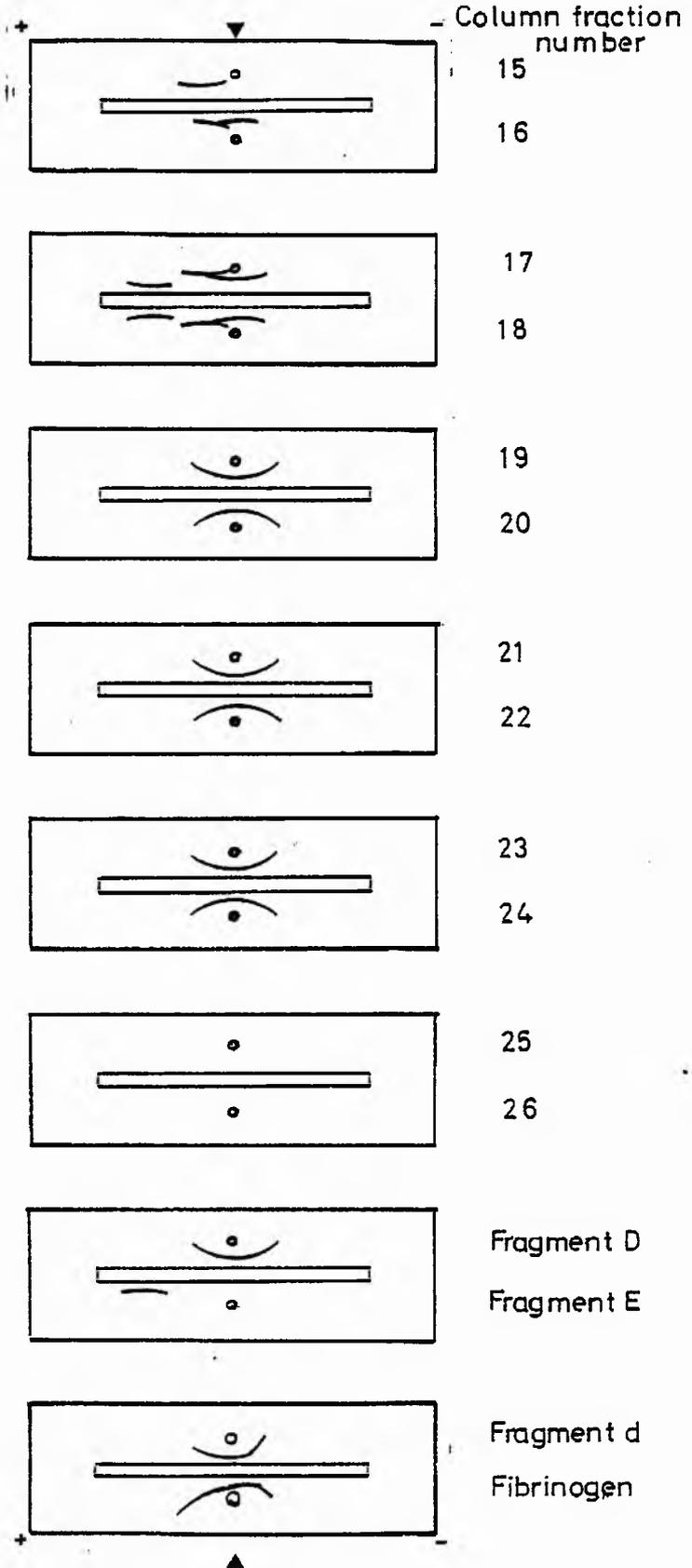


Fig. 5.3 Agar gel immunoelectrophoresis

Fractions 15–26 isolated from a fibrinogen digest by gel filtration (Fig. 5.2) and samples of fibrinogen and fragments D, E and d were examined by immunoelectrophoresis. Anti-human fibrinogen antiserum was present in the central trough. The origins are indicated by the arrows. The cathode is on the right.



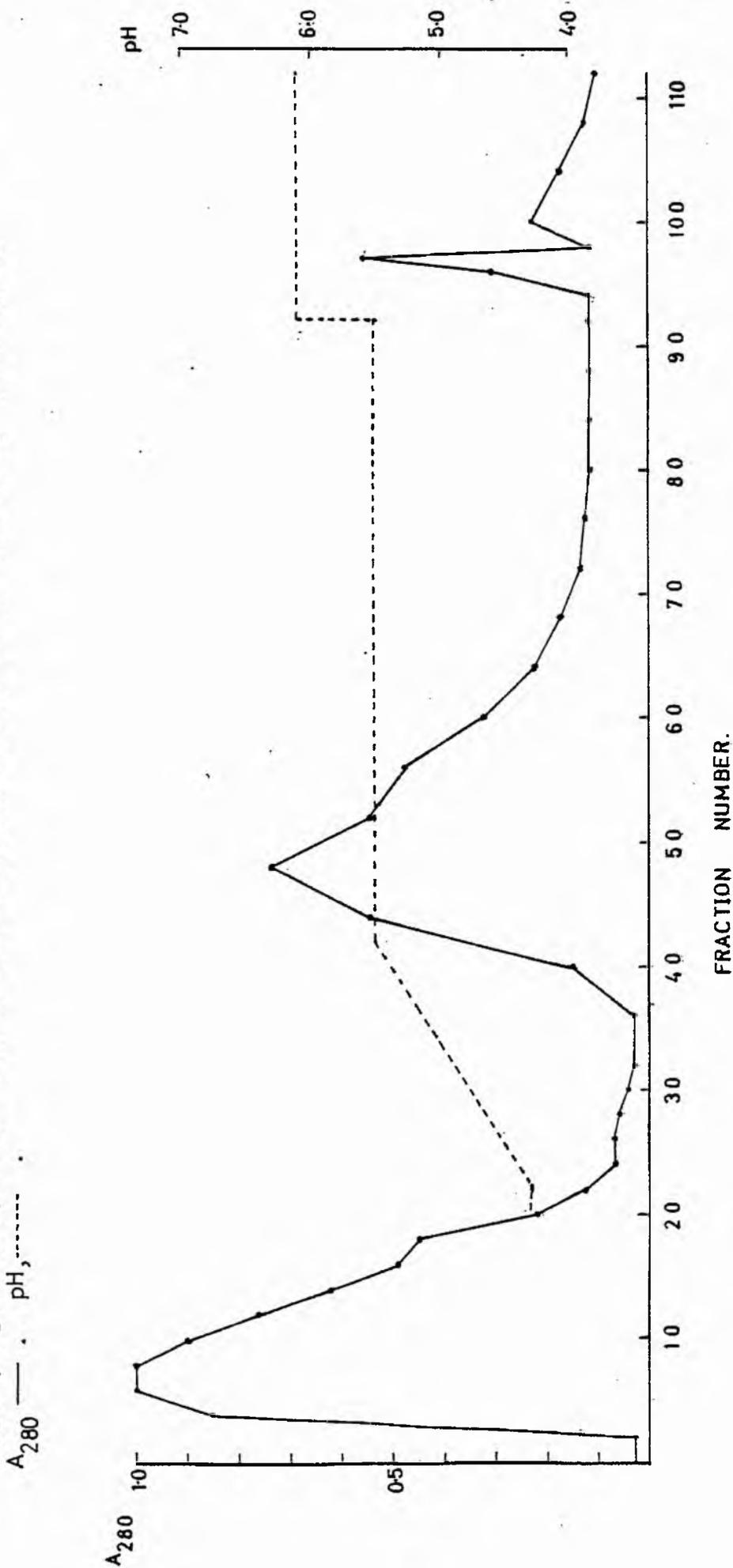
This implies that the reversible nature of the Trasylol-induced inhibition of plasmin, referred to previously (Section 4.2.2), may be less evident at 20°C than 37°C. Polyacrylamide gel electrophoresis of fraction C (the high salt washings of the lysine-Sepharose 4B gel pellet after the removal of the digest-containing supernatant) revealed four faint components of apparent molecular weights 114,000; 94,000; 85,000 and 56,000. These may represent a combination of plasmin(ogen) and streptokinase selectively removed by the gel from the digest solution. However, previous results (Section 4.3.2) suggest that the possibility of fibrinogen digest components being entrapped by the gel must also be considered.

5.3.2 Isolation of fragments D and E from the fibrinogen digest by gel filtration.

Fragments D and E were prepared from the fibrinogen digest by chromatography on Sephadex G-200. The elution diagram is shown in Fig. 5.2. The result of immunoelectrophoretic analysis of aliquots removed from fractions 15 to 26 using anti-human fibrinogen antiserum is shown in Fig. 5.3 (samples of fibrinogen, fragment D, fragment E and fragment d were included for comparison). These results predict the location of both fragments D and E in the early column fractions (15-18) and fragment D, also in fractions 19-24. Aliquots removed from fractions 25 and 26 did not react with the antisera. Considering the results of

Fig 5.4 Sulphopropyl-Sephadex C-25 chromatography of a fragment D and E mixture

A mixture of fragments D and E, isolated by gel filtration (Fig. 5.2), was separated by ion-exchange chromatography. Column dimensions: 2.5 x 30cm. Flow rate 20 ml/h. Fractions; 15 min. The column was eluted with 0.02 M-sodium acetate buffer pH 4.3 containing 0.18 M-NaCl and then by a linear pH gradient (pH 4.3 - 5.5) in 0.02 M-sodium acetate buffer containing 0.27 M-NaCl.



Furlan & Beck (1973) it would seem that these two fractions contained a degraded form of fragment D. Fractions 16-22 were pooled and subjected to further chromatography.

5.3.3 Separation of fragments D and E

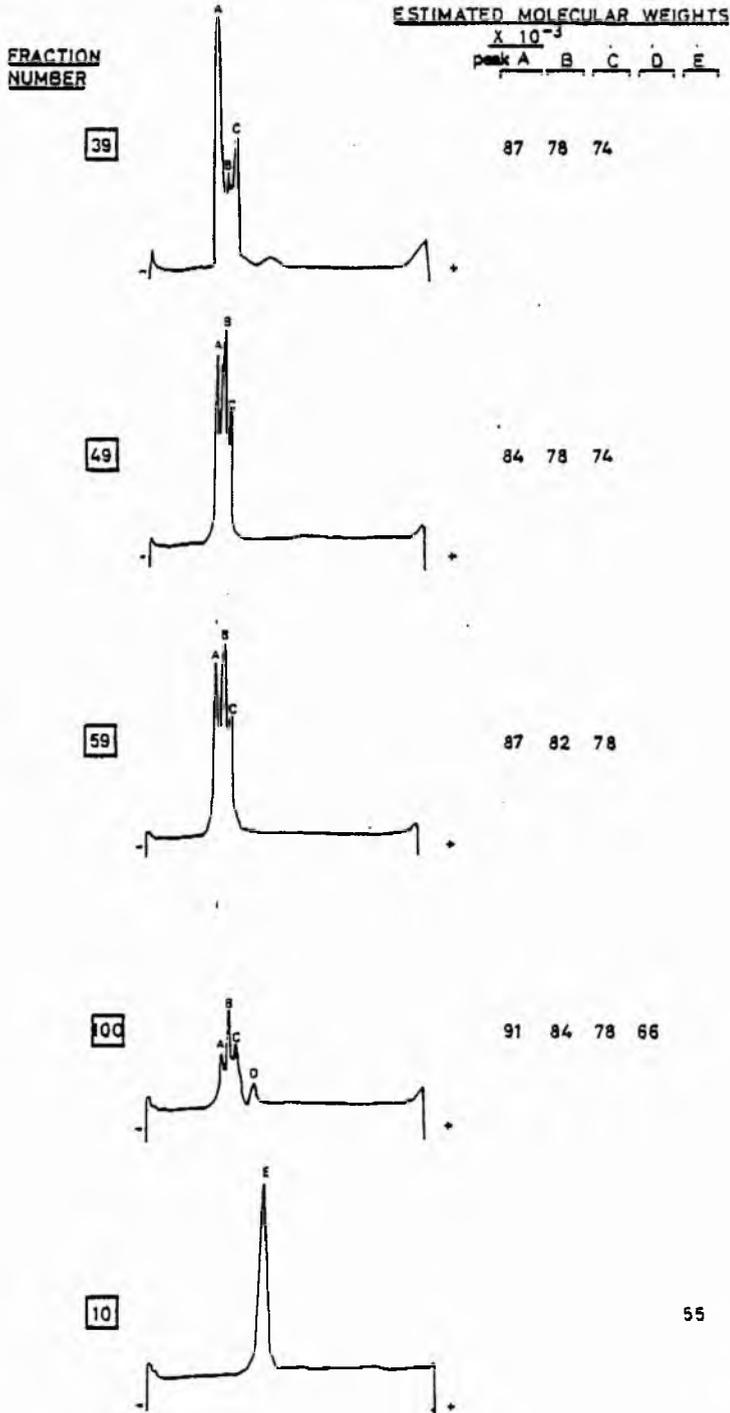
The elution diagram corresponding to the separation of the fragment D and E - containing sample on sulphopropyl-Sephadex C-25 is shown in Fig. 5.4. The results of SDS-polyacrylamide gel electrophoresis of aliquots removed from eluted fractions are shown in Fig. 5.5. The initial peak (fractions 2-22) contained fragment E (apparent molecular weight 55,000) while those fractions comprising the second peak, eluted following the application of a pH gradient, contained three fragment D components of apparent molecular weights (approximately) 87,000; 78,000 and 74,000. The highest molecular weight form of fragment D was most evident in the initial fractions eluted from the column while the intensity of the two lower molecular weight forms increased in the later fractions. The elution promoted by the buffer of pH 6.1 (the third peak) contained four components (fraction 100 Fig. 5.5). The values calculated for their apparent molecular weights suggests that this elution may contain fragment D and a degraded form of the same.

5.4 Discussion

The foregoing experiments describe the isolation of fragment D from a plasmic digest of fibrinogen. Two

Fig. 5.5 Densitometric traces of fractions eluted from a column of sulphopropyl-Sephadex C-25 and separated by SDS polyacrylamide gel electrophoresis

Samples removed from the various column fractions were examined (unreduced) on 5% gels. Apparent molecular weights are shown.



chromatographic procedures were employed. Firstly the separation of fragments D and E from both lower and higher molecular weight fibrinogen degradation products was achieved by gel filtration. The preparation of a fragment D free from fragment E was then accomplished employing ion-exchange chromatography. At pH 4.3 fragment E passed straight through the column of ion-exchanger while the retained fragment D was eluted by increasing the pH of the eluting buffer to 5.5. Although fragments D and E differ in apparent molecular weight by 20,000 to 30,000 they are not resolved by gel filtration and it has been proposed that these two fragments are non-covalently bound in a bimolecular complex (Budzynski et al., 1967). A further step, employing in this case a cation exchanger, is required to achieve their separation.

Those fractions eluted from the sulphopropyl-Sephadex C-25 column by increasing the pH of the buffer contained electrophoretically distinct forms of fragment D. Those of lowest electrophoretic mobility i.e. highest molecular weight were most prevalent in the early fractions. No contamination of these fractions by fragment E was apparent.

The isolation of a heterogeneous preparation of three electrophoretically distinct forms of fragment D is in accordance with the results reported by Furlan et al. (1975); Ferguson et al. (1975) and Pizzo et al. (1973). The

molecular weights reported by these authors for fragment D (calculated by analysis of the subunit composition) ranged from 82,000 to 102,000.

SECTION 6

THE DEGRADATION OF DENATURED FRAGMENT D BY PLASMIN

6.1 Introduction

The studies reported in this Section of work were designed to investigate the structure of fibrinogen fragment D by monitoring the plasmin degradative pathway of fragment D to fragment d employing the technique of SDS-polyacrylamide gel electrophoresis.

Before proceeding with these investigations it was considered appropriate to establish the SDS-gel pattern produced by electrophoresis of each of the fragment D digestion "cofactors" namely plasmin(ogen), streptokinase and Trasylol. This information was employed in this and later fragment D studies in the interpretation of the various SDS-gel band patterns.

6.2 Methods

6.2.1 SDS-polyacrylamide gel electrophoresis of fragment D digestion "cofactors"

The following six samples were examined by SDS-gel electrophoresis;

- (i) Plasminogen (0.044 CA units) incubated in the presence of streptokinase (16 units) for 1h at 37°C.

- (ii) Plasminogen prepared as in (i) and added, after the 1h incubation period, to Trasylol (20 K.I.U.)
- (iii) Plasminogen (0.044 CA units) added to Trasylol (10 K.I.U.)
- (iv) Plasminogen (0.044 CA units)
- (v) Streptokinase (500 units)
- (vi) Trasylol (10 K.I.U.)

Each sample was examined unreduced on a 5% polyacrylamide gel.

6.2.2 Plasmic degradation of denatured fragment D

Fibrinogen fragment D (1.5mg), isolated as described in Section 5, was dialysed against 0.02 M-sodium phosphate buffer, pH 7.5, for 18h at 4°C and then denatured by incubation for 30min at 37°C in the same buffer containing 2 M-urea. Digestion was initiated by the addition of plasmin (0.15 CA units, activated by streptokinase (150 units/CA unit)). Samples were removed from the digest solution at various time intervals and, following the addition of Trasylol (6000 K.I.U. per CA unit of plasmin), were examined by the technique of SDS-polyacrylamide gel electrophoresis.

Where digestion was allowed to proceed for time periods exceeding 8h the antibacterial agent chloramphenicol (final concentration 25µg/ml) was added to the fragment D solution.

6.3 Results

6.3.1 SDS-polyacrylamide gel electrophoresis of fragment D digestion "cofactors"

The purpose of these preliminary SDS-electrophoretic studies was to establish the SDS-gel band pattern appropriate to each of the agents employed as cofactors in the fragment D digestion studies. The SDS-electrophoresis results are presented in Fig. 6.1. The sample of plasminogen produced a major band of apparent molecular weight 69,000 (gel (iv)). Three minor bands are also evident. Violand & Castellino (1976) reported a value of 87,000 to 92,000 for the molecular weight of plasminogen. The lower value predicted from the present study may reflect the proven inaccuracies associated with the calculation of the electrophoretic mobility of a highly disulphide bonded, glycoprotein molecule (Fish et al., 1970). The protein streptokinase (gel (v)) produced six gel bands. The value calculated for the apparent molecular weight of the major band, 51,000, is similar to that reported by Brockway & Castellino (1974) (45,500). The additional bands obvious in this gel may represent streptokinase oligomers and/or contaminants of the streptokinase preparation.

The gel corresponding to the streptokinase-activated plasminogen sample (gel (i)) contains two bands

in addition to those already described for each of these proteins examined individually. The apparent molecular weights are 97,000 (this band has a biphasic appearance) and 40,000. Walther et al. (1974) proposed that the activation of plasminogen by streptokinase is accompanied by the loss of a peptide of molecular weight 8,200 from the plasminogen molecule. The plasmin(ogen) band of gel (i) does indeed display a slightly increased electrophoretic mobility. The band of apparent molecular weight 40,000 may likewise represent a modified form of streptokinase from which a peptide of molecular weight 8,000 has been removed as a consequence of plasminogen interaction (Brockway & Castellino, 1974). The band of apparent molecular weight 97,000 may represent a plasmin/streptokinase complex (Reddy & Markus, 1972).

Trasylol (gel (vi)) is a reversible inhibitor of plasmin and it is believed that it attaches to the active site of the enzyme. The molecular weight reported in the present study for this inhibitor protein (13,000-14,000) is approximately twice the value calculated from the primary sequence data (6512). It may be that either Trasylol forms dimeric structures or the result merely reflects the known inaccuracies associated with molecular weight determinations in this high mobility region of the polyacrylamide gel.

The gel pattern of gel (ii) was produced by

approximately 30 times the level of plasminogen, 70 times the level of streptokinase and twice the level of Trasylol routinely present in a fragment D digest sample analysed on a 5% SDS-gel.

6.3.2 Plasmic degradation of denatured fragment D

Samples were removed during the course of a plasmic digest of denatured fragment D as follows and were then examined, unreduced, on 5% SDS-polyacrylamide gels;

Sample A: Fragment D solution prior to incubation
in urea

Sample B: Fragment D digest sample immediately after
the addition of plasmin

Sample C: Digest sample 40min after the addition
of plasmin

Sample D: Digest sample 2h 40min after the addition
of plasmin

Sample E: Digest sample 18h after the addition of
plasmin

Sample F: Digest sample 22h after the addition of
plasmin

Sample G: Digest sample 42h after the addition of
plasmin

Fig. 6.2 SDS-polyacrylamide gel electrophoresis of plasmin digests of denatured fragment D

Samples were removed from a plasminic digest of denatured fragment D after periods of B 0h D 2h 40min and F 22h. Trasylol was added. Each sample along with sample A (removed from the fragment D solution prior to urea and plasmin treatment) was examined by SDS-gel electrophoresis (unreduced) on a 5% gel. The corresponding densitometer scans are shown. For further details see text.

Fig. 6.2

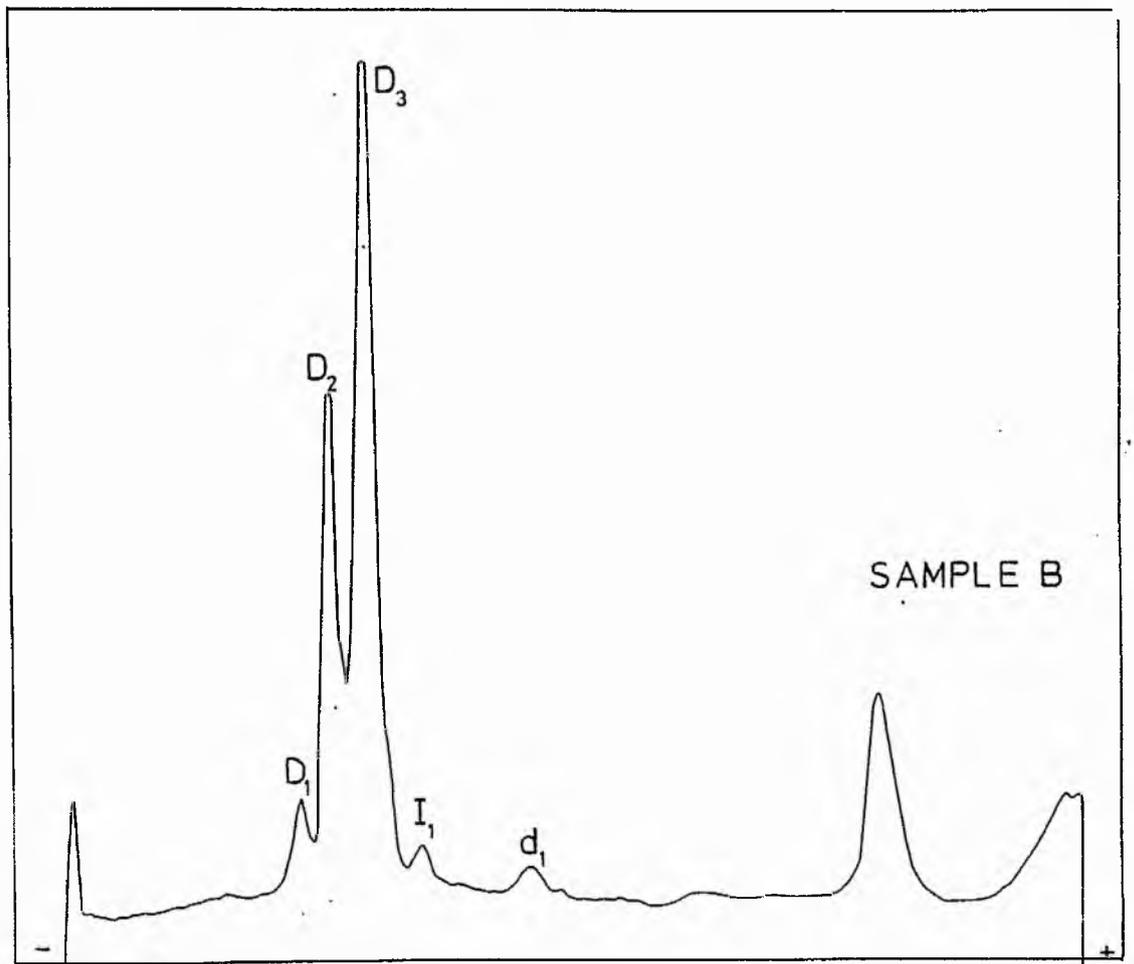
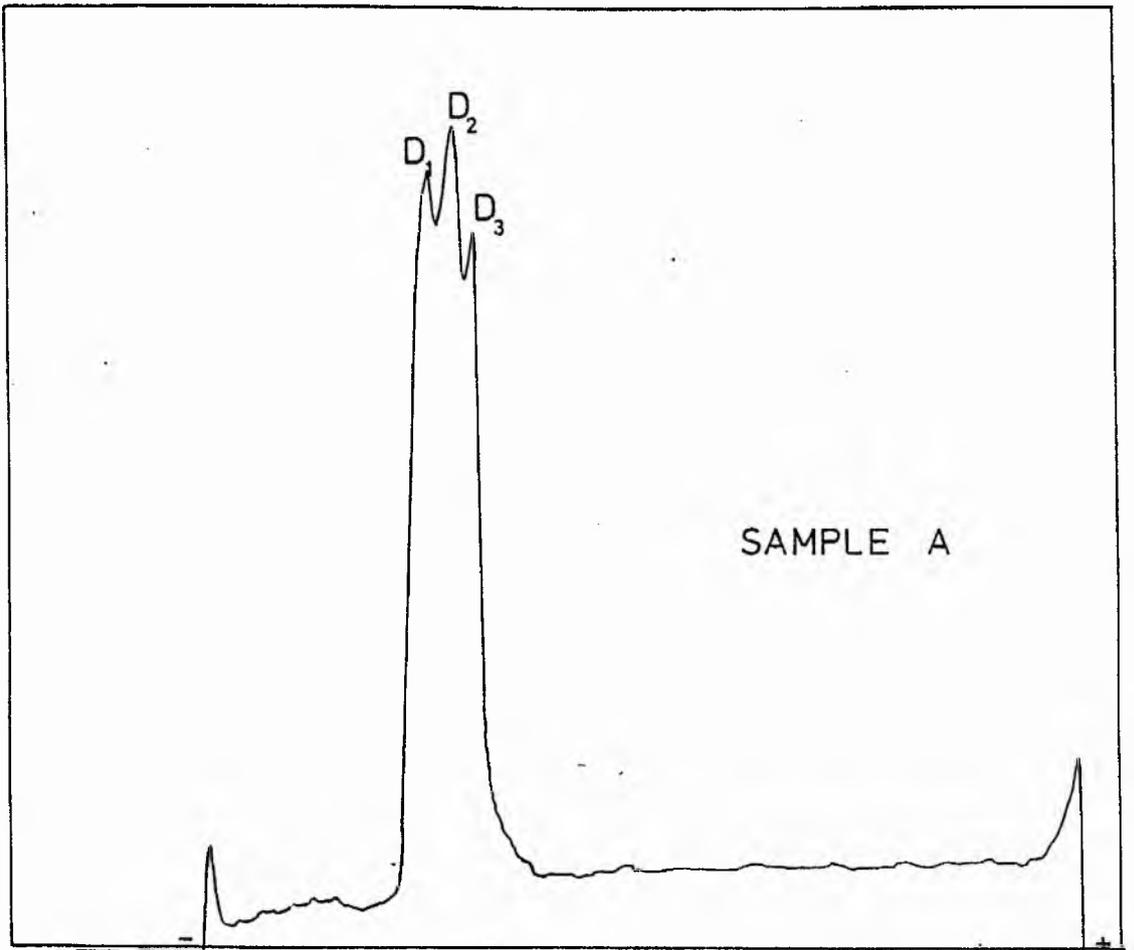
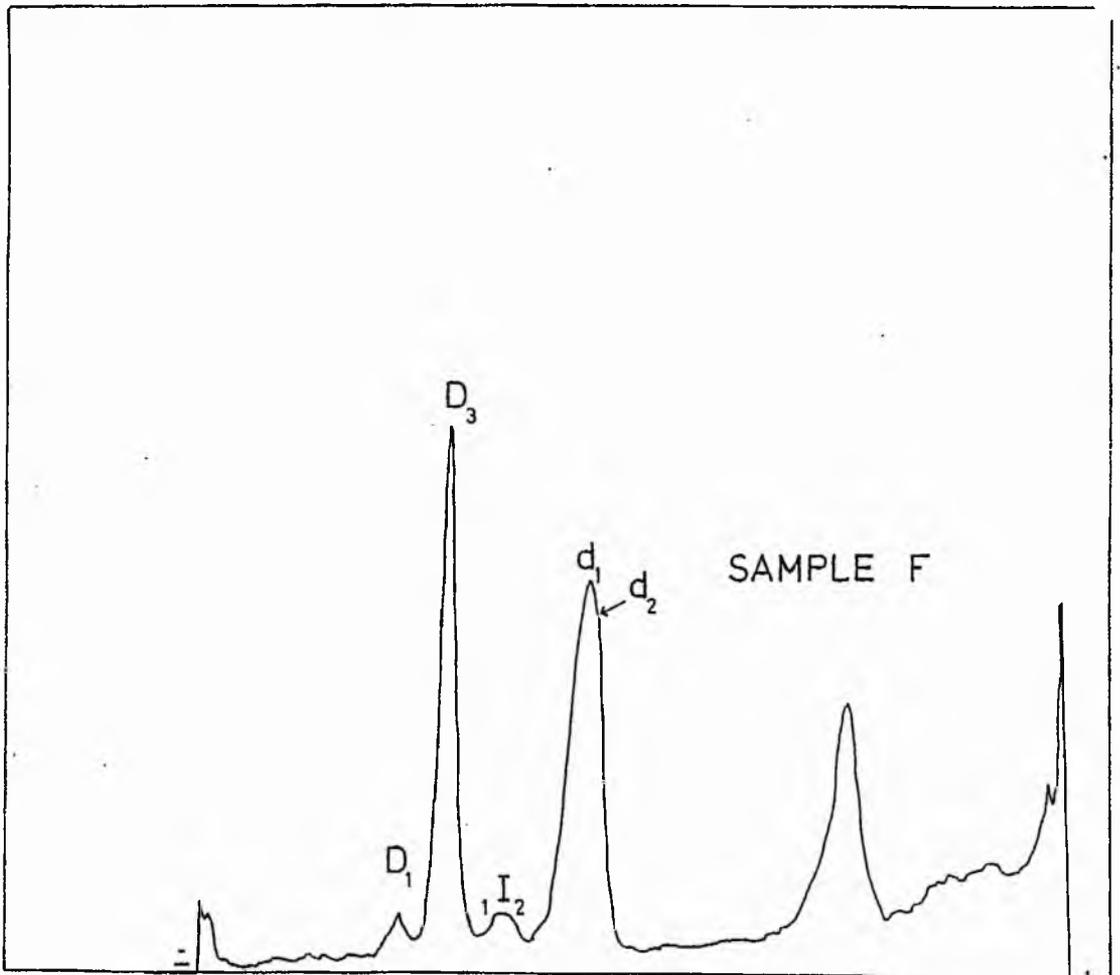
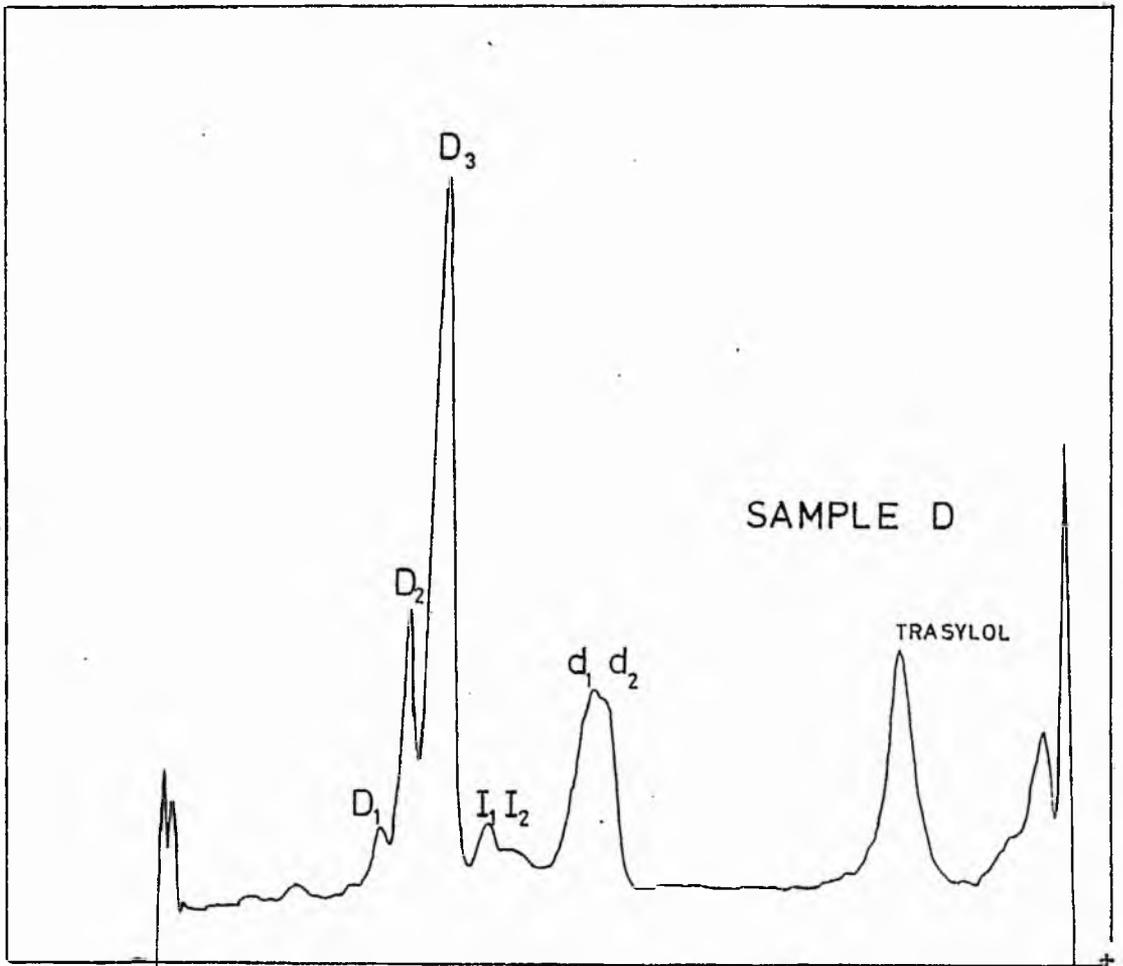


Fig. 6.2



Sample H: Digest sample 66h after the addition of plasmin.

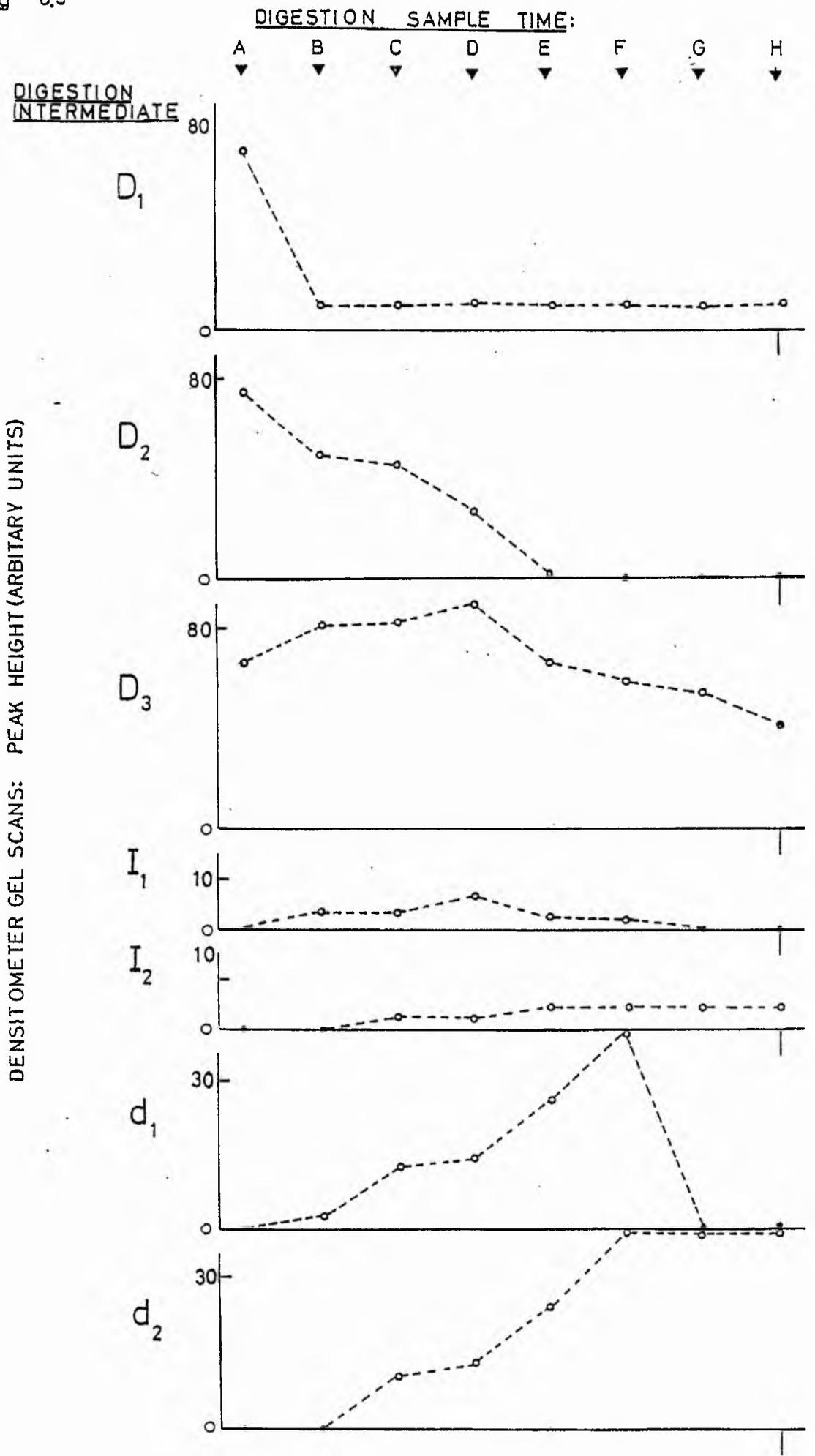
Densitometer scans corresponding to the SDS-gels obtained by analysis of samples A, B, D and F are presented in Fig. 6.2.

The sample of fragment D which had not been exposed to urea or to plasmin, sample A, displays the characteristic three peak pattern typical of the fragment D preparation when isolated by ion-exchange chromatography (as described in Section 5). The effect of urea on the various fragment D species is illustrated by the densitometer scan corresponding to sample B. A sharp fall in the intensity of the peak denoted fragment D_1 is obvious. The height of the peak corresponding to fragment D_2 has also decreased. In addition two minor peaks immediately below the major fragment D peaks are obvious. The sample removed from the fragment D digest solution after an incubation period of 2h 40min (sample D) contains increased amounts of both of these lower molecular weight species and, in addition, each peak is resolved into two components. The peaks of the higher mobility doublet have been designated d_1 and d_2 (since the calculated molecular weights are similar to that of the fragment d described by Kemp *et al.*, 1973) while the two peaks of intermediate mobility between fragments D and d will be referred to as I_1 and I_2 .

Fig. 6.3 The degradation of denatured fragment D
by plasmin

Samples were removed from a plasmic digest of denatured fragment D after periods of B 0h, C 40min, D 2h 40min, E 18h, F 22h, G 42h and H 66h. Sample A was removed from the fragment D solution prior to urea and plasmin treatment. Each sample was examined by SDS-polyacrylamide gel electrophoresis (unreduced, 5% gels) and a quantitative estimate of the amount of the digest components fragments D_1 , D_2 and D_3 , I and I_2 , and fragments d_1 and d_2 present at each stage of the digestion process was thereby obtained from the corresponding densitometer scans. For further details see text.

Fig 6.3



The apparent molecular weight of each of these peaks is as follows; I_1 59,000, I_2 55,000, d_1 45,000, d_2 40,000.

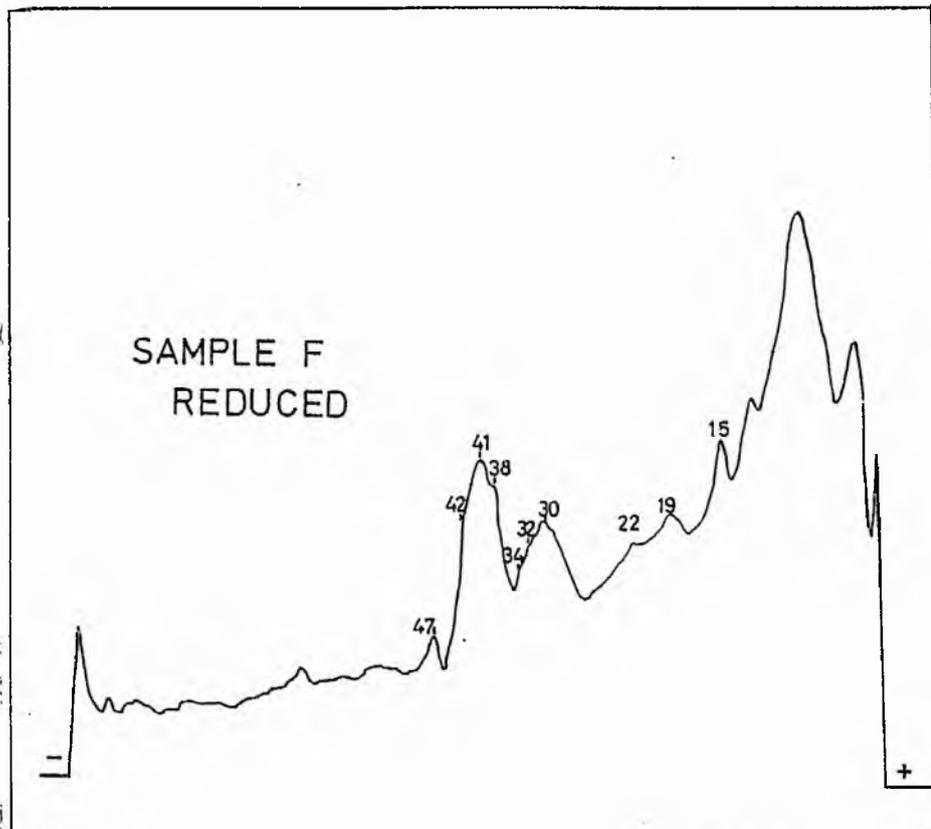
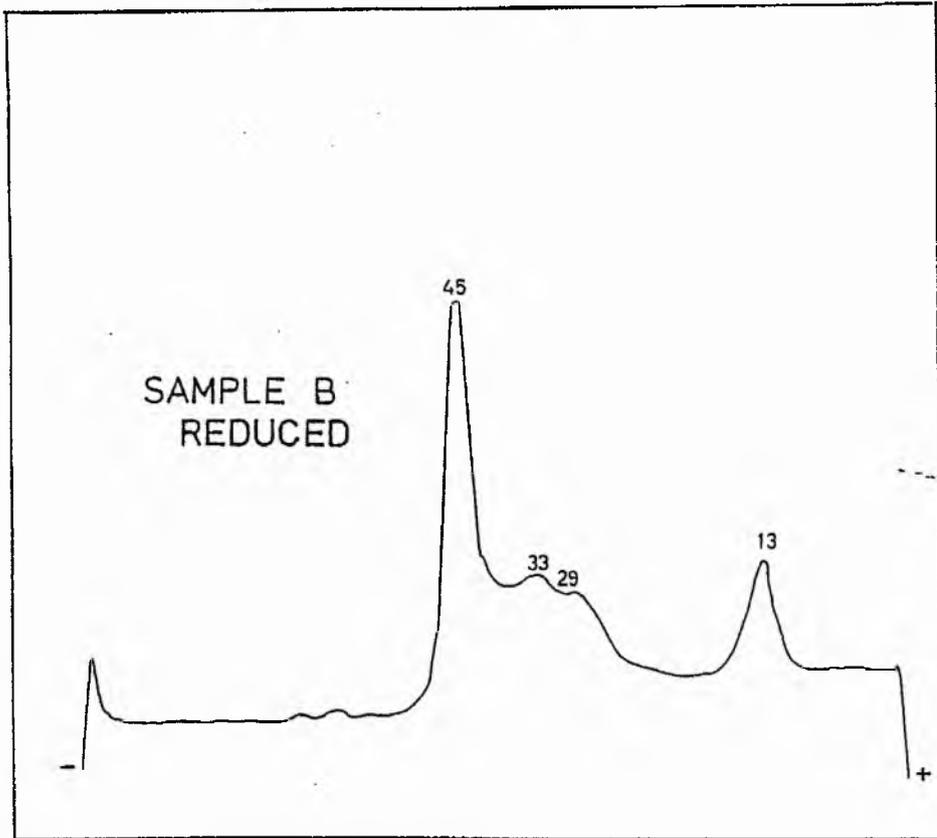
The sample removed from the fragment D digest after a period of 22h (sample F) contains considerable amounts of fragment D_3 . Fragment D_1 is represented by a small peak while the peak corresponding to fragment D_2 is absent. In addition the peaks attributed to fragments d_1 and d_2 appear more intense.

These changes in the electrophoretic pattern displayed by the various fragment D digest samples A-H, are presented diagrammatically in Fig. 6.3. This figure was prepared by obtaining a quantitative estimate of the digest components (fragments D_1 , D_2 and D_3 , I_1 , I_2 and fragments d_1 and d_2) from the polyacrylamide gel densitometer scans corresponding to each digest sample. The initial fall in the amount of the fragment D_1 component following the addition of plasmin to the de:natured fragment D sample already noted is evident. However a small but constant level of this species is maintained throughout the course of the digestion period. (It is unlikely that this minor component may be attributable to residual plasmin(ogen). Electrophoretic analysis of a sample containing twice the amount of plasminogen employed in the digest samples produced only a faint band of mobility appropriate to fragment D_2).

Fig. 6.4 SDS-polyacrylamide gel electrophoresis
of plasmin digests of denatured
fragment D after reduction

Samples were removed from a plasmin digest of denatured fragment D after periods of B 0h and F 22h (according to the scheme of Fig. 6.3). Trasylol was added. Each sample was examined, reduced, on a 5% gel and the corresponding densitometer scans are shown. Apparent molecular weights of the various peaks ($\times 10^{-3}$) are indicated.

Fig. 6.4



The amount of fragment D_2 present in the digest samples gradually decreases. No fragment D_2 is detected in the digest samples removed after an incubation period of 18h. By way of contrast the amount of fragment D_3 initially increases and then lessens gradually. The component denoted I_1 is detected in the digest samples before I_2 . However no significant accumulation of either of these digest components occurs at any stage of the fragment D digestion process and I_1 is absent from samples examined after the 42h incubation period.

In a similar fashion fragment d_1 is detected before fragment d_2 . The intensity of the gel peaks corresponding to these two species is greater than that of I_1 and I_2 . However with increasing incubation time the peak designated d_2 becomes the more intense of the fragment d doublet.

Samples removed from the fragment D digest solution at times B and F were also examined, reduced on 5% polyacrylamide gels. The corresponding densitometer scans are shown in Fig. 6.4. Reduction of sample B produced four components; a major peak of apparent molecular weight 45,000 and three less intense peaks of increasing mobility. The corresponding scan obtained by analysis of sample F exhibits a more complex peak pattern. At least ten peaks of varying intensities are obvious. The intensity of the peak corresponding to the highest molecular weight species present, and to the most prevalent

component of sample B, is much reduced in this later digest sample.

6.4 Discussion

A time course study of the plasmic digestion of a fragment D preparation denatured by exposure to urea has been monitored by the technique of SDS-polyacrylamide gel electrophoresis. An important conclusion to be drawn from these studies is that the susceptibility of each of the three fragments D to the combined action of urea and plasmin differs significantly.

The highest molecular weight form of fragment D was particularly unstable. However two observations are worthy of comment. Firstly despite the rapid decrease in the levels of fragment D_1 promoted by the urea incubation treatment, a small component of the fragment D_1 peak proved to be resistant to further degradation and was still detected in the samples removed from the digest solution after an incubation period of 66h. Secondly, the observed rapid initial fall in the levels of fragment D_1 was not accompanied by a rapid rise in the levels of fragment D_2 implying that fragment D_1 may have been degraded directly to fragment D_3 or to an even lower molecular weight product. Bands of mobility appropriate to I_1 and fragment d_1 were detected at this stage of the fragment D digest.

The levels of fragment D_2 were completely decreased

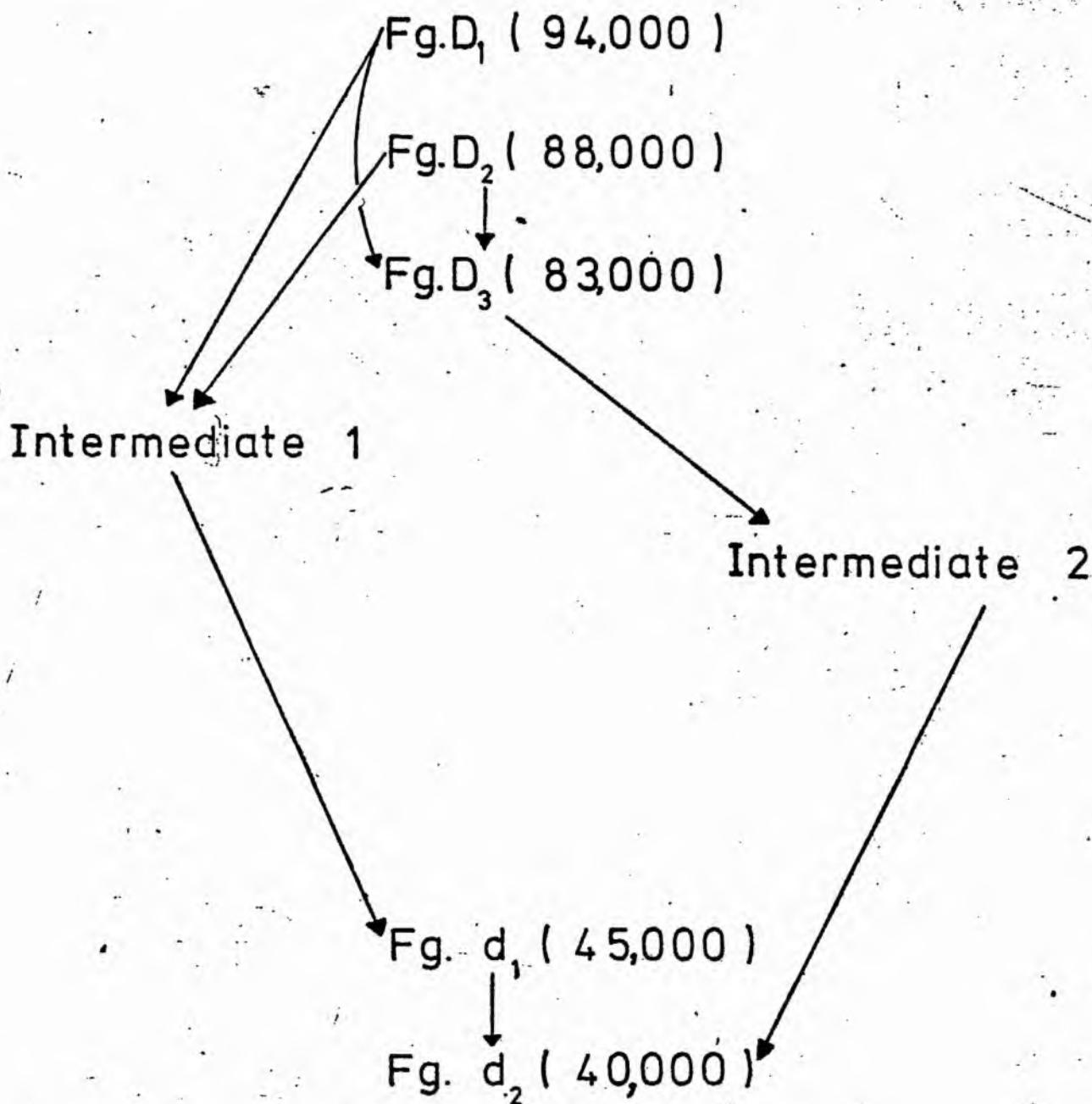
on a time-dependent basis following the addition of plasmin. This change was accompanied initially by a parallel intensification of the levels of fragment D_3 .

In sharp contrast to the behaviour described for fragments D_1 and D_2 , fragment D_3 appeared to be relatively resistant to degradation by plasmin. Even after an incubation period of 66h a considerable amount of this high electrophoretic mobility form of fragment D was detected. However the results obtained from analysis of the reduced digest samples suggest that the situation may be more complex. Reduction of an early fragment D digest sample produced four components of apparent molecular weights 45,000, 33,000, 29,000 and 13,000. Furlan et al. (1975) concluded from their studies of fragment D that the three major molecular weight forms contain identical β and α chain remnants of apparent molecular weights 45,000 and 10,000 but differ in the molecular weight of the constituent γ chain. They reported molecular weight values of 39,000, 33,000 and 28,000 for the three stages of γ chain digestion. By analogy the reduced chain components detected in sample B of the present study may be identified as follows; MW45,000 = D_β , MW33,000 = $D_{\gamma-2}$, MW29,000 = $D_{\gamma-3}$ and MW13,000 = D_α . This interpretation of the results corresponding to the reduced digest sample is consistent with the presence of two peaks designated fragments D_2 and D_3 in the unreduced sample. However, the reduced

chain pattern of the later digest sample (F) is not readily reconcilable with the published data. Several additional components were detected and furthermore, the intensity of the highest molecular weight peak, the major component of the early digest sample and assumed to represent the β constituent chain of fragment D, was much reduced. Thus the apparently intact structure predicted for the fragment D_3 molecule from analysis of an unreduced digest sample must be incorrect. The molecule must have undergone extensive degradation of the constituent chains. Four components of molecular weights greater than that of the largest γ chain component of the reduced earlier digest sample were present in sample F. Therefore four degraded forms of the 45,000 molecular weight D_β chain must have been present in sample F. Thus degradation of the β chain component of fragment D_3 had occurred - a conclusion consistent with the detection of drastically reduced levels of the 45,000 molecular weight form of the β chain in sample F.

To summarise the subunit structure predicted for the fragment D_3 molecule is more complex than that suggested by Furlan et al. (1975). After prolonged exposure to plasmin the susceptibility of the denatured molecule to attack by plasmin may not be restricted to the γ chain. The present results suggest that the β chain component is also degraded. It can be concluded

Fig. 6.5



PROPOSED PATHWAYS FOR THE DEGRADATION OF DENATURED
FRAGMENT 'D' BY PLASMIN.

The molecular weights of fragments D_1 , D_2 and D_3 are based on the values reported by Furlan et al. (1975)² For other details see text.

therefore, that although the fragment D_3 molecule may be extensively degraded from plasmic attack at both β and δ constituent chains, the overall structure of the molecule must remain intact. This proposal may be explained, in part, by the presence of a large number of disulphide bonds within the fragment D molecule. However, since this resistance to degradation was not displayed by the two lower mobility forms of fragment D, some additional factor must be responsible for the atypical stability of the fragment D_3 molecule. This point will be reconsidered later in this Discussion.

The digestion of fragment D to fragment d may follow the scheme outlined in Fig. 6.5. It is proposed that rapid degradation of fragment D_1 occurs either to fragment D_3 or via an intermediate structure, I_1 to fragment d_1 . The digestion of fragment D_2 may proceed either by the pathway followed by fragment D_1 or by degradation to fragment D_3 and thereby via I_2 to fragment d_2 .

This scheme is substantiated by the detection of two molecular weight forms of fragment d and I. Significantly the molecular weight difference between the two fragment d molecules is similar to that calculated for the two I components. The transient accumulation of the I_1 and I_2 species during the process of fragment D digestion is consistent with the assumption that they represent an intermediate stage in the conversion of

fragment D to d. The disappearance of I_1 from the digest samples followed the completion of the digestion of fragment D_2 .

Furlan et al. (1975) also reported, from a similar study, the presence of two molecular weight forms of fragment d which differed in molecular weight by 4,000 - a value similar to that predicted by the present study.

While a portion of the fragment D_3 component presented in Fig. 6.5 may have arisen from degradation of fragment D_2 the majority of the fragment D_3 molecules proved to be particularly resistant to degradation. It appears reasonable to propose therefore that more than one conformationally different form of the fragment D molecule exhibiting, consequently, differing susceptibilities to degradation, must exist. With hindsight it is interesting to consider whether this theory can be reconciled with the report by Haverkate & Timan (1977) that the fragment D molecule prepared in the presence of Ca^{2+} displays a resistance to degradation by plasmin even in the presence of 2 M-urea. It will be shown later in this work that Ca^{2+} , bound to fragment D confers a stability on the molecule. Thus the varying stabilities displayed by the fragment D molecules investigated in the present case may reflect the limited availability of free Ca^{2+} during the preparation of fragment D resulting from the use of a phosphate based buffer system.

In conclusion it must be conceded that several

aspects of the theory represented by Fig. 6.5 require further verification since the scheme is based partly on evidence drawn from the quantitative analysis of densitometer scans - a method of disputable accuracy. However the fact remains that the three major molecular weight forms of fragment D exhibit differing susceptibilities to attack by plasmin.

SECTION 7

THE NH₂-TERMINAL AMINO ACID COMPOSITION OF FRAGMENT D

7.1 Introduction

This final Section of Part A reports the results of a study to determine the NH₂-terminal amino acids of fibrinogen fragment D.

7.2 Methods

7.2.1 NH₂-terminal amino acid analysis

NH₂-terminal amino acid analysis of fragment D-containing fractions isolated from a fibrinogen digest by gel filtration and ion-exchange chromatography (as described in Section 5) was performed according to the method detailed in Section 2.2.5.

7.3 Results

7.3.1 The NH₂-terminal amino acid composition of fragment D

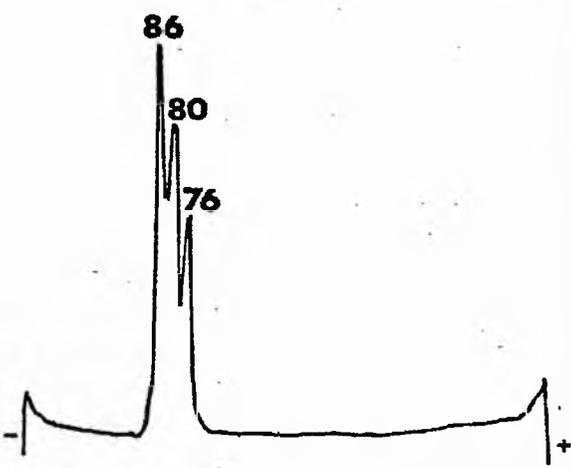
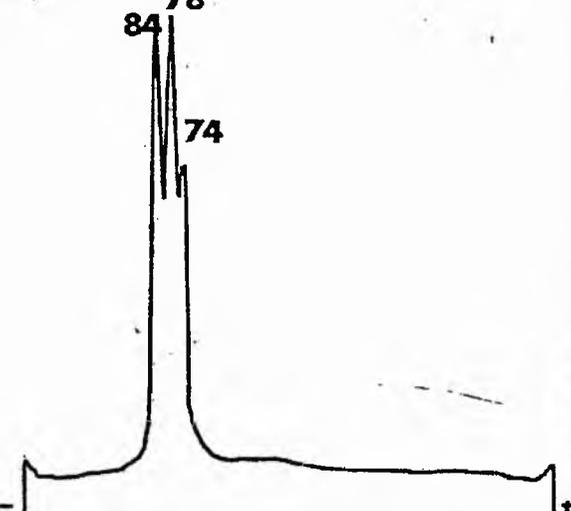
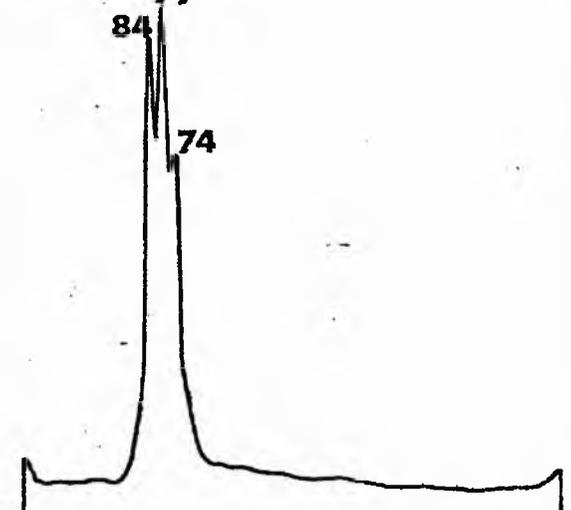
The results obtained from the NH₂-terminal analysis of samples removed from three fragment D-containing column fractions are presented in Fig. 7.1. Each sample was also subjected (unreduced) to SDS-polyacrylamide gel electrophoresis. The corresponding densitometer scans are shown.

The NH₂-terminal amino acids detected in column

Fig. 7.1 Characterisation of fibrinogen fragment D

Fragment D isolated from a plasmic digest of fibrinogen by gel filtration and sulphopropyl-Sephadex C-25 ion-exchange chromatography was examined employing the techniques of SDS-polyacrylamide gel electrophoresis (5% gels, unreduced samples) and NH₂-terminal amino acid analysis. Apparent molecular weights ($\times 10^{-3}$) are indicated. Dansyl amino acids which were not resolved are indicated thus, Ser/Thr. Col. Frac. refers to the sulphopropyl-Sephadex C-25 column fraction number (see Section 5.3.3, Fig. 5.4).

Fig. 7.1

COL. FRAC.	DENSITOMETRIC SCAN OF ACRYLAMIDE GEL	NH ₂ -TERMINAL AMINO ACIDS
47	 <p>A densitometric scan showing three distinct peaks. The tallest peak is at 86, followed by a slightly shorter peak at 80, and a third peak at 76. The baseline is relatively flat with minor noise. The x-axis is marked with a minus sign (-) on the left and a plus sign (+) on the right.</p>	Val Leu Ala
50	 <p>A densitometric scan showing three distinct peaks. The tallest peak is at 84, followed by a peak at 78, and a shorter peak at 74. The baseline is flat. The x-axis is marked with a minus sign (-) on the left and a plus sign (+) on the right.</p>	Val Leu Met Ser/Thr
52	 <p>A densitometric scan showing three distinct peaks. The tallest peak is at 84, followed by a peak at 79, and a shorter peak at 74. The baseline is flat. The x-axis is marked with a minus sign (-) on the left and a plus sign (+) on the right.</p>	Val Leu Met Ser/Thr Phe Tyr

fraction 47 were valine, leucine and alanine. However in the two later column fractions alanine was not detected but two additional NH_2 -terminal residues were demonstrated, serine/threonine (the dansyl spot corresponding to these amino acids was not resolved) and methionine. Furthermore the sample corresponding to the highest column fraction number studied (fraction 52) also contained the NH_2 -terminal amino acids tyrosine and phenylalanine.

The SDS-gel electrophoresis results predict a similar fragment D composition for each of the three column fractions studied. In each case three peaks corresponding to the three molecular weight forms of fragment D are obvious. The two later column fractions contain proportionately more of the intermediate fragment D, i.e. D_2 (molecular weight 78-80,000) peak than does the lowest number ("early") column fraction.

7.4 Discussion

The NH_2 -terminal amino acids of fragment D have been studied by several investigators. Collen et al. (1975) suggest that the NH_2 -terminal residue of the D_α -chain is aspartate while Doolittle et al. (1977a) propose that six residues are split from the NH_2 -terminus resulting in the valine NH_2 -terminal residue reported by Furlan et al. (1975). Therefore it seems probable that the

valine residue detected in each of the fragment D-containing column fractions may correspond to the NH_2 -terminal residue of the α subunit chain.

In the case of the δ constituent chain of fragment D, Takagi & Doolittle (1975) suggest that the early fragment D δ -chain contains an alanine NH_2 -terminal residue which is replaced as a consequence of further plasmic attack by a serine residue. According to Furlan et al. (1975) this NH_2 -terminal sequence of the D δ -chain remains intact while the δ chain is progressively degraded by plasmin from the COOH-terminus. An NH_2 -terminal alanine residue was detected only in the fragment D sample corresponding to the earliest column fraction. However the above reports predict that the NH_2 -terminal serine/threonine amino acid peculiar to the two later column fractions may in fact represent the serine residue subsequently exposed by plasmic degradation of the δ chain NH_2 -terminus.

This comparison of the present results with published data implies that the third NH_2 -terminal residue detected in the fragment D sample corresponding to column fraction 47 (i.e. leucine) must represent the NH_2 -terminal amino acid of the β constituent chain of fragment D. However this conclusion is not in agreement with the reports by Collen et al. (1975) and by Furlan et al. (1975)

that the NH_2 -terminal residue of the fragment $\text{D } \beta$ chain is aspartate. The present results considered in conjunction with the fibrinogen β chain sequence data published by Henschen & Lottspeich (1977) predict the existence of a plasmin-susceptible lysine-leucine bond within the β chain at a position approximately 48 amino acid residues nearer to the COOH-terminus than the lysine-aspartate cleavage point reported by Collen et al. (1975) and Furlan et al. (1975).

The apparent differences in the NH_2 -terminal amino acid compositions of the three fragment D-containing column fractions merit further discussion. Hitherto it has been assumed that the three molecular weight forms of fragment D isolated in Section 5 represent three discrete stages of fragment D digestion by plasmin. The heterogeneity of fragment D preparations was investigated by Furlan et al. (1975) who concluded that the three molecular weight forms of fragment D contained the same α and β chain remnants while the molecular weight heterogeneity of the preparation was caused by the sequential attack of plasmin at the COOH-terminus of the δ chain. However in the present study the fragment D samples removed from the two "late" column fractions displayed NH_2 -terminal amino acids in addition to those assigned to the NH_2 -termini of the α , β and δ chains. The identification of an NH_2 -terminal methionine

residue suggests that one of the constituent chains of fragment D contains a lysine-methionine cleavage site and indeed Doolittle et al. (1977a) have identified a plasmin-susceptible peptide bond of this description within the COOH-terminal region of the constituent α chain of fragment D. Lysis of this peptide bond should release a 24 amino acid polypeptide from the D α chain.

The final column fraction studied contained two additional NH_2 -terminal amino acids, phenylalanine and tyrosine. Plasminic cleavage points within the constituent chains of fragment D involving these amino acids have not been reported elsewhere. It should be noted however that while the foregoing NH_2 -terminal analysis results predict a more degraded structure for the fragment D molecule present in the later column fractions, this conclusion is not confirmed by an examination of the respective SDS-gel electrophoresis results. Despite the presence of a less intense peak corresponding to fragment D_2 in the first of the three column fractions studied, the three SDS-gel results appear similar. In no case is there evidence of low molecular weight degraded material having co-eluted with the fragments D. It can be assumed therefore that the apparent degradation of the fragment D molecules present in the later column fractions must have occurred at sites close to the COOH or NH_2 -

termini of the constituent chains (therefore the release of a peptide would not significantly alter the apparent molecular weight of the remaining fragment D structure) or alternatively at sites within the disulphide bonded region of the molecule (cleavage of a constituent chain would therefore not effect the release of a peptide and consequently the molecular weight of the intact fragment D molecule would be unaltered).

To summarise, the studies reported in this Section indicate that the explanation of the apparent heterogeneity of the fragment D preparation isolated from a plasmic digest of fibrinogen by column chromatography may be more complex than first appreciated. The three molecular weight forms of fragment D detected in each of the column fractions cannot represent simply the same three stages of fragment D degradation by plasmin since it has been demonstrated that the NH_2 -terminal amino acid composition of the fragment D molecules eluted in the "early" and "late" column fractions differed. A more degraded structure is envisaged for the latter fragment D molecules.

Two possible explanations of this observation are as follows. Firstly the fragment D preparation subjected to column chromatography may have contained a heterogeneous population of fragment D molecules representing multiple stages of plasmic degradation; the more degraded molecular forms of fragment D being isolated in the later column

fractions. Secondly while it is unlikely that the "extra" NH_2 -termini detected in the fragment D preparation can be attributed to the direct effect of contamination by streptokinase, Trasylol or plasmin(ogen) the indirect effect of contaminating plasmin(ogen) cannot be ignored. It will be recalled that the fragment D isolation procedure described in Section 5 involved the use of lysine-Sepharose 4B to remove plasmin from the fibrinogen digest. However an affinity between the fragment D and plasmin molecules may exist (to be described in Part B, Division 2, Section 4.3.4) and it is feasible that the apparently pure fragment D preparation may have been contaminated by plasmin. Therefore degradation of the fragment D molecules, exposing additional NH_2 -termini, may have occurred during the isolation procedure.

However the major conclusion from these studies is that the heterogeneity of the fragment D preparation was more complex than could be predicted from SDS-gel electrophoresis results.

SECTION 8

DISCUSSION

The most important conclusion to emerge from the foregoing studies must be that the fragment D preparation isolated by plasmic digestion of fibrinogen was composed of a heterogeneous population of fragment D molecules. This characteristic was revealed by SDS-gel electrophoresis, NH_2 -terminal analysis and by plasmic degradative studies employing denatured fragment D. In the latter case the differing susceptibilities of the various fragment D molecules to the degradative action of urea and plasmin was explained by postulating the existence of at least two conformationally different forms of the molecule. Thus the well-documented heterogeneity of fragment D preparations must be more complicated than has hitherto been assumed (e.g. by Ferguson et al., 1975 and Furlan et al., 1975). The isolation of a population of fragment D molecules heterogeneous with respect to size and properties must be viewed as a serious complicating factor in studies designed to investigate the structure of fragment D.

In conclusion it is clear that an essential requirement for further fragment D characterisation studies is the development of a method for the isolation of a homogeneous preparation of fragment D. However the physiological implications of this statement should

not be overlooked. Obviously the most important form of the fragment D molecule is that which is likely to exist in vivo. The characterisation of this structure would make a significant contribution to the understanding of the biological activity of fragment D and to the investigation of the structure and conformation of the parent molecule, fibrinogen. The origin of the heterogeneity of the fragment D preparation requires investigation.

PART B

PART B

DIVISION 1

SECTION 1
INTRODUCTION

The foregoing work, Part A was concerned with the investigation of the structure of fibrinogen fragment D. However, the interpretation of these digestion studies was complicated by the initial heterogeneity of the fragment D preparation. Thus the publication by Haverkate & Timan (1977) of their findings which suggested that degradation of fibrinogen by plasmin at calcium chloride concentrations of "about 2 mM" produced a single, high molecular weight form of fragment D was of considerable significance to the present work. Their results implied that this fragment D preparation would be free from the problem of heterogeneity encountered in Part A and would therefore be an ideal starting point for further characterisation studies. Furthermore since this fragment D is prepared from fibrinogen in the presence of Ca^{2+} concentrations approximating to those existing in vivo the investigation of its structure can be more meaningfully related to its physiological importance.

The study of the fragment D prepared in the presence of Ca^{2+} necessitated the modification of many of the previously described techniques to produce systems more compatible with the presence of free Ca^{2+} . This will be described in the initial sections of Division 1. In general a phosphate buffer system was replaced by one

based on Tris. This first Division will conclude with a repetition of the fibrinogen digestion studies reported by Haverkate & Timan. Several aspects of this work will be examined in greater detail.

The discussion of the work reported in Part B prompted the development of a nomenclature system for the various types of fibrinogen fragment D. This will be outlined below.

SECTION 2
NOMENCLATURE

The following fibrinogen fragment D nomenclature rules will be applied throughout Part B of this work:

- (i) Fragment D will be applied as a general term to refer to all fragment D species produced by plasmic degradation of fibrinogen.
- (ii) Fragment $D_{Ca^{2+}}$ will refer to the fragment D produced following the incubation and subsequent digestion of fibrinogen in the presence of 2 mM- $CaCl_2$.
- (iii) Fragment D_{EDTA} As for (ii) but in the presence of 5 mM-EDTA.
- (iv) Fragment D_B will be applied to the fragment D species produced from fibrinogen in a buffer solution to which neither EDTA nor $CaCl_2$ had been added.
- (v) Fragment $Ca^{2+}D_{EDTA}$ will refer to the fragment D produced by plasmic digestion of fragment $D_{Ca^{2+}}$ following exposure to EDTA.

Heterogeneities within each fragment D type will be

denoted by subscripts i.e. Fragment D_1 refers to a species of lower electrophoretic mobility than Fragment D_2 .

SECTION 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

This list supplements that of Part A (Section 2.2.2). Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Dimethyl suberimidate was supplied by Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., while dimethyl adipimidate was a gift from Dr J.R. Coggins, Univ. of Glasgow. Ampholine (pH range 3.5-10) was purchased from LKB, Stockholm, Sweden.

3.1.2 Buffer solution

The following buffer was used routinely throughout this work and was prepared as described below. The pH of the solution was measured at 20°C.

0.05 M-Tris/HCl, pH 7.5 was prepared by mixing 250ml of 0.2 M-Tris, 425ml of 0.1 M-HCl and 300ml distilled water. The pH was adjusted to 7.5 with 6 M-HCl and the final volume brought to 1 litre.

3.2 Methods

3.2.1 SDS-polyacrylamide gel electrophoresis

Acrylamide stock solutions were prepared by dissolving acrylamide and methylenebisacrylamide directly

Table 3.1 Composition of gels of various porosities

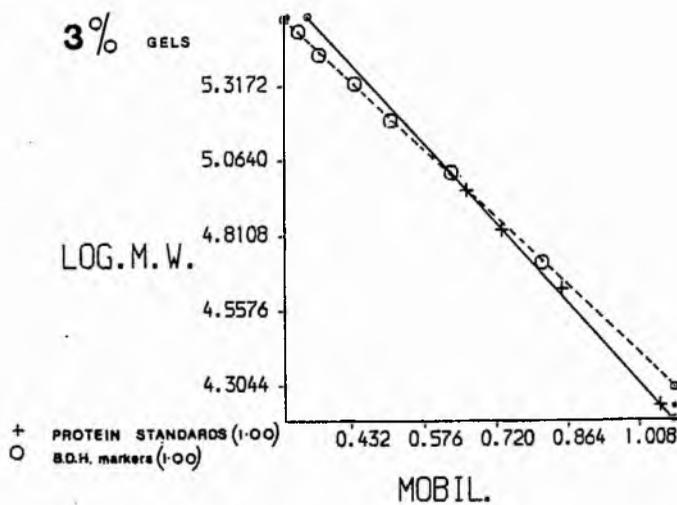
TOTAL ACRYLAMIDE CONCENTRATION (w/v)	METHYLENEBIS ACRYLAMIDE (as a percentage of total acrylamide)	AMMONIUM PERSULPHATE (w/v)	TEMED (v/v)
3%	3%	0.075%	0.15%
4%	3%	0.075%	0.15%
5%	5%	0.0625%	0.125%
10%	3%	0.05%	0.10%

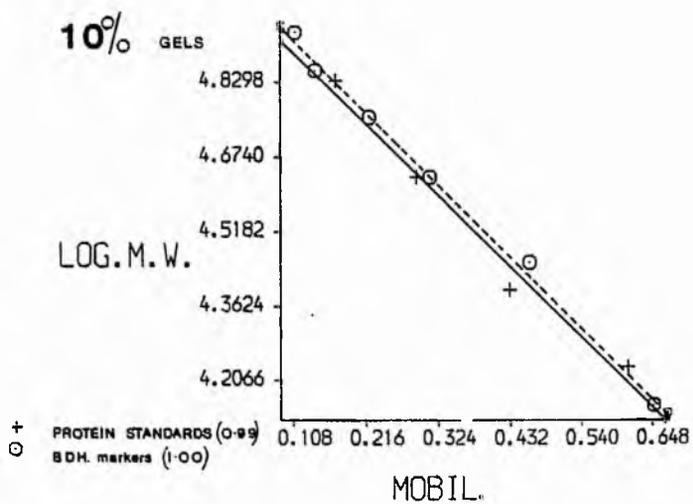
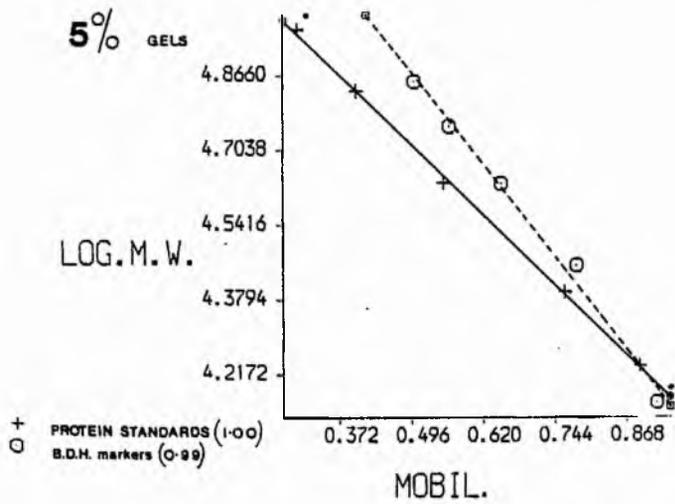
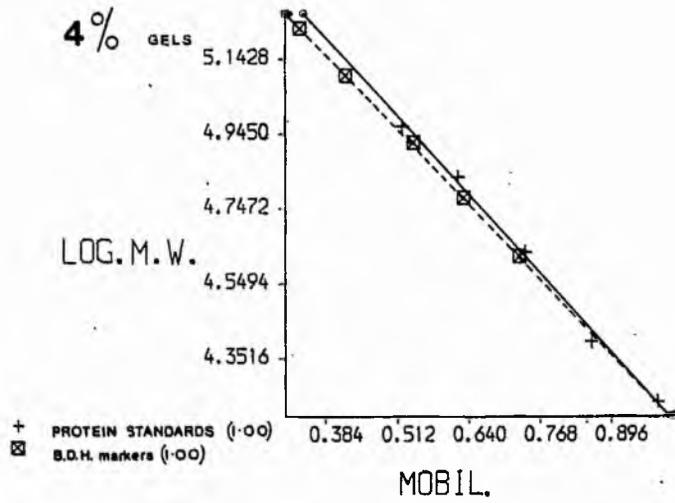
in the following gel buffer solution; 0.1 M-Tris/HCl buffer, pH 7.5, containing 6 M-urea and 0.2% (w/v) sodium dodecyl sulphate (SDS). 19ml of the required stock acrylamide solution was mixed with 1ml of freshly prepared ammonium persulphate solution (in water) and N, N, N', N', tetramethylethylenediamine (TEMED). Final gel concentrations are shown in Table 3.1. Gels were cast in acid-cleaned glass tubes (0.4 x 6cm) and were overlaid with water, this being replaced by gel buffer when the gels had set. 5% and 10% gels were used after 4h but, 3% and 4% gels were stored at 4°C for 18h prior to use.

Samples for electrophoresis were mixed with an equal volume of a 3% (w/v)-SDS, 8 M-urea solution. An aliquot (10-90 μ l) was mixed with a few drops of glycerol and 10 μ l of tracking dye (0.05% (w/v)-bromophenol blue in water) before application to the gel. Reduced samples were prepared by mixing with an equal volume of a 3% (w/v)-SDS, 8 M-urea, 3% (v/v)-2-mercaptoethanol solution and heating at 100°C for 5min. BDH protomer molecular weight markers stock solution prepared as described previously (Part A, Section 2.2.1) was treated thus. To 100 μ l was added an equal volume of the urea/SDS/2-mercaptoethanol solution, 20 μ l of tracking dye and 2 drops of glycerol. 100 μ l of this solution was applied to a gel. Protein molecular weight standards

Fig. 3.1 SDS polyacrylamide gel electrophoresis. Typical calibration plots -1

The logarithm of the molecular weights of protein standards (solid line) and of BDH molecular weight markers (broken line) are plotted as a function of electrophoretic mobility. For 3% and 4% gel systems the BDH high molecular weight markers (range 53,000 to 265,000) were employed while for 5% and 10% gel systems the low molecular weight markers (range 14,300 to 71,500) were used. The figures in brackets are the correlation coefficients.





(phosphorylase a (EC 2.3.1.1) from rabbit muscle; bovine serum albumin; ovalbumin; chymotrypsinogen (EC 3.4.4.5) from bovine pancreas and myoglobin) were dissolved in 0.05 M-Tris/HCl buffer, pH 7.5, containing 0.2% (w/v)-SDS (final concentration 2.5mg/ml). To 100 μ l of this solution was added an equal volume of the urea/SDS/2-mercaptoethanol solution. This mixture was heated at 100°C for 5min and then stored at 4°C prior to use. 10 μ l of each protein standard solution was mixed with 1 drop of glycerol and 10 μ l of tracking dye before application to the gel surface.

The electrophoresis reservoir buffer (0.1 M-Tris/HCl, pH 7.5, containing 0.2% (w/v) SDS) was layered on top of each sample and electrophoresis was performed at a constant current of 8 mA/gel for 2-4h. The electrophoresis apparatus employed and the measurement of gels were as previously described (Part A, Section 2.2.1).

3.2.2 Staining and destaining of polyacrylamide gels

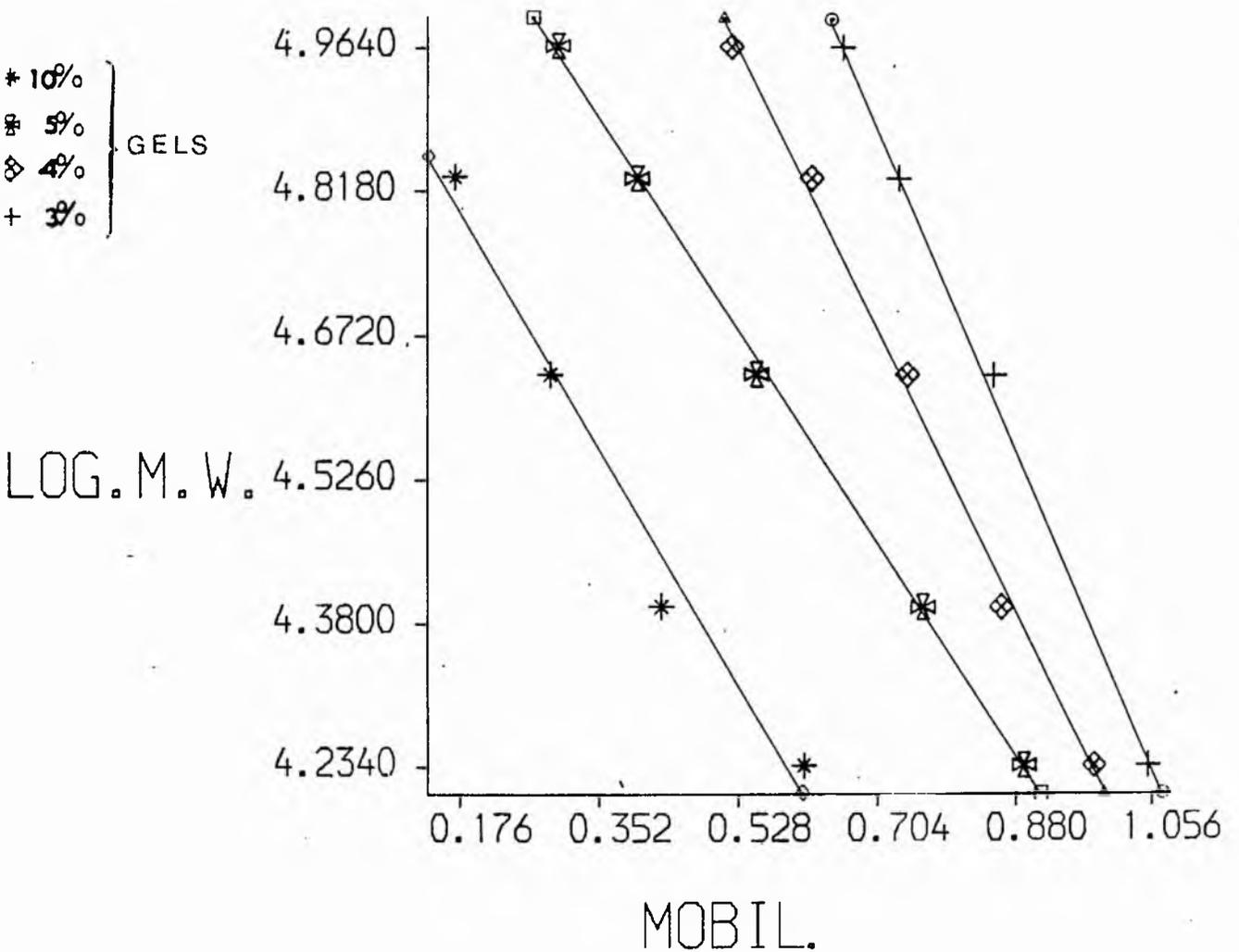
Gels were stained and destained, as previously described, (Part A, Section 2.2.2) and then scanned at 570nm using a Vitatron TLD-100 densitometer or photographed.

3.2.3 Addendum

Graphs were plotted, as previously described (Part A, Section 2.2.3). Fig.3.1 illustrates a sample of the

Fig. 3.2 SDS polyacrylamide gel electrophoresis. Typical calibration plots-2

A plot of the logarithm of the molecular weights of the protein standards against electrophoretic mobility for each gel system (3%, 4%, 5% and 10%).



graphs obtained for (a) 3% (b) 4% (c) 5% and (d) 10% polyacrylamide gel systems by plotting the logarithm of the molecular weights of the protein standards as a function of their calculated electrophoretic mobilities. The results obtained with both BDH protomer molecular weight markers and a series of protein molecular weight standards are shown. These two sets of calibration proteins produced very similar graphs for all but the 5% polyacrylamide gel system. In accordance with the reasoning of Part A, Section 2.2.3 a series of single protein standards was used routinely throughout the following three sections and typical standard graphs for all four gel types are shown in Fig. 3.2.

3.2.4 Immuno-electrophoresis

3.2.5 Estimation of NH₂-terminal amino acids

3.2.6 Estimation of protein concentration

These three techniques were performed exactly as described in Part A (Sections 2.2.4-2.2.6).

SECTION 4

THE REMOVAL OF CONTAMINATING PLASMINOGEN FROM HUMAN FIBRINOGEN AND THE PREPARATION OF PLASMINOGEN FROM HUMAN PLASMA

4.1 Introduction

The purpose of the procedures to be described in this section was (i) to remove contaminating plasminogen from fibrinogen and (ii) to isolate and then assay plasminogen prepared from human plasma. The rationale behind these techniques and the methods employed have already been discussed in Part A, Section 3. One modification was made. An alternative method of desalting the plasminogen eluted from the lysine-Sepharose 4B column was applied.

4.2 Methods

4.2.1 The removal of contaminating plasminogen from fibrinogen

Human fibrinogen was freed of contaminating plasminogen by the affinity chromatography method employing lysine-Sepharose 4B described in Part A, Section 3.2.1. One modification was made to the procedure, namely 0.05 M-sodium phosphate buffer, pH 7.5, containing 3 mM-EDTA was replaced by 0.05 M-Tris/HCl buffer pH 7.5. The eluted fibrinogen was stored in suitable volumes at -20°C. An aliquot of the "purified"

fibrinogen was compared with one of fibrinogen which had not been subjected to the column procedure as follows;

(i) the reduced chain composition of each fibrinogen was examined on 10% polyacrylamide gels;

(ii) after incubation in the presence of streptokinase (50 units/mg) for 1h at 37°C both types of fibrinogen were examined (unreduced and reduced) on 10% polyacrylamide gels;

(iii) the protein eluted from the lysine-Sepharose 4B column by 6-amino-n-hexanoic acid was dialysed against 0.05 M-Tris/HCl buffer, pH 7.5, for 48h at 4°C. An aliquot was added to a sample of each type of fibrinogen and the resulting solutions were incubated in the presence of streptokinase (50 units/mg) for 1h at 37°C. Each type of fibrinogen sample was examined (reduced) on 10% polyacrylamide gels.

4.2.2 Isolation of plasminogen from human plasma

The first stage in the preparation of plasminogen from human plasma was accomplished as described in Part A, Section 3.2.2 by lysine-Sepharose 4B affinity chromatography. However one modification was made to the procedure, viz. 0.05 M-sodium phosphate buffer, pH 7.5, containing 3 mM-EDTA was replaced by 0.05 M-Tris/HCl buffer, pH 7.5.

Those fractions eluted by the NaCl-containing

buffer were pooled, dialysed against distilled water (24h, 4°C) and then freeze dried. The immunoreactivity of the reconstituted sample against anti-human plasminogen antiserum was examined using the technique of immunoelectrophoresis.

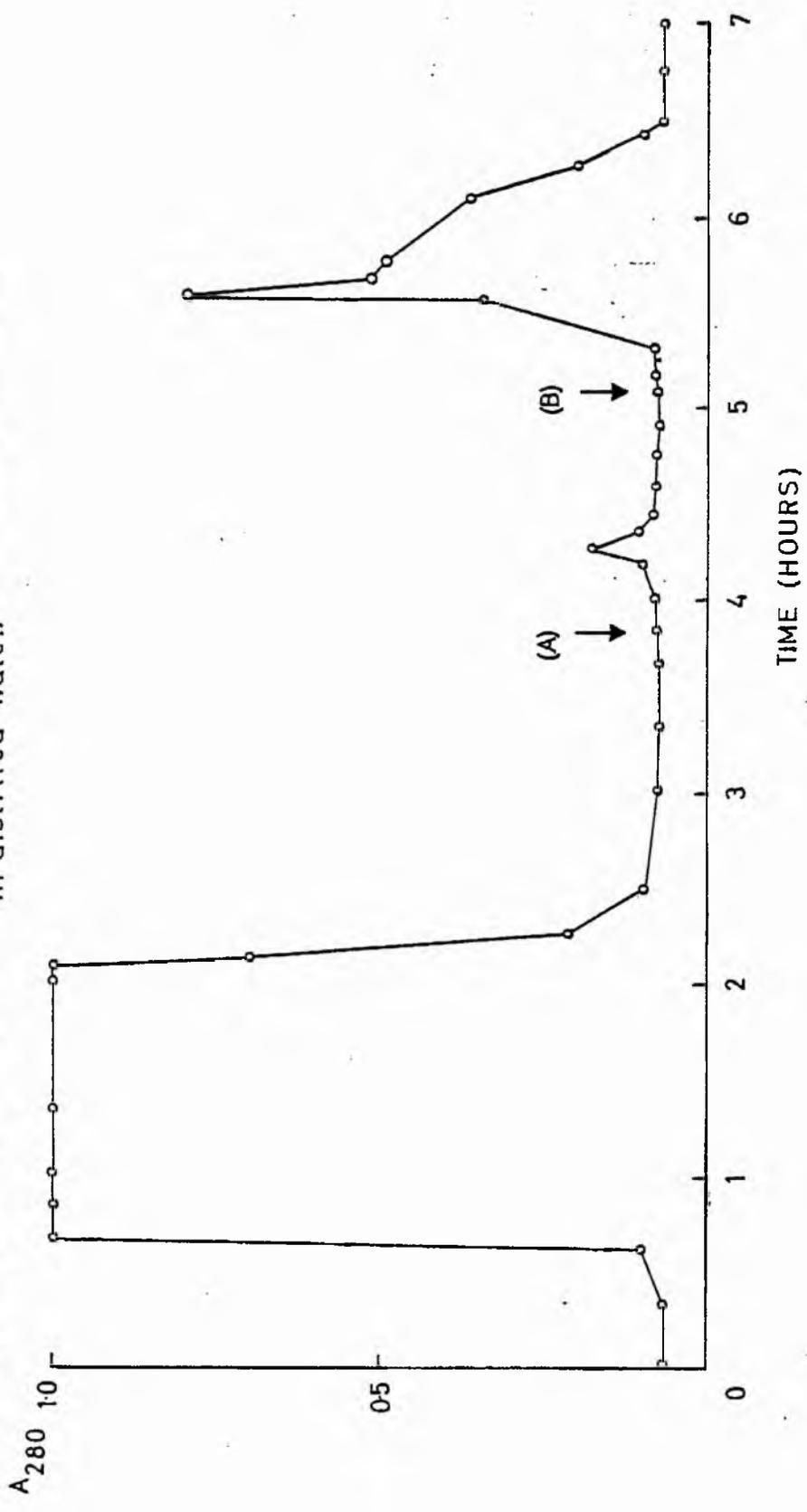
The (plasminogen-containing) fractions eluted by the 6-amino-n-hexanoic acid solution were pooled and treated as described below.

4.2.3 Desalting of plasminogen

The pooled, plasminogen-containing fractions isolated above were desalted at room temperature by application to a column (2.5 x 20cm) filled with Sephadex G-25. The equilibration of the column and the subsequent elution of the sample were performed with 0.005 M-Tris/HCl buffer, pH 7.5. The desalted plasminogen sample was dialysed against 0.05 M-Tris/HCl buffer, pH 9.0, containing 0.1 M-NaCl and 0.02 M-lysine monohydrochloride. An aliquot was removed from the dialysate and the immunoreactivity against anti-human plasminogen antiserum investigated by immunoelectrophoresis. The apparent molecular weight (unreduced) was estimated on a 5% polyacrylamide gel.

Fig. 4.1 Chromatography of human fibrinogen on lysine-Sepharose 4B

Contaminating plasminogen was removed from fibrinogen by lysine-Sepharose 4B chromatography. Column dimensions: 1.0 x 15cm. Flow rate: 35ml/h. Elution procedure: 0.05 M-Tris/HCl buffer at pH 7.5 followed by (A) 0.5 M-NaCl in the same buffer and (B) 0.2 M-amino-n-hexanoic acid in distilled water.



4.2.4 Assay of plasminogen

Plasminogen was activated to plasmin and assayed by the caseinolytic procedure described by Johnson et al. (1969) and employed in Part A, Section 3.2.3.

4.3 Results

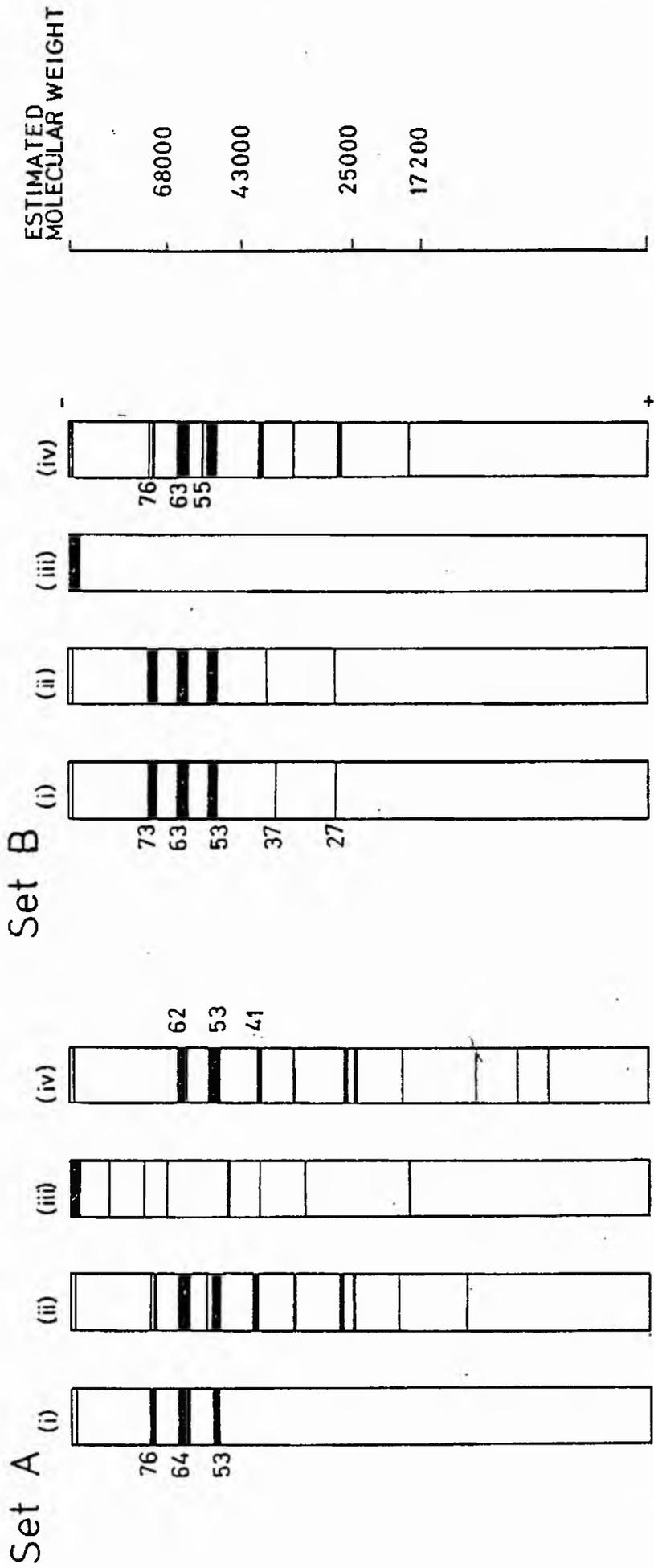
4.3.1 The removal of contaminating plasminogen from fibrinogen

The elution profile obtained following the application of human fibrinogen to a column containing lysine-Sepharose 4B is illustrated by Fig. 4.1. It appears similar to that of the analogous procedure performed in Part A, Section 3.3.1 where a phosphate buffer system was used.

Experiments were performed using both "purified" and "unpurified" fibrinogen samples to assess the respective levels of inherent plasminogen contamination and thereby to provide an index of the efficiency of the column purification procedure. The polyacrylamide gel patterns obtained after examination of the various fibrinogen samples are illustrated in Fig. 4.2. Upon reduction, both "unpurified" (gel A (i)) and "purified" (gel B (i)) fibrinogen samples display the characteristic reduced fibrinogen $A\alpha$, $B\beta$ and γ chain pattern. A slight splitting of the $A\alpha$ band was obvious in gel A (i) implying that the "unpurified"

Fig.4.2 SDS-polyacrylamide gel electrophoresis of fibrinogen

Fibrinogen both before (SetA) and after (SetB) lysine Sepharose treatment were examined on 10% gels (i) reduced, (ii) reduced but following their incubation in the presence of streptokinase, (iii) as for (ii) but unreduced and (iv) as for (ii) but also present during incubation was a sample of the 6-amino-n-hexanoic acid eluate.



fibrinogen had undergone slight degradation. However gel B (i) displays two faint bands below that attributed to the δ chain of the "purified" fibrinogen. Thus limited degradation of this sample may also have occurred. Degradation of "unpurified" fibrinogen was promoted by the addition of the plasminogen activator, streptokinase. Analyses of both reduced (gel A (ii)) and unreduced (gel A (iii)) samples has revealed evidence of fibrinogen breakdown products. Further degradation of this fibrinogen was induced by the combined action of streptokinase and an aliquot of the fraction eluted from the lysine-Sepharose 4B column by 6-amino-n-hexanoic acid (gel A (iv)), complete degradation of the fibrinogen A α chain being promoted.

The addition of streptokinase to "purified" fibrinogen did not promote further degradation of the intact fibrinogen molecule (gel B (iii)) or of its constituent chains (gel B (ii)). Significant degradation of "purified" fibrinogen was however, induced following the addition of streptokinase and an aliquot of the 6-amino-n-hexanoic acid-promoted eluate from the lysine-Sepharose 4B column (gel (iv)). The "purified" fibrinogen sample does not appear to contain significant levels of contaminating plasminogen.

4.3.2 Isolation of plasminogen from human plasma

Figure 4.3 illustrates the elution profile obtained following the application of human plasma to a column of

Fig.4.3 The isolation of plasminogen from human plasma by lysine-Sepharose 4B chromatography

Chromatography of plasma on lysine-Sepharose 4B. Column dimensions: 2.5 x 13 cm, Flow rate: 45 ml/h. Sample: 260 ml plasma, Elution procedure as for Fig. 4.1.

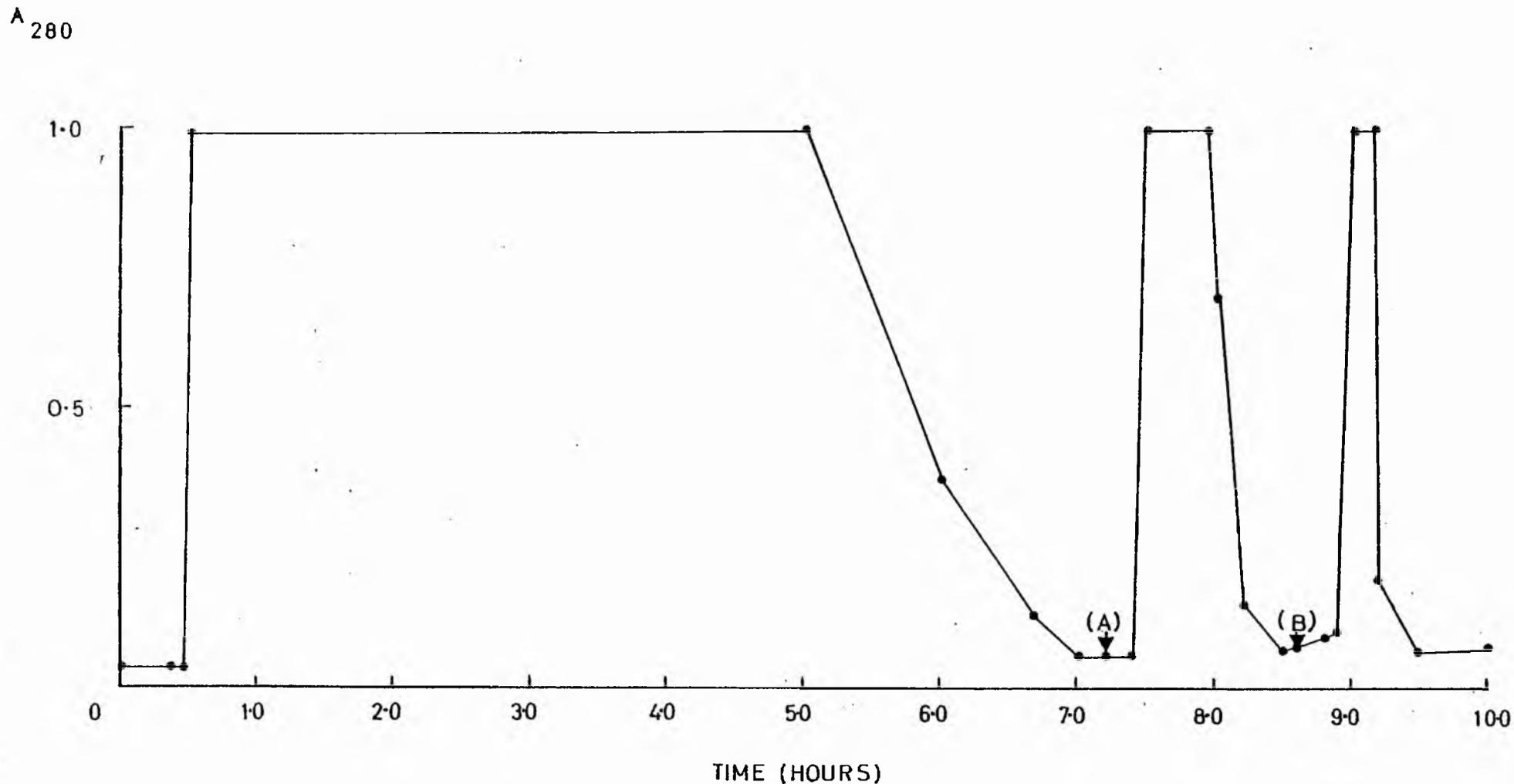
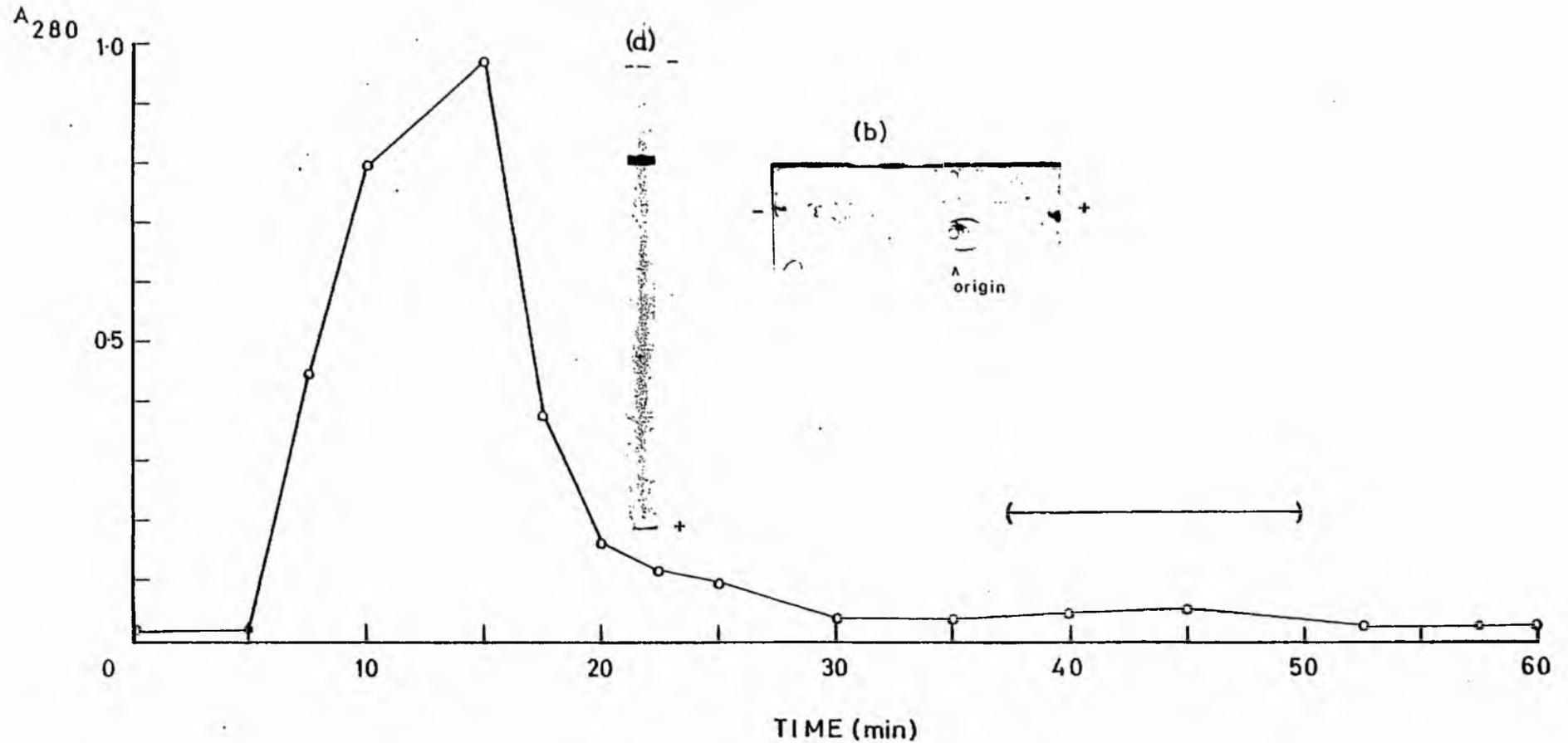


Fig. 4.4 Sephadex G-25 gel filtration of human plasminogen

Human plasminogen isolated by lysine-Sepharose chromatography was desalted by Sephadex G-25 gel filtration. Column dimensions; 2.5 x 20cm. Flow rate:150ml/h. Elution buffer:0.005 M-Tris/HCl buffer, pH 7.5. The horizontal bar indicates the elution of 6-amino-n-hexanoic acid. Inset; the results of (a) SDS-gel electrophoresis (unreduced, 5% gel) and (b) immunoelectrophoresis (against anti-human plasminogen antiserum) of the desalted sample.



lysine-Sepharose 4B. This pattern of elution differs in two respects from that obtained in Part A, Section 3.3.2, using an identical procedure but employing a phosphate buffer system. In the latter instance the elution promoted by the 6-amino-n-hexanoic acid solution was greater than that shown in Fig. 4.3 while a decreased elution was promoted by the NaCl-containing buffer. Thus in the present case less plasminogen appears to have been removed from the plasma during its passage through the lysine-Sepharose 4B gel. To ascertain if this finding could be explained by the partial elution of the plasminogen by the NaCl-containing buffer the immunoreactivity of this eluted solution was tested against anti-human plasminogen antiserum. No reaction was obvious.

An alternative method was employed to desalt the plasminogen preparation to that performed in Part A, Section 3.2.2 i.e. Sephadex G-25 gel filtration was preferred to prolonged dialysis. The column procedure was rapid; the plasminogen eluted from the lysine-Sepharose 4B column being desalted within 25 minutes (Fig. 4.4). The results of SDS-polyacrylamide gel electrophoresis (5% gel, unreduced sample) and of immunoelectrophoresis (against anti-human plasminogen antiserum) of the desalted plasminogen preparation are shown inset into Fig. 4.4. A positive reaction of the preparation against this antiserum is obvious. The

Table 4.1 The caseinolytic activity of plasminogen isolated from human plasma

Human plasminogen was isolated by lysine-Sepharose 4B affinity chromatography and then desalted by Sephadex G-25 gel filtration. Assay was by the caseinolytic procedure of Johnson *et al.* (1969).

Volume of Plasma (mls)	258
LYS-Sepharose Elution Volume (mls)	39
A ₂₈₀	1.14
G-25 Elution Volume (mls)	51
A ₂₈₀	0.86
Caseinolytic Activity (units/ml)	2.17
µg Plasminogen per ml of plasma	101
Caseinolytic Activity per ml of plasma	0.43
SPECIFIC ACTIVITY (units/mg)	4.25

results of SDS-gel analysis suggests that the preparation contained a single protein of apparent molecular weight 72,000.

The caseinolytic activity of the isolated plasminogen preparation was estimated and the results are presented in Table 4.1. A yield of 43 CA units/100ml of plasma was obtained.

4.4 Discussion

This section of work had two aims; firstly to prepare fibrinogen free from contaminating plasminogen and secondly to isolate plasminogen from human plasma for use in future digestion studies.

The evidence presented from streptokinase-induced digestion studies is consistent with the removal of a plasminogen-containing fraction from fibrinogen. However two faint protein bands of apparent molecular weights 37,000 and 27,000 (i.e. of lesser molecular weight than the constituent chains of fibrinogen) were detected in the reduced "purified" fibrinogen sample prior to the addition of streptokinase. Such bands were not visible in the analogous "unpurified" fibrinogen sample. It is possible therefore that the affinity chromatography process in itself may cause limited degradation of fibrinogen.

To conclude, no deleterious effect on the efficiency of the fibrinogen purification procedure was induced by

substituting a phosphate based buffer system with that of Tris.

"Purified" fibrinogen prepared in this manner was employed exclusively in Part B of this work.

The plasminogen preparation isolated from human plasma by lysine-Sepharose 4B affinity chromatography reacted against anti-human plasminogen antiserum. Furthermore it exhibited a single band upon electro-:phoretic examination (unreduced) of apparent molecular weight 72,000. Molecular weights of 87,000 to 92,000 for human plasminogen and 85,000 for plasmin have been reported (Violand & Castellino, 1976). Plasminogen is a glycoprotein containing 19-22 disulphide bonds (Robbins et al., 1975). These structural characteristics of a protein molecule may affect the observed electro-:phoretic mobility by their influence on SDS-binding and on the ability of the molecule to assume an extended conformation (Fish et al., 1970); factors which may explain the relatively low value obtained in the present investigation for the molecular weight of plasminogen. The caseinolytic activity of the preparation expressed as CA units/100ml of human plasma was 43. This figure is equivalent to 53% of the yield obtained in Part A, Section 3.2.2 where a phosphate buffer system was employed in place of the present Tris system. A possible explanation considered for the low yield of proenzyme was the partial

elution by the NaCl-containing buffer from the lysine-Sepharose 4B gel. However, this solution did not react against anti-human plasminogen antiserum. An alternative explanation for the low recovery of plasminogen may be construed from the reference made by Deutsch & Mertz (1970) to the fact that the replacement of a phosphate buffer system with one based on Tris produced a lower yield of plasminogen and a decreased specific activity. Thus the introduction of a buffer system more compatible with the presence of free Ca^{2+} may have been made at the expense of the efficiency of the lysine-Sepharose 4B chromatographic procedure to isolate plasminogen.

The activity reported for the plasminogen preparation has been assumed in all subsequent experiments.

SECTION 5

THE EFFECT OF Ca^{2+} ON THE PLASMIC DIGESTION
OF FIBRINOGEN

5.1 Introduction

The significance of the report by Haverkate & Timan (1977) to the present work has been discussed. These authors described the production of a single, high molecular weight form of fragment D from fibrinogen in the presence of Ca^{2+} . This fragment D (hereafter referred to as $\text{D}_{\text{Ca}^{2+}}$) was found to be resistant to further proteolysis by plasmin even in the presence of 2 M-urea. The purpose of the ensuing work was to investigate these claims. Additional properties displayed by fragment $\text{D}_{\text{Ca}^{2+}}$ and also the effect of 4 M-urea on the process of fibrinogen digestion will be described.

5.2 Methods

5.2.1 Preparation of fibrinogen

Human fibrinogen dissolved (5mg/ml) in 0.05 M-Tris/HCl buffer, pH 7.5, was prepared for digestion by dialysis for 18h against this buffer alone (fibrinogen-Blank) or this buffer containing either 5 mM-EDTA (fibrinogen-EDTA) or 2 mM- CaCl_2 (fibrinogen- CaCl_2). This nomenclature for fibrinogen solutions will be employed throughout Part B.

5.2.2 Activation of plasminogen

Plasminogen was activated to plasmin by incubation at 37°C for 15min in the presence of streptokinase (170 units/CA unit of plasminogen) and glycerol (25%, v/v). The resulting enzyme solution contained 0.85 CA units/ml.

5.2.3 Plasmin digestion of fibrinogen

The method of fibrinogen digestion by plasmin is based upon that described by Haverkate & Timan (1977). Digestion of fibrinogen was initiated by the addition of plasmin in a ratio of 0.17 CA units/mg of fibrinogen. NaN_3 (0.02%, w/v) was added to the digestion solution. Where digestion was performed in the presence of CaCl_2 or EDTA the concentrations of these reagents was maintained at 2 mM and 5 mM respectively. The fibrinogen digest solution was incubated at 37°C for 6h and, following the addition of the same amount (0.17 CA units/mg) of plasmin solution, for a further 18h. At this point the susceptibility of each of the fragments D in the digestion mixture to further degradation by plasmin under denaturing conditions was investigated by the addition of the appropriate volume of a 10 M-urea solution and further plasmin solution (0.085 CA units/mg). The digest solution was then maintained at 37°C for a further period of 24h. Each addition to the fibrinogen digest solution was accompanied by the appropriate volume from a stock solution of CaCl_2 or EDTA (or buffer) to ensure a constant level of

these compounds in each incubation solution.

Samples were removed from the digest solution at various time intervals and digestion was terminated by the addition of Trasylol (5500 K.I.U./CA unit plasmin).

The results from two further digestion studies will be described.

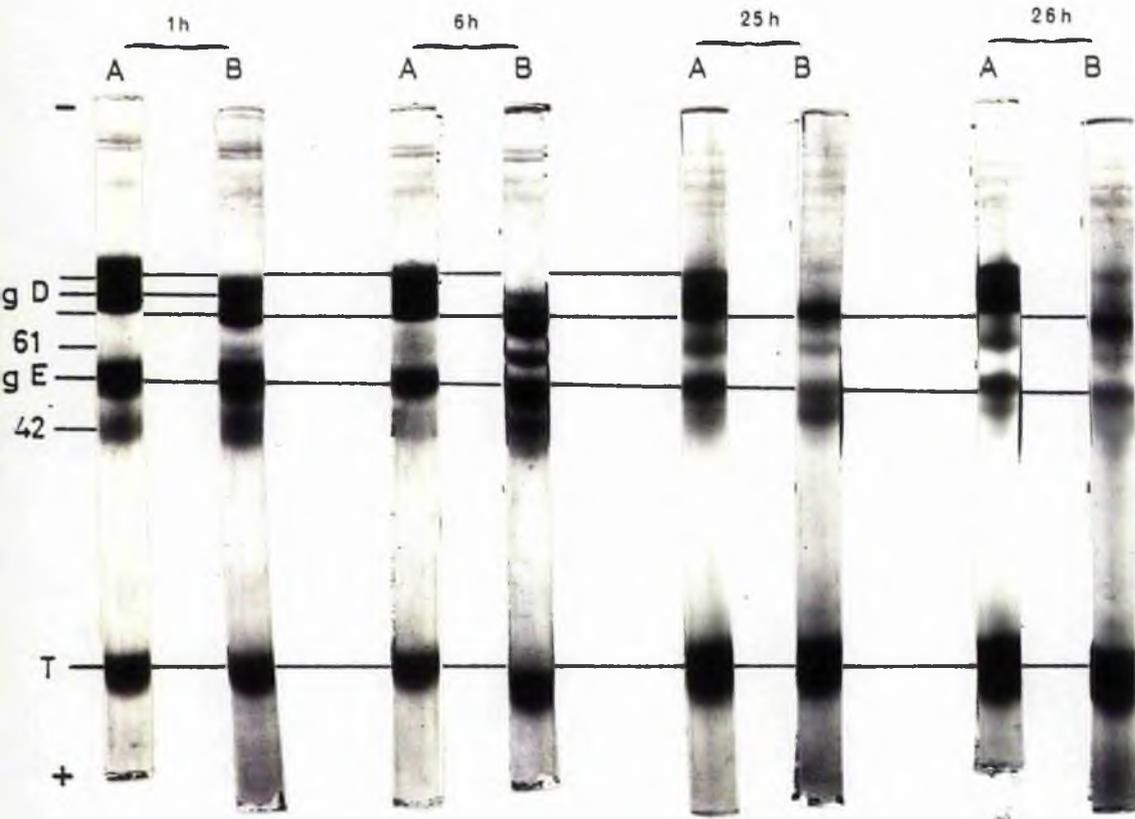
In the first case one modification was made to the above method. Fibrinogen samples were preincubated for 15min at 20°C and then digested, in the presence of 4 M-urea.

In the second, the investigation of the early stages of fibrinogen digestion by plasmin was performed by reducing both the temperature of incubation of the digest solution (from 37°C to 23°C) and the level of plasmin employed (from 0.17 to 0.11 CA/mg of fibrinogen). All other conditions of fibrinogen digestion were exactly as described above.

5.2.4 The effect of CaCl₂ on the caseinolytic activity of plasmin

The caseinolytic activity of plasmin both in the presence and absence of added CaCl₂ (2 mM) was assayed according to the method of Johnson et al. (1969) which was described in Part A, Section 3.2.3.

Fig 5-1 Polyacrylamide gel electrophoresis of fibrinogen digest samples



Fibrinogen was digested by plasmin both in (A) the presence and (B) the absence of added CaCl₂. Samples were removed from the digest after periods of 1h and 6h. Following the addition of urea (final conc. 2 M) at 24h two further samples were removed at 25h and 26h. Each sample was examined (unreduced) on 5% polyacrylamide gels. Apparent mol. wts. ($\times 10^{-3}$) are shown. T = Trasylol.

5.2.5 The effect of CaCl_2 on the electrophoretic mobility of protein molecular weight standards

Bovine serum albumin and ovalbumin, proteins employed routinely as molecular weight standards in SDS-gel electrophoresis were prepared for gel application (as described in Section 3.2.1) both in the presence and absence of 3 mM- CaCl_2 , and their electrophoretic mobility in 5% polyacrylamide gels calculated.

5.3 Results

5.3.1 Plasmic degradation of fibrinogen-Blank and fibrinogen- CaCl_2

Two samples of fibrinogen, fibrinogen- CaCl_2 and fibrinogen-Blank were digested by plasmin. Samples were removed after 1, 6, 25 and 26 hours. The last two samples represent, respectively, 1 and 2 hour stages of digestion after the addition of urea (to a final concentration of 2 M). Each digest sample was examined (unreduced) on 5% SDS-polyacrylamide gels and photographs corresponding to each gel are shown in Fig. 5.1. Differences are apparent in the composition of the fragment D-containing band of each fibrinogen digest sample. The degradation products of the initial (time 1h) fibrinogen-Blank digest sample (labelled B in Fig. 5.1) consist of two bands within the mobility region expected for fragment D and of one in the region expected for fragment E. A faint band of apparent molecular weight

61,000 is obvious between these two major bands. An additional band of apparent molecular weight 42,000 is evident between fragment E and the band attributable to Trasylol. The fragments D present in these samples will be referred to as fragment D_{B-1} and fragment D_{B-2} . In the gel corresponding to the later stage of digestion (time 6h) the intensity of the fragment D_{B-1} band is decreased. This change is accompanied by an obvious intensification of the band of apparent molecular weight 61,000 and of the band immediately below that of fragment E. These changes in band pattern suggest that fragment D_B is susceptible to the degradative action of plasmin and indeed the combined action of urea and plasmin (times 25 & 26h) accomplished the almost complete destruction of fragments D_B . A concomitant intensification of the band of apparent molecular weight 42,000 is obvious - a value similar to that of the fragment d produced under comparable conditions and described by Furlan et al. (1975) and also in Part A. Fragment D_B does not appear to be resistant to the action of plasmin, its degradation to fragment d being accelerated under denaturing conditions.

The results corresponding to the analogous samples removed from the fibrinogen- CaCl_2 digest solution are shown in Fig. 5.1, labelled A. The initial digest sample (time 1h) also displays two bands within the fragment D mobility region. One band is of similar

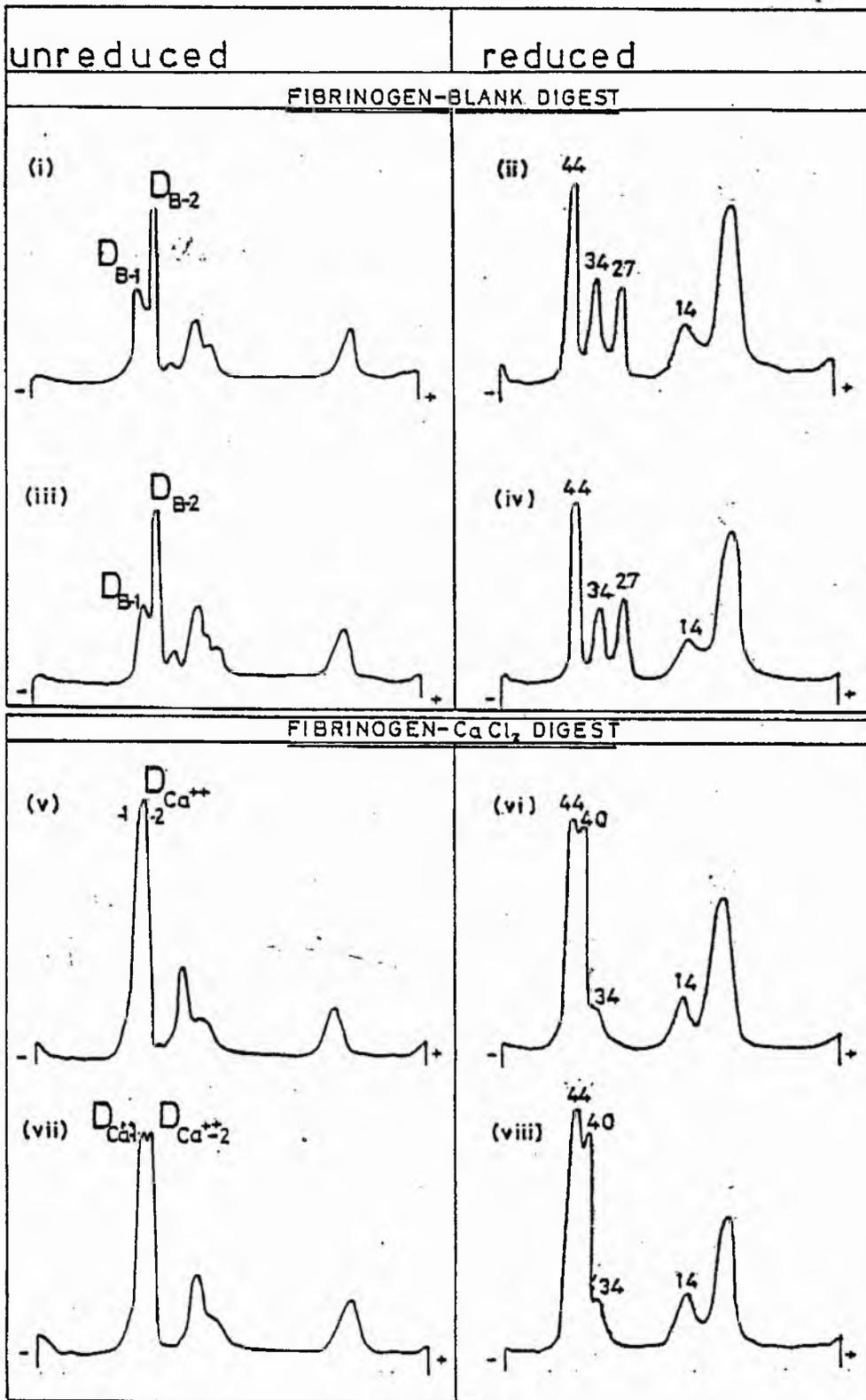
mobility to that of fragment D_{B-1} , while the other is of lower electrophoretic mobility. The two bands of fragment $D_{Ca^{2+}}$ are very close and poorly resolved. Unlike the situation with fragment D_B the continued incubation of fragment $D_{Ca^{2+}}$ with plasmin did not promote further degradation of the molecule (time 6h). Thus a resistance of fragment $D_{Ca^{2+}}$ to the action of plasmin is obvious. Again in sharp contrast to the behaviour of the fragment D_B -containing samples, the combined action of urea and plasmin did not promote a rapid breakdown of fragment $D_{Ca^{2+}}$ (time 25h). However the resistance to plasmin attack was not complete. The incubation of fragment $D_{Ca^{2+}}$ in the presence of urea and plasmin for a further one hour period (time 26h) promoted an intensification of the bands of apparent molecular weights 61,000 and 42,000. Therefore although fragment $D_{Ca^{2+}}$ displays a greater resistance to the action of plasmin under denaturing conditions than does fragment D_B , degradation by plasmin can be induced and indeed SDS-gel analysis of a sample removed from the fragment $D_{Ca^{2+}}$ digest solution following incubation in 2 M-urea for a period of 24h, revealed a considerable reduction in the intensity of both fragment $D_{Ca^{2+-1}}$ and $D_{Ca^{2+-2}}$ bands (not shown).

The apparent molecular weights calculated for the various intact fragment D species were as follows;

Fig. 5.2 Densitometric scans of fibrinogen digest samples examined by SDS-polyacrylamide gel electrophoresis

Fibrinogen was digested by plasmin both in the absence (fibrinogen-Blank) and presence (fibrinogen-CaCl₂) of added Ca²⁺. Samples removed from the digest solutions after periods of 2h and 3h were examined unreduced (5% gels) and reduced (10% gels). Scans (i) to (iv) correspond to the fibrinogen-Blank digest samples and scans (v) to (viii) to the fibrinogen-CaCl₂ digest samples. Apparent molecular weights ($\times 10^{-3}$) are shown.

Fig. 5.2



fragment $D_{Ca^{2+}-1}$ (82,000), fragment $D_{Ca^{2+}-2}$ (78,000), fragment D_{B-1} (77,000) and fragment D_{B-2} (72,000).

Digestion of fibrinogen by plasmin in the presence of 2 mM-MgCl₂ produced an identical pattern to that described above for fibrinogen-Blank digest samples. Thus the effect of Ca²⁺ on the digestion scheme of fibrinogen was not imitated by another divalent metal ion. A specific action of Ca²⁺ in the process of fibrinogen digestion is implied.

Two additional samples removed from the fibrinogen-CaCl₂ and fibrinogen-Blank digests at times 2h and 3h were examined, unreduced (5% gels) and reduced (10% gels), by SDS-electrophoresis. The corresponding densitometric scans are shown in Fig. 5.2. These results reveal further differences between the two types of fragment D.

Upon reduction of samples containing both forms of fragment D_B (Fig. 5.2, (i) and (iii)) four main peaks are obvious (Fig. 5.2, (ii) and (iv)). The calculated apparent molecular weights are shown ($\times 10^{-3}$) and comparison with previously published data (Furlan et al., 1975; Ferguson et al., 1975) suggests that the MW 44,000 peak corresponds to the β component chain of fragment D_B, the MW 34,000 and 27,000 peaks to successive digestion stages of the component D- γ chain, while the peak corresponding to the α chain remnant of fragment D_B is located near the end of the gel along with the reduced constituent chains of fragment E. The presence of two γ chain remnants in the reduced samples is consistent with the detection of two

molecular weight forms of the intact fragment D_B in the corresponding unreduced samples since the heterogeneity of a fragment D preparation has been concluded by various investigators (e.g. Furlan et al., 1975) to reflect differing extents of degradation of the component γ chain.

Reduction of the fragment $D_{Ca^{2+}}$ -containing digest samples (Fig. 5.2 (v) and (vii)) produced three main peaks (Fig. 5.2 (vi) and (viii)). The results obtained above suggest that the MW 44,000 peak represents the β subunit chain of fragment $D_{Ca^{2+}}$ and the high mobility peak the α subunit chain. The remaining peak, apparent molecular weight 40,000, must, therefore represent the γ chain component of fragment $D_{Ca^{2+}}$. Thus a higher molecular weight form of the γ chain has indeed been identified in a fragment D prepared from fibrinogen digested in the presence of Ca^{2+} as reported by Haverkate & Timan (1977). The inference drawn from analyses of both types of unreduced fragment D, that fragment $D_{Ca^{2+}}$ samples contained a lower electrophoretic mobility i.e. higher molecular weight fragment D component than the fragment D_B samples has thus been confirmed by examination of their respective reduced chain compositions.

However, Fig. 5.2 displays a further point of dissimilarity between the two digest samples. Reduction of a sample containing two electrophoretically distinct

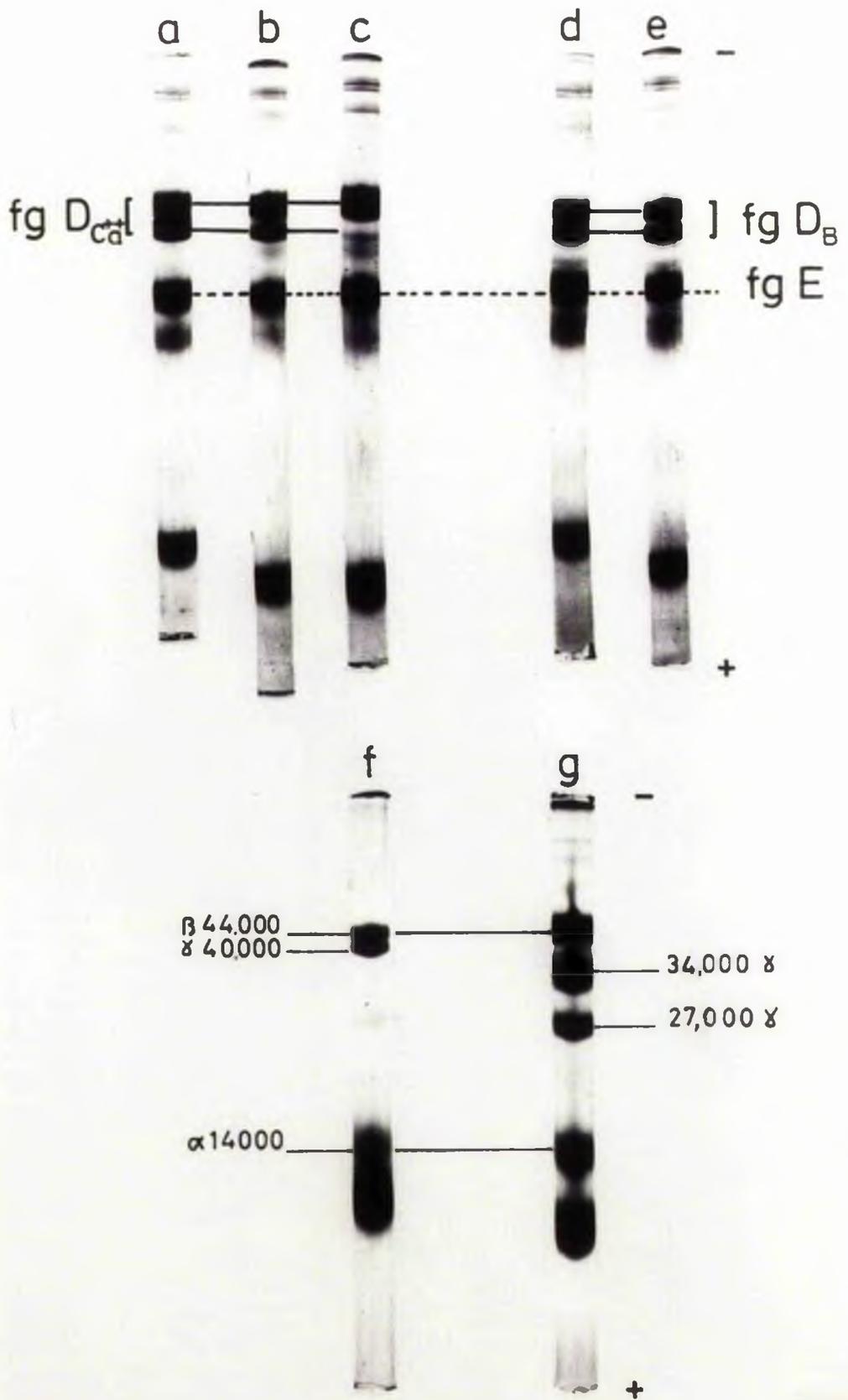
Fig. 5.3 The effect of EDTA and storage on the electrophoretic mobility of fibrinogen-CaCl₂ and fibrinogen-Blank digest samples.

A sample removed from a fibrinogen-CaCl₂ digest was examined by SDS-gel electrophoresis (a) unreduced, (b) unreduced but following incubation in the presence of 4 M-urea and 1.5% SDS at 20°C for 24h and (c) unreduced but following incubation in the presence of 10 mM-EDTA at 20°C for 10min. Gel (f) illustrates the composition of the sample applied to gel (c) following reduction.

The comparable sample removed from a digest of fibrinogen-Blank was examined (d) unreduced and (e) unreduced but following incubation in the presence of 10 mM-EDTA at 20°C for 10min. Gel (g) illustrates the reduced chain composition of a sample of fragment D_B following incubation in the presence of 5 mM-CaCl₂ at 20°C for 10min.

Gels (a) to (e) are 5% polyacrylamide gels while gels (f) and (g) are 10% gels. Apparent molecular weights ($\times 10^{-3}$) are shown.

Fig. 5.3



forms of fragment D_B has revealed two δ chain components while the reduction of a sample containing also, apparently, two fragment $D_{Ca^{2+}}$ species has revealed only one δ chain peak. A faint peak is apparent as a shoulder below the δ chain 40,000 molecular weight peak, and displays an apparent molecular weight of 34,000 (Fig. 5.2 (vi) and (viii)). However, the obvious weak intensity of this peak is not consistent with it having arisen from one of the two major peaks of fragment $D_{Ca^{2+}}$ demonstrated in the unreduced sample i.e. the amount of this 34,000 molecular weight peak is not sufficiently significant to alter the suggestion made above that reduction of two major electrophoretic forms of fragment $D_{Ca^{2+}}$ produces only one form of constituent δ chain.

5.3.2 The effect of EDTA and of storage on the electrophoretic mobility of fibrinogen- $CaCl_2$ and fibrinogen-Blank digest samples

The results of studies designed to investigate the effect of EDTA on the electrophoretic mobility of the constituents of both fibrinogen-Blank and fibrinogen- $CaCl_2$ digest samples revealed a further difference in the properties of the two types of fragments D. These results are shown in Fig. 5.3. The electrophoretic mobility displayed by the various constituents of the fibrinogen-Blank digest (gel d) was apparently unaffected

by the addition of EDTA (final concentration 10 mM) prior to electrophoresis (gel e). By contrast the mobility of the fragment $D_{Ca^{2+}}$ component of the fibrinogen- $CaCl_2$ digest sample (gel a) was altered by its exposure to EDTA (gel c). Two effects are obvious. The intensity of the higher mobility fragment $D_{Ca^{2+}}$ band has decreased while that of the lower mobility band has increased. These effects occurred in the absence of significant amounts of higher molecular weight fibrinogen digestion products and in the presence of Trasylol. It therefore is unlikely that the increase in the amount of this low mobility fragment $D_{Ca^{2+}}$ band could be the result of ongoing digestion of higher molecular weight precursors. Similarly the disappearance of the high mobility fragment $D_{Ca^{2+}}$ band was not accompanied by the appearance of significant amounts of lower molecular weight fragments. (However a small portion of the fragment $D_{Ca^{2+}}$ component of the digest sample did not exhibit this decrease in mobility upon addition of EDTA. This atypical faint band is obvious immediately below the major low mobility fragment $D_{Ca^{2+}}$ band in gel (c). It has been noted that upon reduction of fragment $D_{Ca^{2+}}$ a faint band of apparent molecular weight 34,000 is obvious immediately below that of the band attributed to the γ chain. These two faint atypical components of the fragment $D_{Ca^{2+}}$ sample may be related. The high mobility, EDTA-resistant, component of the unreduced

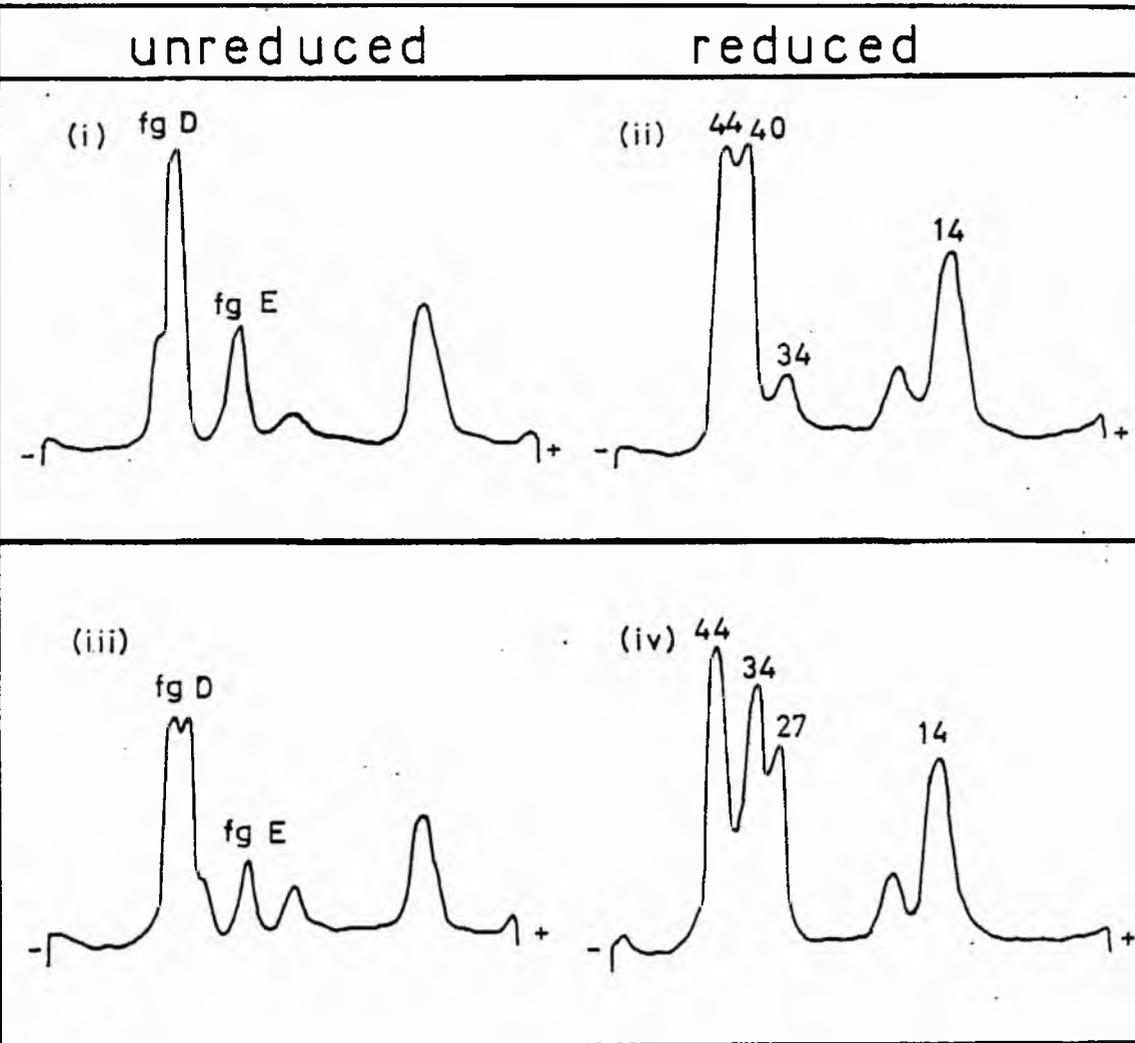
fragment $D_{Ca^{2+}}$ sample may contain the high mobility δ chain of molecular weight 34,000).

This EDTA-induced decrease in the mobility of the fragment $D_{Ca^{2+}}$ band was also promoted by the incubation of the sample at 20°C in the presence of SDS and urea for 24h. However in this case the transition of the high to the low mobility band was incomplete (gel b). This treatment, like that of EDTA addition, did not alter the mobility of the fragment D_B band and produced a gel pattern similar to that displayed by gel d. The bands of apparent molecular weights 61,000 and 42,000 were in fact of greater intensity in this sample which suggests that fragment D_B is degraded by the incubation treatment.

The mobility of the fragment E band was unaffected by either the addition of EDTA or by the incubation treatment.

Therefore the molecular weight of fragment $D_{Ca^{2+}}$ may apparently be increased either by its treatment with EDTA or by its incubation under denaturing conditions. No such effect was induced in a fragment D prepared in the absence of added Ca^{2+} . EDTA-treatment of the fragment $D_{Ca^{2+}}$ sample prior to reduction did not alter the reduced chain pattern (gel f). Neither the reduced chain pattern nor the pattern displayed by the unreduced fragment D_B -containing sample was affected by pretreatment with $CaCl_2$ (5 mM). (Reduced sample: gel g;

Fig. 5.4 SDS-polyacrylamide gel electrophoresis of
fibrinogen-CaCl₂ digest samples



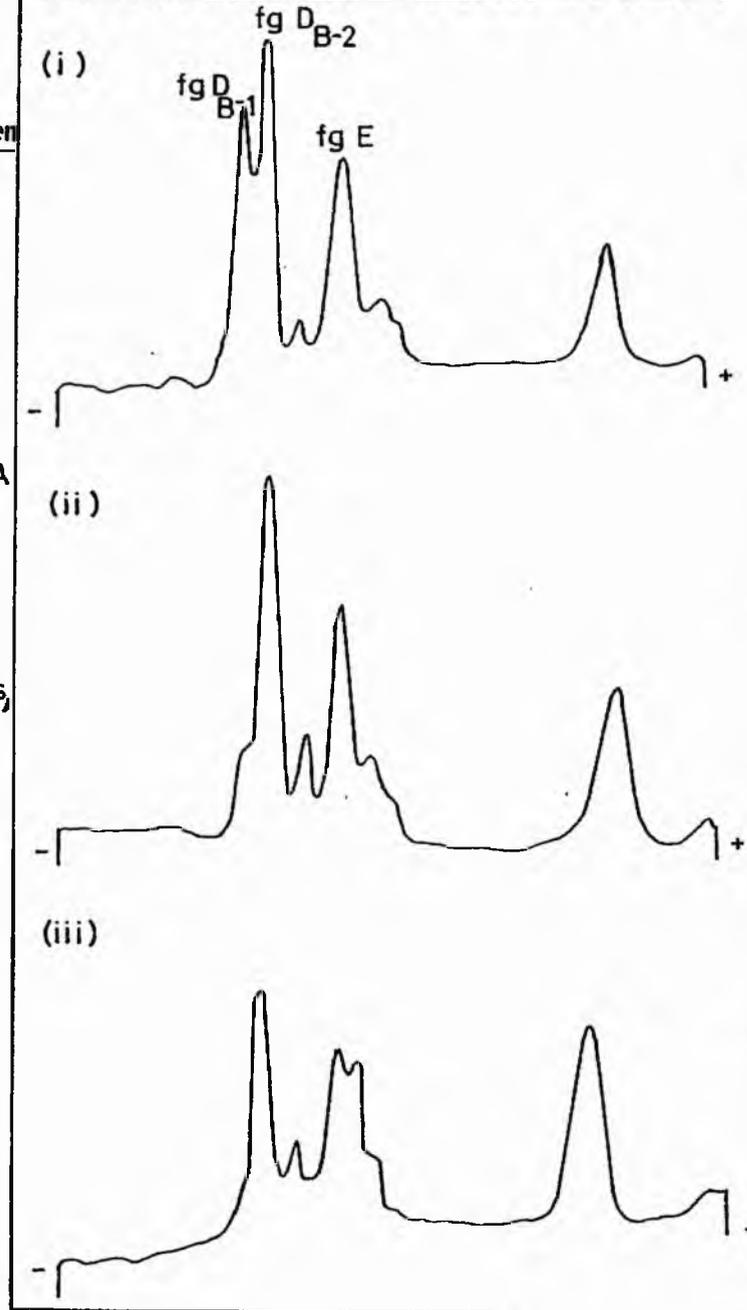
A fibrinogen-CaCl₂ digest sample containing fragment D_{Ca⁺⁺} was examined by polyacrylamide gel electrophoresis (i) unreduced (ii) reduced. An identical sample was subjected to EDTA-treatment (5mM) and further digestion by plasmin for 3.5h. It was then re-examined (iii) unreduced and (iv) reduced. Apparent mol.wts. ($\times 10^{-3}$) are shown. Diag.(i) and (iii) are 5% polyacrylamide gel densitometric scans, (ii) and (iv) are 10% gel scans.

Fig. 5.5

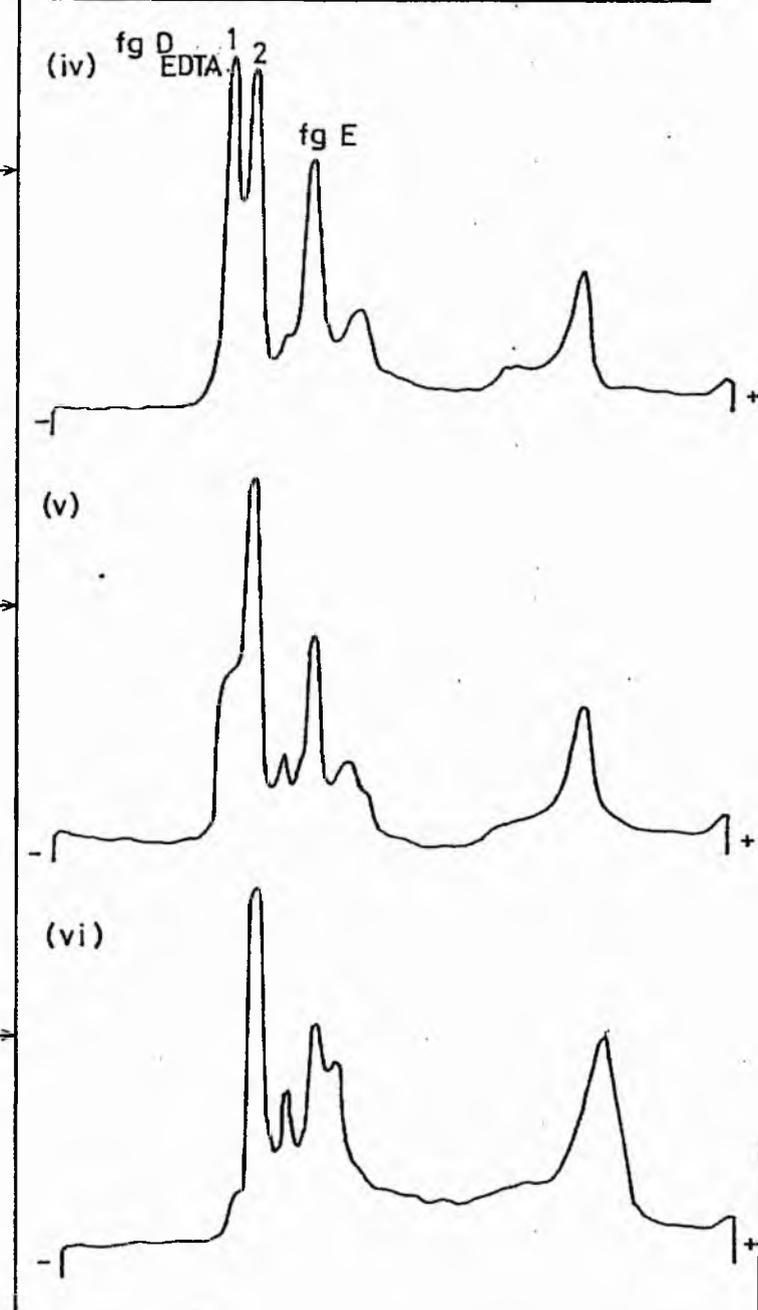
SDS-polyacrylamide gel
electrophoresis of fibrinogen
digest samples

Fibrinogen was digested by plasmin in 0.05 M-Tris/HCl buffer, pH 7.5 (fibrinogen-Blank digest) and in the same buffer containing 5mM-EDTA (fibrinogen-EDTA digest). The densitometer scans corresponding to samples removed from each digest after periods of 1h, 2h and 6h are shown (5% gels, unreduced samples).

FIBRINOGEN-BLANK DIGEST



FIBRINOGEN-EDTA DIGEST



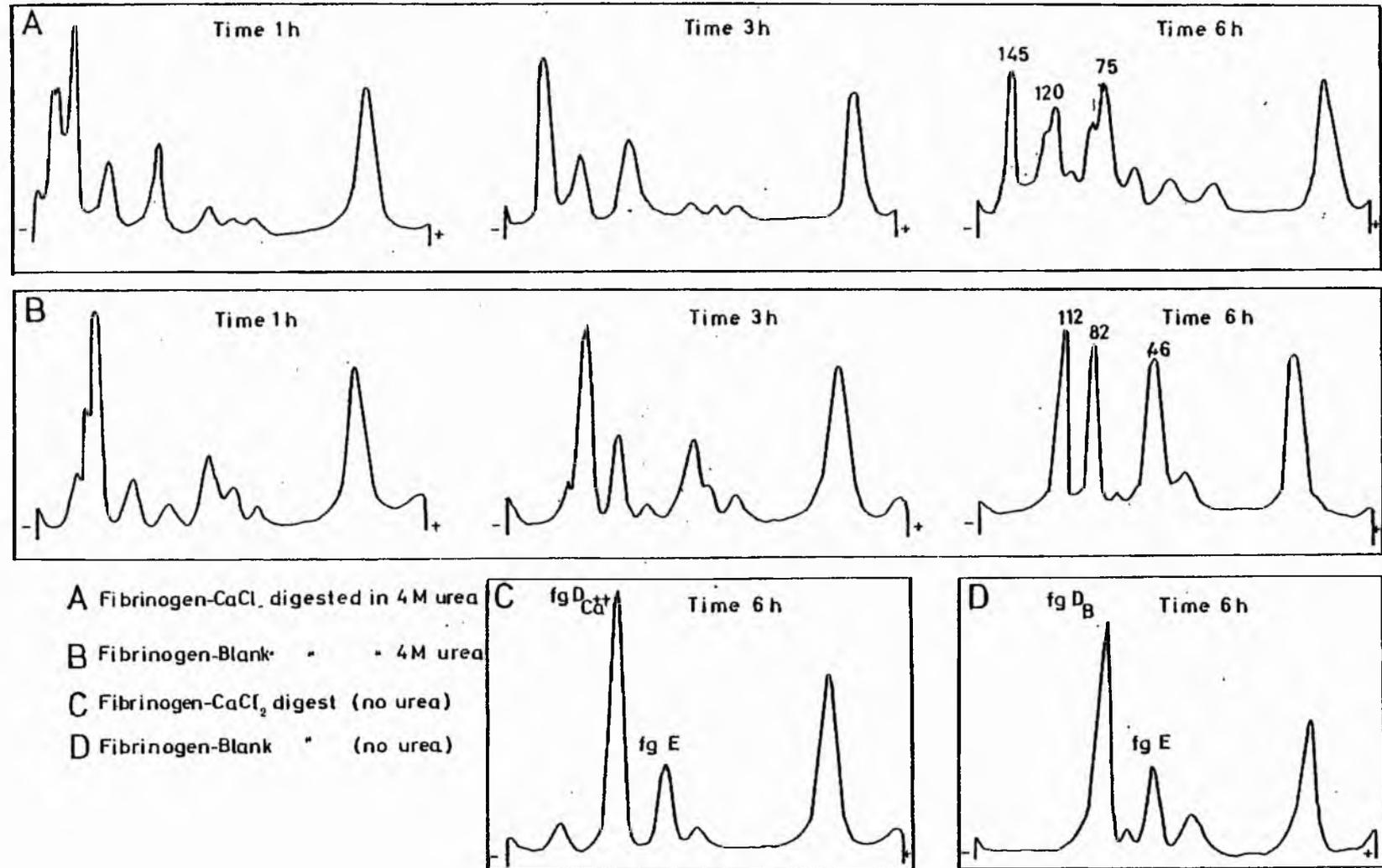
unreduced sample produced a pattern identical to that of gel e). Thus the peculiar properties of the fragment $D_{Ca^{2+}}$ -containing samples cannot be attributed simply to the presence of $CaCl_2$ within the buffer during analysis. However fragment $D_{Ca^{2+}}$ could be converted to a fragment D whose reduced and unreduced electrophoretic pattern was identical to that of fragment D_B (Fig. 5.4) by its treatment with EDTA and then plasmin. It follows that the combined action of a chelating agent and plasmin is required to achieve the degradation of the hitherto plasmin-resistant fragment $D_{Ca^{2+}}$ molecule. Degradation progressed via intermediates, the subunit apparent molecular weights of which, were identical to those of fragment D_B .

5.3.3 The plasmic digestion of fibrinogen-Blank and fibrinogen-EDTA

In an identical manner to that described in Section 5.3.1 the pathway of plasmic digestion of fibrinogen-EDTA was compared to that of fibrinogen-Blank. Aliquots removed after periods of 1h, 2h and 6h, were examined (unreduced) by SDS-gel electrophoresis. The corresponding densitometric scans are shown in Fig. 5.5. The mobility values of the fragment D and E-containing peaks are identical in each case. The analogous results obtained by polyacrylamide gel analysis of the reduced digest samples implied an identical subunit chain composition for

Fig. 5.6 Polyacrylamide gel electrophoresis of fibrinogen digested by plasmin

Fibrinogen-Blank and fibrinogen-CaCl₂ were digested by plasmin both in the presence and absence of 4 M-urea. Digest samples were examined, unreduced, on 5% gels. The corresponding densitometer scans are shown. Apparent mol.wts. are indicated ($\times 10^{-3}$).



fragments D_B and D_{EDTA} . However inspection of the relative amounts of the fragment D peaks in each digest (Fig. 5.5) reveals that digestion of fragment D_{EDTA} may proceed more slowly. In scans (iv) (v) and (vi) the intensity of the fragment D_{EDTA} peaks is greater than those of the comparable fragment D_B -containing samples. With this proviso, the results of Fig. 5.5 imply that the digestion of fibrinogen-EDTA and of fibrinogen-Blank by plasmin follow an identical pathway.

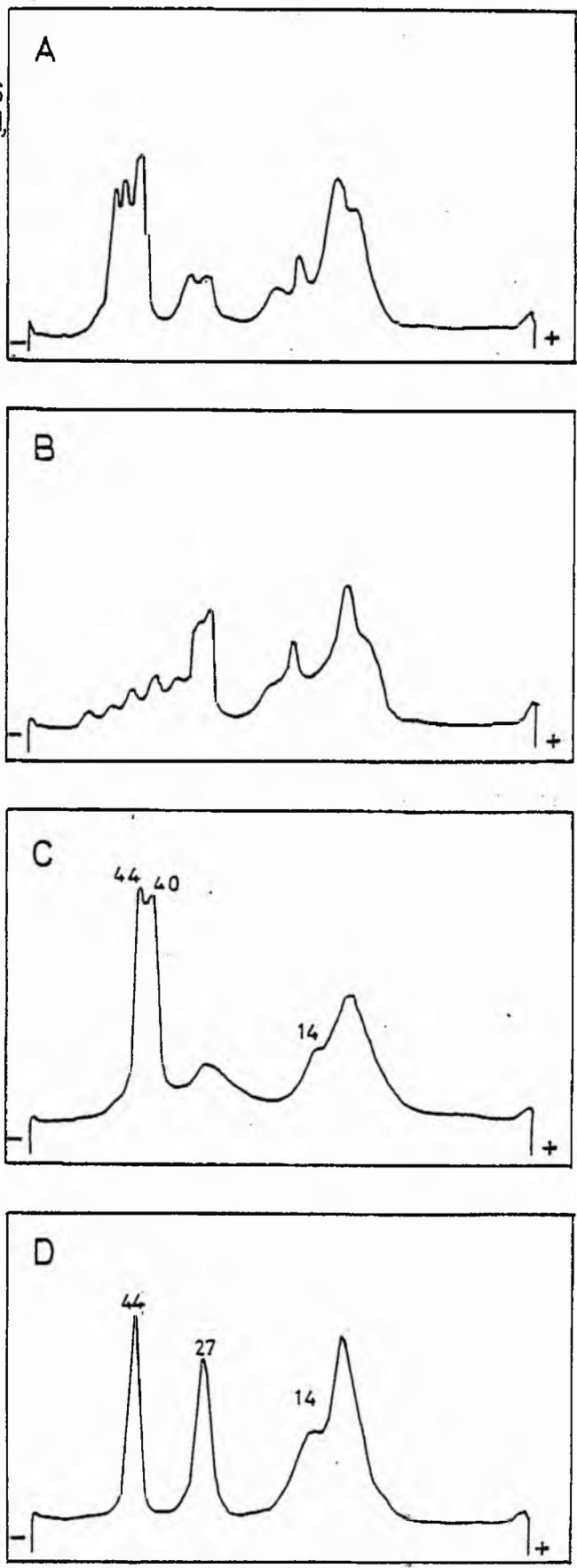
5.3.4 The degradation of fibrinogen-Blank and fibrinogen- $CaCl_2$ in the presence of 4 M-urea

Haverkate & Timan (1977) reported that the plasmin resistance displayed by the fragment $D_{Ca^{2+}}$ molecule was evident even in the presence of 2 M-urea. This experiment describes the effect of digesting both fibrinogen- $CaCl_2$ and fibrinogen-Blank in the presence of twice this concentration of denaturing agent.

The densitometric scans obtained by SDS-gel electrophoresis of samples removed from each of the digests after periods of 1h, 3h and 6h are shown in Fig. 5.6 (unreduced, 5% gels). The comparison with the corresponding samples removed from each digest performed in the absence of urea (scans C and D) reveals that the digestion of each type of fibrinogen in the presence of urea progressed via novel and different intermediates. However, in the digest containing Ca^{2+} (scans A) the higher molecular weight species are preserved to a more

Fig. 5.7 SDS-polyacrylamide gel electrophoresis of fibrinogen digested in the presence and absence of urea

Samples removed from each of the four fibrinogen digests described in Fig. 5.6 at Time 3h were examined, reduced, on 10% gels. Apparent mol.wts. ($\times 10^{-3}$) are shown.



advanced stage of digestion than those of the fibrinogen-Blank digest (scans B). Thus Ca^{2+} may protect the high molecular weight fibrinogen degradation products from plasmin attack even in the presence of 4 M-urea. Further evidence for this proposal is supplied by the results of the SDS-gel analysis of the various reduced samples. The fibrinogen- CaCl_2 digest sample prepared in the presence of 4 M-urea (Fig. 5.7, A) displays several peaks within the molecular weight range 40-50,000. The corresponding fibrinogen-Blank sample (Fig. 5.7, B) contains only faint peaks within this molecular weight range. The major peaks are evident within a molecular weight range of 24-34,000.

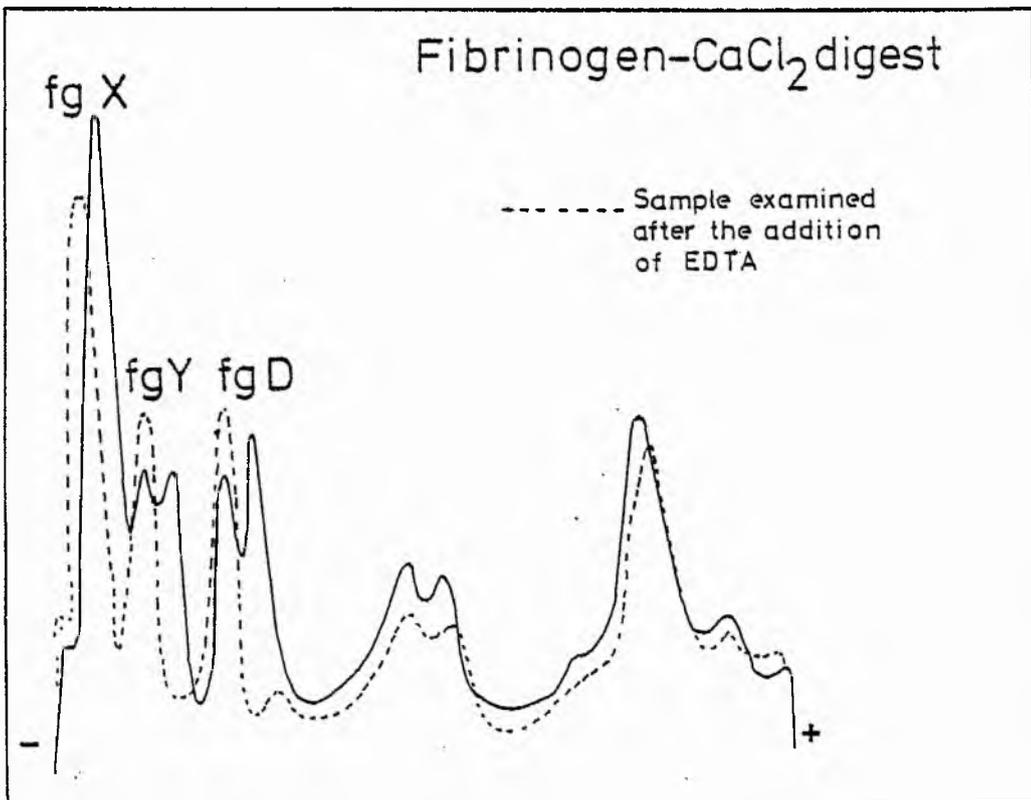
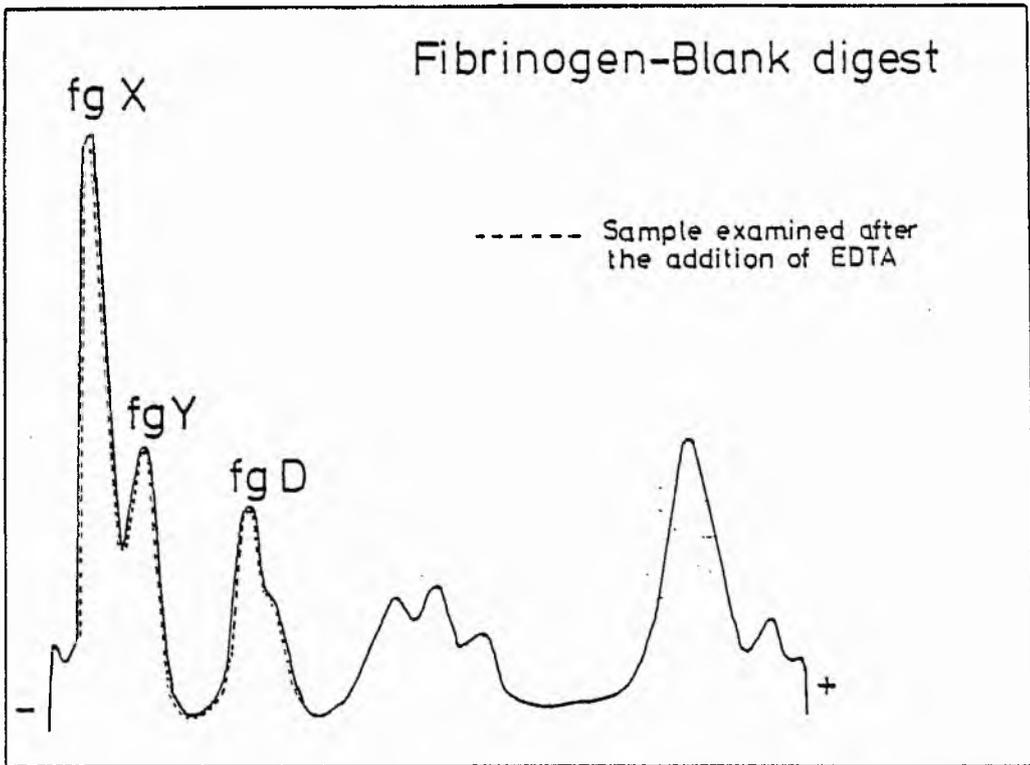
5.3.5 The early stages of the digestion of fibrinogen- CaCl_2 and fibrinogen-EDTA by plasmin

According to the scheme of fibrinogen digestion proposed by Marder et al. (1969) fibrinogen is converted to the two terminal digestion products fragments D and E via two high molecular weight intermediates termed fragments X and Y. The purpose of investigating the early stages of fibrinogen digestion, through reducing both the temperature of incubation and the level of plasmin in the digest solution, was to determine if the precursors of fragment $\text{D}_{\text{Ca}^{2+}}$ in the fibrinogen digestion scheme also displayed the anomalous electrophoretic mobility properties described for fragment $\text{D}_{\text{Ca}^{2+}}$. The densitometric scans

Fig. 5.8 SDS-polyacrylamide gel electrophoresis of samples removed from the early stages of fibrinogen digestion by plasmin

Fibrinogen-CaCl₂ and fibrinogen-Blank were digested by plasmin at 23°C. Samples removed from each of the digest solutions after a period of 5min were examined, unreduced on 5% polyacrylamide gels. An identical sample from each digest solution was similarly examined following incubation in the presence of 10 mM-EDTA. The densitometric scans corresponding to each of these four gels are shown.

Fig. 5.8



corresponding to a sample removed from a fibrinogen- CaCl_2 and a fibrinogen-Blank digest are shown in Fig. 5.8. The peaks corresponding to fragments X, Y and D are labelled. In the present system fragments X and fibrinogen were not resolved and ran as a broad peak at the top of the gel. The densitometric scan corresponding to the fibrinogen-Blank digest sample contains a single fragment Y peak between the broad peaks of fragment X and fragment D_B . Fragment D_B appears as a doublet peak. The mobilities of each of these four peaks were unaffected by the addition of EDTA to the digest sample prior to electrophoresis. This pattern of peaks differs from that obtained with the fibrinogen- CaCl_2 digest sample. As before fragment $D_{\text{Ca}^{2+}}$ appears as a doublet peak, one peak is of similar mobility to the low mobility peak of the fragment D_B described above, the other peak, as before, displays an even lower electrophoretic mobility value. Analysis of the fibrinogen- CaCl_2 digest sample has also revealed an additional peak within the fragment Y region. The addition of EDTA to this sample prior to electrophoresis produced three distinct changes in the electrophoretic pattern. Firstly, fragment $D_{\text{Ca}^{2+}}$ appears as a single peak of increased magnitude and, as has previously been described, this change was accompanied by the "loss" of the high mobility fragment $D_{\text{Ca}^{2+}}$ peak. Secondly the additional high mobility peak observed within the fragment Y region is no longer

Table 5.1 The effect of CaCl₂ on the caseinolytic activity of plasmin

CaCl ₂ conc. (mM)	Caseinolytic Activity (CA units/ml)
0	1.6 ± 0.05 (2)
2	2.2 ± 0.02 (4)

The caseinolytic activity of a human plasmin preparation was estimated both in the absence and presence of added CaCl₂. The results are expressed as the mean activity ± SEM (n).

evident while the remaining peak has intensified. Finally the peak attributed to fibrinogen and fragment X is noticeably sharper.

These results have demonstrated that the anomalous dual mobility displayed by fragment $D_{Ca^{2+}}$ is also a characteristic of the two higher molecular weight fibrinogen- $CaCl_2$ digestion products, fragments X and Y.

It is interesting to note in passing that the SDS-gel analysis of two digest samples removed at an even earlier digestion stage than that illustrated in Fig. 5.8 revealed that the initial form of fragment D_B evident was the low mobility i.e. high molecular weight form. Conversely the higher mobility i.e. lower molecular weight form of fragment $D_{Ca^{2+}}$ was initially evident.

5.3.6 The effect of $CaCl_2$ on the caseinolytic activity of plasmin

The possibility that the presence of Ca^{2+} in the fibrinogen digestion solution favoured the production of a higher molecular weight i.e. less degraded form of fragment D by inhibiting the action of plasmin was investigated by studying the effect of Ca^{2+} on the caseinolytic activity of plasmin. The results are presented in Table 5.1 and in fact $CaCl_2$ induced a significant ($p=0.001$) increase in the caseinolytic activity of plasmin.

5.3.7 The effect of CaCl_2 on the electrophoretic mobility of protein molecular weight standards

The addition of CaCl_2 to protein standard solutions did not alter the electrophoretic mobility of either the reduced or unreduced forms of the proteins. Thus it is unlikely that the anomalous mobility effects described for fragment $\text{D}_{\text{Ca}^{2+}}$ can be attributed to a non-specific effect of CaCl_2 on the mobility of a protein during SDS-gel electrophoresis.

5.4 Discussion

The initial purpose of the foregoing experiments was to investigate the report made by Haverkate & Timan (1977) that plasminic degradation of fibrinogen in the presence of 2 mM- CaCl_2 produces one major molecular weight form of fragment D. The significance of this claim to the present work has been discussed.

A single high molecular weight fragment D (designated $\text{D}_{\text{Ca}^{2+}}$) was indeed produced from fibrinogen in the presence of Ca^{2+} . The molecular weights of its constituent chains were β -44,000, δ -40,000 and α -14,000. The comparable values reported by Haverkate and Timan were 43,000, 38,000 and 12,000. In the absence of added Ca^{2+} a fragment D was detected which was identical to fragment $\text{D}_{\text{Ca}^{2+}}$ apart from the lower molecular weight forms of its constituent δ chain (molecular weights 34,000 and 27,000). However this conclusion requires

qualification. The homogeneity of the fragment $D_{Ca^{2+}}$ preparation predicted from analysis of its reduced chain composition was not confirmed by examination of the intact, unreduced molecule until it had either been (i) incubated under denaturing conditions for a period of 24h or (ii) treated with EDTA. Both treatments produced a conversion of the biphasic fragment $D_{Ca^{2+}}$ band obvious in the SDS-gel to a single low mobility band. This finding is not in agreement with the statement made by Haverkate & Timan (1977) that the addition of EGTA (20 mM) to their fragment $D_{Ca^{2+}}$ sample did not alter its electrophoretic mobility. However they may be referring to the reduced chain electrophoretic pattern of fragment $D_{Ca^{2+}}$ or alternatively, they may have left their samples at room temperature prior to electrophoresis thereby effectively incubating them under denaturing conditions.

In accordance with the finding of Haverkate & Timan fragment $D_{Ca^{2+}}$ appears resistant to further degradation by plasmin. The reduced chain composition of fragment $D_{Ca^{2+}}$ (more significantly the plasmin-vulnerable δ chain) was essentially unaffected by continued incubation in the presence of plasmin. This was not true for the fragment D_B sample, degradation of the constituent δ chain being detected. However the present results do not confirm unreservedly the claim by Haverkate & Timan that fragment $D_{Ca^{2+}}$ was resistant to plasmin even in the

presence of 2 M-urea. A slight degradation of fragment $D_{Ca^{2+}}$ was detected. This was in sharp contrast to the result obtained with fragment D_B - the virtually complete digestion of the molecule being induced.

A specific role for Ca^{2+} in the production and the properties of fragment $D_{Ca^{2+}}$ seems an attractive possibility. However several alternative explanations of the present results must first be considered. Ca^{2+} may have a non-specific effect on the electrophoretic mobility of proteins thereby promoting the calculation of incorrect molecular weight values. However this suggestion is not compatible with the finding that (i) the mobility of fragment D_B was unaffected by subsequent incubation in the presence of Ca^{2+} , (ii) fragment E displayed an identical electrophoretic mobility whether prepared in the absence or the presence of Ca^{2+} and (iii) the electrophoretic mobility of protein molecular weight markers could not be altered by pre-incubation in the presence of Ca^{2+} .

Alternatively Ca^{2+} may have decreased the level of plasmin activity, slowing the digestion of fibrinogen and thereby preserving the higher molecular weight forms of fragment D. Thus the effect of Ca^{2+} would be quite unrelated to the proposed specific interaction of the ion with fibrinogen. This possibility has been rejected by Haverkate & Timan (1977) because Ca^{2+} did not influence the activity of plasmin on the synthetic substrate,

D-valyl-leucyl-lysine p-nitroanilide (S-2251). The present results employing α -casein (which may be a more reliable method of estimating the fibrinolytic activity of plasmin since Jollès et al. (1978) have demonstrated a structural relationship between bovine casein and the human fibrinogen δ chain) confirm the reasoning of Haverkate & Timan but, in addition suggest that Ca^{2+} may stimulate the caseinolytic activity of plasmin. This observation may explain the finding that the digestion of fibrinogen-EDTA progressed more slowly than that of fibrinogen-Blank. EDTA may have chelated Ca^{2+} essential to the maximal plasmin activity.

The present results favour the proposal of Haverkate & Timan that Ca^{2+} bound to fibrinogen fragment D induced a plasmin-resistant conformation of fragment D. Fragment $\text{D}_{\text{Ca}^{2+}}$ differed from fragments D_{B} and D_{EDTA} only in the length of its constituent δ chain and could be converted to a species identical to those lower molecular weight fragments D by exposure to EDTA and then plasmin. This is consistent with the reports by Ferguson et al. (1975) and by Furlan et al. (1975) that the plasminic degradation of fragment D is restricted to attack at the COOH-terminal region of the δ chain. Thus available evidence signifies an important relationship between Ca^{2+} and the δ chain of fragment $\text{D}_{\text{Ca}^{2+}}$. Since the plasmin-susceptible region of this chain is protected from digestion by Ca^{2+} it seems reasonable to propose that

Ca^{2+} is bound to the COOH-terminal region of the δ chain thereby inducing a plasmin resistant conformation. This conformational effect of Ca^{2+} may alter the ability of the molecule to unfold in the presence of SDS and urea and as a consequence, the fragment containing the bound Ca^{2+} would appear smaller and therefore exhibit an increased electrophoretic mobility. This reasoning offers an explanation of the anomalous electrophoretic mobility displayed by fragment $\text{D}_{\text{Ca}^{2+}}$ and its fibrinogen digestion precursors. The dual electrophoretic mobilities demonstrated for these molecules may represent on the one hand the molecule exhibiting the altered conformation due to bound Ca^{2+} (the high mobility form) and on the other hand an identical molecule, but without this conformational restraint due to the loss of Ca^{2+} (the low mobility form). This theory predicts that the removal of bound Ca^{2+} should accomplish the conversion of the higher to the lower mobility form and this transition was indeed induced by the treatment of fragment $\text{D}_{\text{Ca}^{2+}}$ with EDTA or by its incubation under denaturing conditions for a period of 24h. Thus each mobility form of fragment $\text{D}_{\text{Ca}^{2+}}$ may be the same molecule - a proposal which is confirmed by the examination of the reduced chain composition of fragment $\text{D}_{\text{Ca}^{2+}}$ samples. Two mobility forms of the intact molecule produced only one molecular weight form of δ chain.

To summarise, a model for the fragment $\text{D}_{\text{Ca}^{2+}}$ molecule

is proposed in which Ca^{2+} is bound towards the COOH-terminal region of the constituent δ chain. The conformational restraint imposed by the bound ion renders fragment $\text{D}_{\text{Ca}^{2+}}$ resistant to further degradation by plasmin and furthermore is responsible for the anomalously high electrophoretic mobility displayed by the molecule.

This reasoning implies that the Ca^{2+} is bound and able to exert its conformational effect on fragment $\text{D}_{\text{Ca}^{2+}}$ even after treatment with urea and SDS prior to electrophoresis. It could be argued that the forces between Ca^{2+} and the fragment $\text{D}_{\text{Ca}^{2+}}$ molecule would have to be exceedingly strong to resist the denaturing action of these two agents and therefore that the explanation of the observed effect of EDTA on the electrophoretic mobility of fragment $\text{D}_{\text{Ca}^{2+}}$ is unlikely to be correct. However studies investigating the scheme of fibrinogen digestion in the presence of 4 M-urea (i.e. an identical concentration of urea to that employed during electrophoresis) revealed that Ca^{2+} may indeed affect the susceptibility of fibrinogen to plasmin under strongly denaturing conditions.

To conclude, the heterogeneity, both in molecular weight and electrophoretic mobility, of fragment D preparations reported by various authors may be attributed to two distinct, but not unrelated, effects of Ca^{2+} on digestion and molecular conformation.

This model of the fragment $\text{D}_{\text{Ca}^{2+}}$ molecule requires

further consideration and several aspects will be investigated in the following Section.

SECTION 6

The effect of Ca^{2+} on the electrophoretic mobility of the δ chain constituent of fibrinogen and fragment D

6.1 Introduction

The results presented in Section 5 support the proposal of Haverkate & Timan (1977) that Ca^{2+} is intimately involved in the production of a high molecular weight form of fragment D by plasmin from fibrinogen. A model was proposed whereby Ca^{2+} was bound to the δ chain of fragment D_{Ca²⁺} and the conformation of the chain altered as a consequence. This effect was envisaged to account for the anomalous electrophoretic mobility displayed by fragment D_{Ca²⁺}. The purpose of the experiments which follow was to establish whether the δ chain of fibrinogen and of fragment D_{Ca²⁺} also displayed this characteristic dual mobility.

6.2 Methods

6.2.1 Preparation of fibrinogen

The fibrinogen solutions termed fibrinogen-Blank, fibrinogen- CaCl_2 and fibrinogen-EDTA were prepared as described in Section 5.2.1.

6.2.2 Fibrinogen digestion

The preparation of plasmin and the manner of fibrinogen

digestion followed identical procedures to those described in Sections 5.2.2 and 5.2.3.

6.2.3 Preparation of reduced samples for SDS-polyacrylamide gel electrophoresis

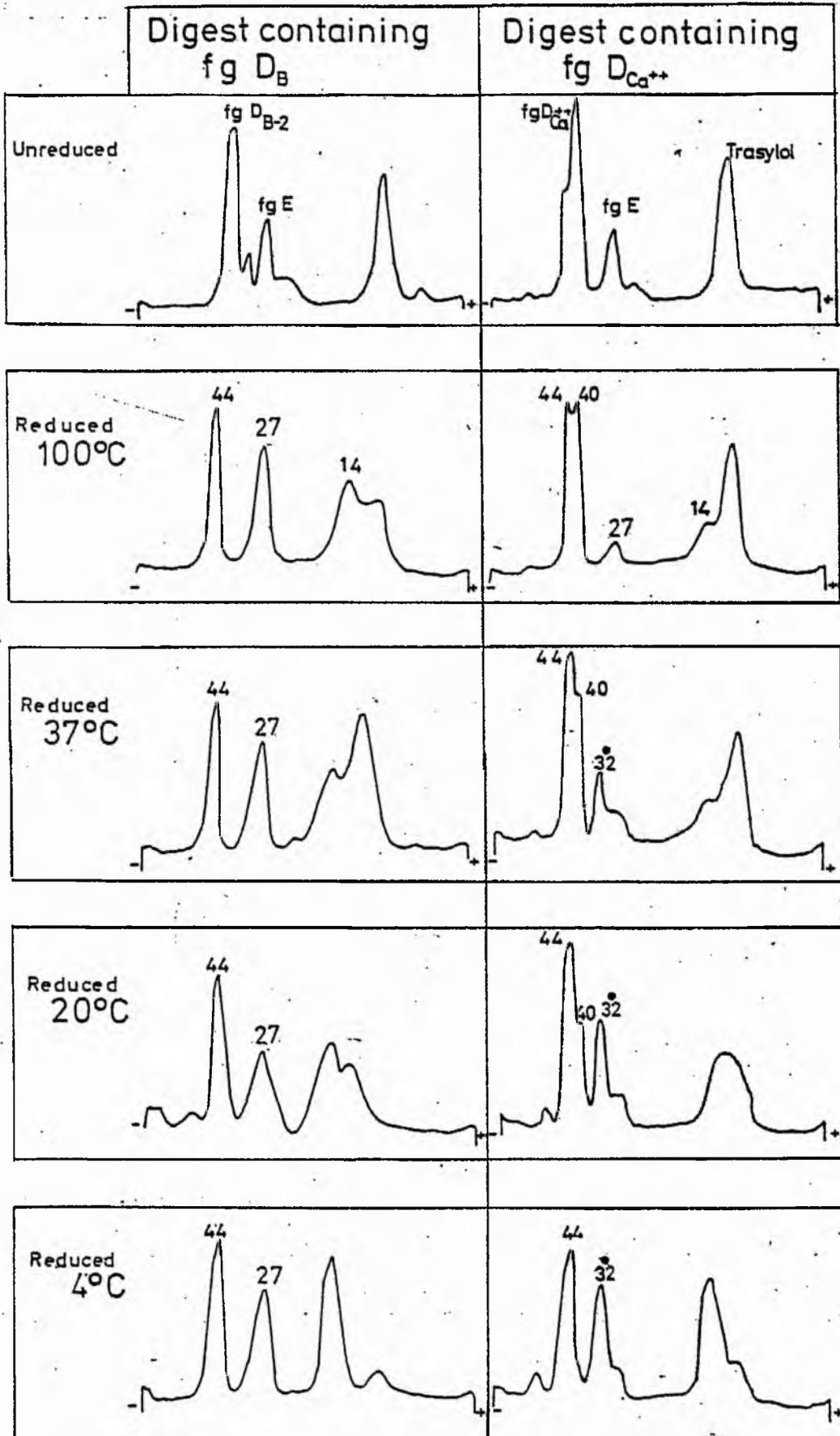
The method of preparing a reduced sample for electrophoresis was varied. Samples were reduced at one of four temperatures; 100°C, 37°C, 20°C or 4°C. The method of reduction at 100°C was identical to the procedure hitherto routinely employed viz. the sample was mixed with an equal volume of 8 M-urea, 3% (w/v) SDS and 3% (v/v) 2-mercaptoethanol and then incubated at 100 C for 5 min. Samples reduced at 37°C, 20°C or 4°C were mixed with an equal volume of the same solution but containing 2%, not 3%, (v/v) 2-mercaptoethanol and were then incubated at the appropriate temperature for 30 minutes.

The effect of EDTA on a reduced sample was investigated as follows. After the 30 minute incubation period the sample was split into two aliquots. To one was added EDTA (final concentrations will be detailed where appropriate) and to the other was added an equivalent volume of buffer (0.05 M-Tris/HCl buffer, pH 7.5). Both aliquots were maintained at 20°C for 10 minutes before application to the SDS-polyacrylamide gel surface.

Fig. 6.1 SDS-polyacrylamide gel electrophoresis
of fragment D_{Ca}2+ and fragment D_B-
containing fibrinogen digest samples

Samples of fibrinogen digests containing either fragment D_{Ca}2+ or fragment D_B were examined unreduced on 5% gels or, following their reduction at 100°C, 37°C, 20°C or 4°C, on 10% gels. The apparent molecular weights ($\times 10^{-3}$) are shown. The additional high mobility peak in the reduced fragment D_{Ca}2+ - containing samples is indicated (●).

Fig 6.1



6.2.4 Preparation of fibrinogen fragment E

A fragment E preparation isolated in Part A, Section 5.2.3 and then freeze-dried was reconstituted in 0.05 M-Tris/HCl buffer, pH 7.5 (0.3mg/ml). Two 1ml samples were removed. One was dialysed against this same buffer, the other against this buffer containing 2 mM-CaCl₂.

6.3 Results

6.3.1 The reduced subunit composition of fragments D_{Ca²⁺} and fragments D_B

Fragments D_{Ca²⁺} and D_B prepared by digestion of fibrinogen were reduced at (i) 100°C (ii) 37°C (iii) 20°C and (iv) 4°C. The densitometer scans obtained following SDS-polyacrylamide gel electrophoresis (10% gels) of each of these samples are presented in Fig. 6.1 along with the scans corresponding to each fragment D sample examined unreduced, on a 5% gel. The patterns displayed by each of the fragments D following their reduction at 100°C are similar to those reported in Section 5. Fragment D_{Ca²⁺} is composed of a β chain (MW 44,000) a γ chain (MW 40,000) and an α chain (MW 14,000). Fragment D_B consists of a β and an α chain of similar molecular weights to those of fragment D_{Ca²⁺} while its constituent γ chain displays a molecular weight of 27,000. The results of Section 5 identify this fragment as fragment D_{B-2}. Neither the relative intensity

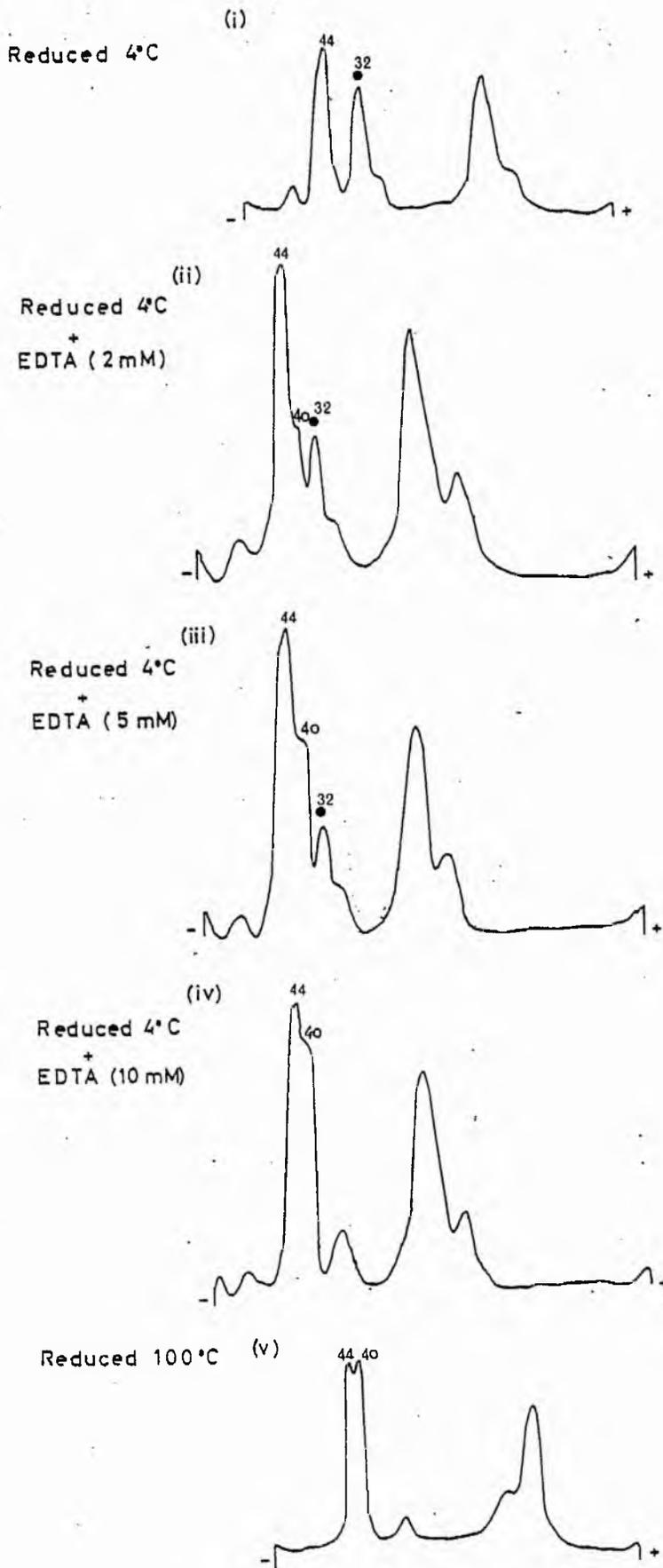
nor the number of these reduced chain components was altered upon reduction of the sample at temperatures ranging from 100°C to 4°C. The α chain remnant and the reduced chains of fragment E are obvious as bands located near the bottom of the gel. (Variations in the relative intensities of the peaks within this region of the gel are obvious in both fragment $D_{Ca^{2+}}$ and D_B -containing samples. Experiments to be described later in this Section indicated that this changing pattern of high mobility peaks is related to the effect of the temperature of reduction on fragment E).

The densitometric scans corresponding to the reduced chain patterns of fragment $D_{Ca^{2+}}$ -containing samples reveal that while the peak attributed to the β chain component is unaffected by varying the temperature of reduction that of the δ chain (MW 40,000) decreases in intensity as the temperature of reduction is lowered. This change is accompanied by a parallel increase in a hitherto undemonstrated peak of apparent molecular weight 32,000. This peak (denoted (•) in Fig. 6.1) is obvious in the sample reduced at 4°C to the complete exclusion of the δ (MW 40,000) chain peak. These results suggest that there is an 'inverse relationship' between the amount of the δ -40,000 peak and the 32,000 molecular weight peak. Therefore, the two peaks may represent two conformationally different forms of the one fragment $D_{Ca^{2+}}$ δ chain.

Fig. 6.2 The effect of EDTA on the reduced chain pattern of fragment D_{Ca²⁺}

Four samples of a fibrinogen digest containing fragment D_{Ca²⁺} were reduced at 4°C. Sample (i) was examined without further treatment while EDTA was added to the remaining samples to a final concentration of (ii) 2 mM, (iii) 5 mM, (iv) 10 mM prior to SDS-gel electrophoresis. A further digest sample (v) was reduced at 100°C. Each of the samples was examined on 10% polyacrylamide gels and the corresponding densitometer scans are shown. The apparent molecular weights ($\times 10^{-3}$) of the various peaks are indicated. The additional high mobility peak is indicated (●).

Fig. 6.2



6.3.2 The effect of EDTA on the reduced chain pattern of fragments $D_{Ca^{2+}}$ and D_{EDTA}

Further studies investigating the effect on the reduced chain pattern of fragment $D_{Ca^{2+}}$ of the chelating agent EDTA, implicate the involvement of Ca^{2+} in the dual mobility demonstrated for the fragment $D_{Ca^{2+}}$ γ chain.

Three samples of fragment $D_{Ca^{2+}}$ which had been reduced at $4^{\circ}C$ were treated with EDTA to a final concentration of 2 mM, 5 mM or 10 mM. After an incubation period of 10min at $20^{\circ}C$ each sample was examined by SDS-polyacrylamide gel electrophoresis (10% gels). The corresponding densitometer scans are shown in Fig. 6.2 (ii)-(iv). With increasing concentration of EDTA the reduced chain pattern of a sample which had not been exposed to EDTA (scan (i)) gradually reverts to that produced by reduction of fragment $D_{Ca^{2+}}$ at $100^{\circ}C$ (scan (v)). The additional peak, labelled (●) immediately below the γ -40,000 peak, decreases in intensity with increasing levels of EDTA and, in the presence of 10 mM EDTA (scan (iv)), is no longer apparent. This decrease in peak height is accompanied by the intensification of the γ -40,000 peak. The difference in apparent molecular weight between these two peaks is 8,000.

The above experimental procedure was repeated using a fragment D_B sample. The results are presented in

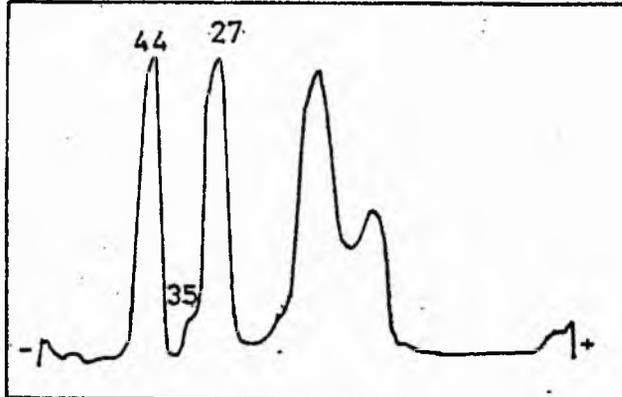
Fig. 6.3 The reduced chain pattern of fragment D_B

Four samples of a fibrinogen digest containing fragment D_B were examined by SDS-polyacrylamide gel electrophoresis following their reduction at (i) 4°C, (ii) 100°C, (iii) 4°C in the presence of 5 mM-CaCl₂ and (iv) 4°C but EDTA was added to this sample (final concentration 10 mM) prior to electrophoresis. The corresponding densitometer scans are shown. The apparent molecular weights ($\times 10^{-3}$) are indicated.

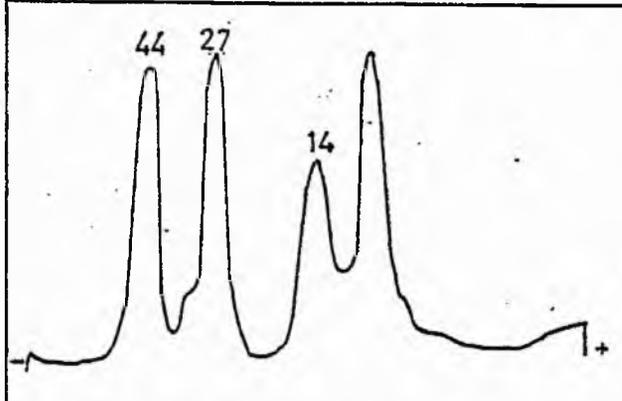
Fig 6.3

fragment D_B

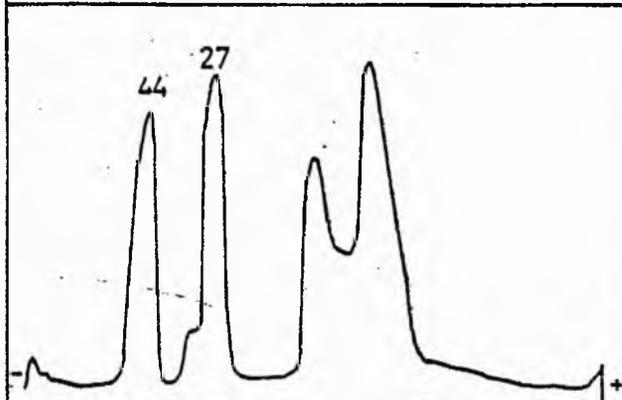
(i)
Reduced at 4°C



(ii)
Reduced at 100°C



(iii)
Reduced at 4°C
in the presence
of 5 mM-CaCl₂



(iv)
Reduced at 4°C
and then EDTA
added (10 mM)

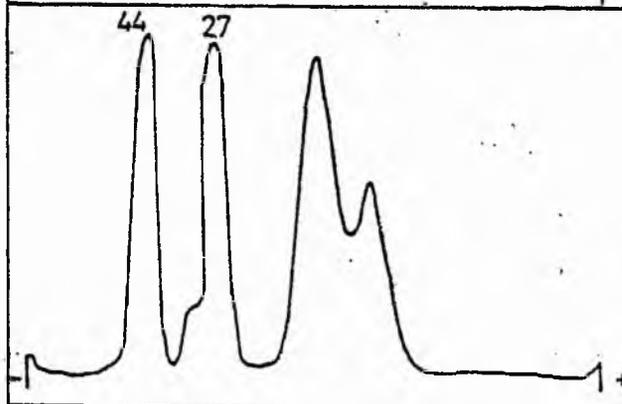
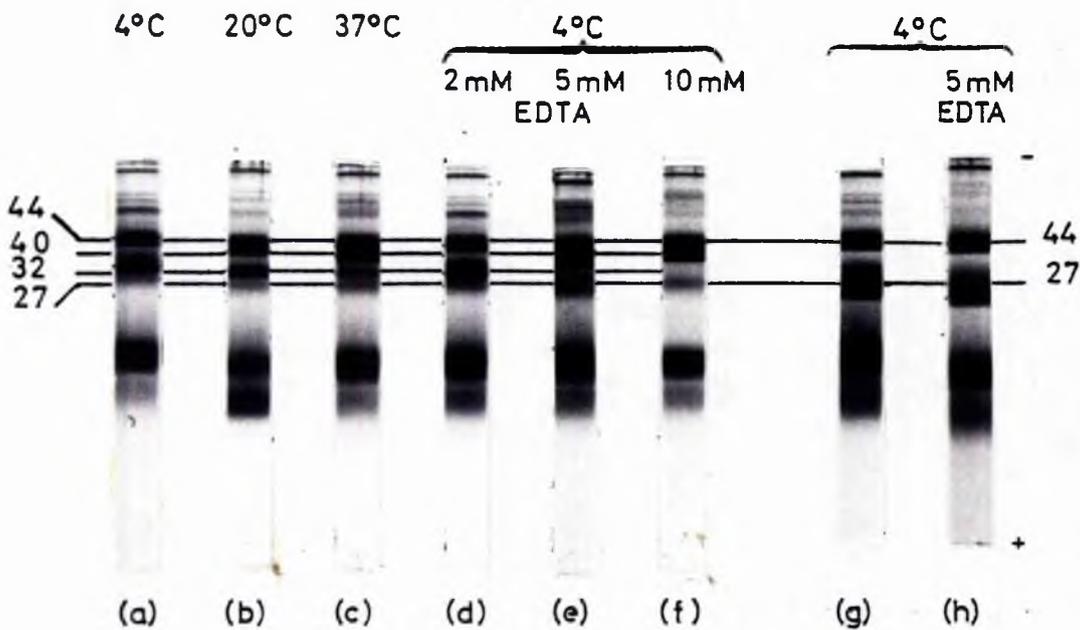


Fig. 64 The reduced chain patterns of fragments

$D_{Ca^{++}}$ and D_B



Samples of fibrinogen digests containing either fragment $D_{Ca^{++}}$ (gels (a) - (f)) or fragment D_B (gels (g) and (h)) were examined by SDS-polyacrylamide gel electrophoresis following their reduction at one of three temperatures as shown. EDTA was added to samples (d)-(f) and (h), final concentrations detailed above, prior to electrophoresis. Apparent molecular weights ($\times 10^{-3}$) are indicated. (10% gels).

Fig. 6.3. The pattern of reduced chains obtained by reduction of fragment D_B at (i) 4°C and (ii) 100°C was identical to that obtained at (iv) 4°C following the addition of EDTA. Furthermore the detection of an extra peak upon examination of a fragment $D_{Ca^{2+}}$ sample reduced at 4°C cannot be attributed to some non-specific effect of Ca^{2+} in the sample solution. The addition of excess Ca^{2+} to a sample of fragment D_B , which was then reduced at 4°C , did not promote the appearance of an additional peak (Fig. 6.3 (iii)).

The results of the two foregoing experiments are summarised in Fig. 6.4 (gels (a)-(f) refer to fragment $D_{Ca^{2+}}$, gels (g) and (h) to fragment D_B) which illustrates two important properties displayed by fragment $D_{Ca^{2+}}$. Reduction of this fragment at low temperatures promotes two effects (i) the disappearance of the band corresponding to the δ chain of molecular weight 40,000 and (ii) the parallel increase in the amount of a species of molecular weight 32,000. This second, high mobility band, could be converted to its lower mobility counterpart either by raising the temperature of reduction or by the addition of EDTA. It is therefore unlikely that the appearance of the high mobility component may be attributed to digestion of fragment $D_{Ca^{2+}}$. These findings are instead, more consistent with a model of the fragment $D_{Ca^{2+}}$ molecule in which Ca^{2+} , bound to the COOH-terminal region of the δ chain, changes the conformation of this region and thereby induces an

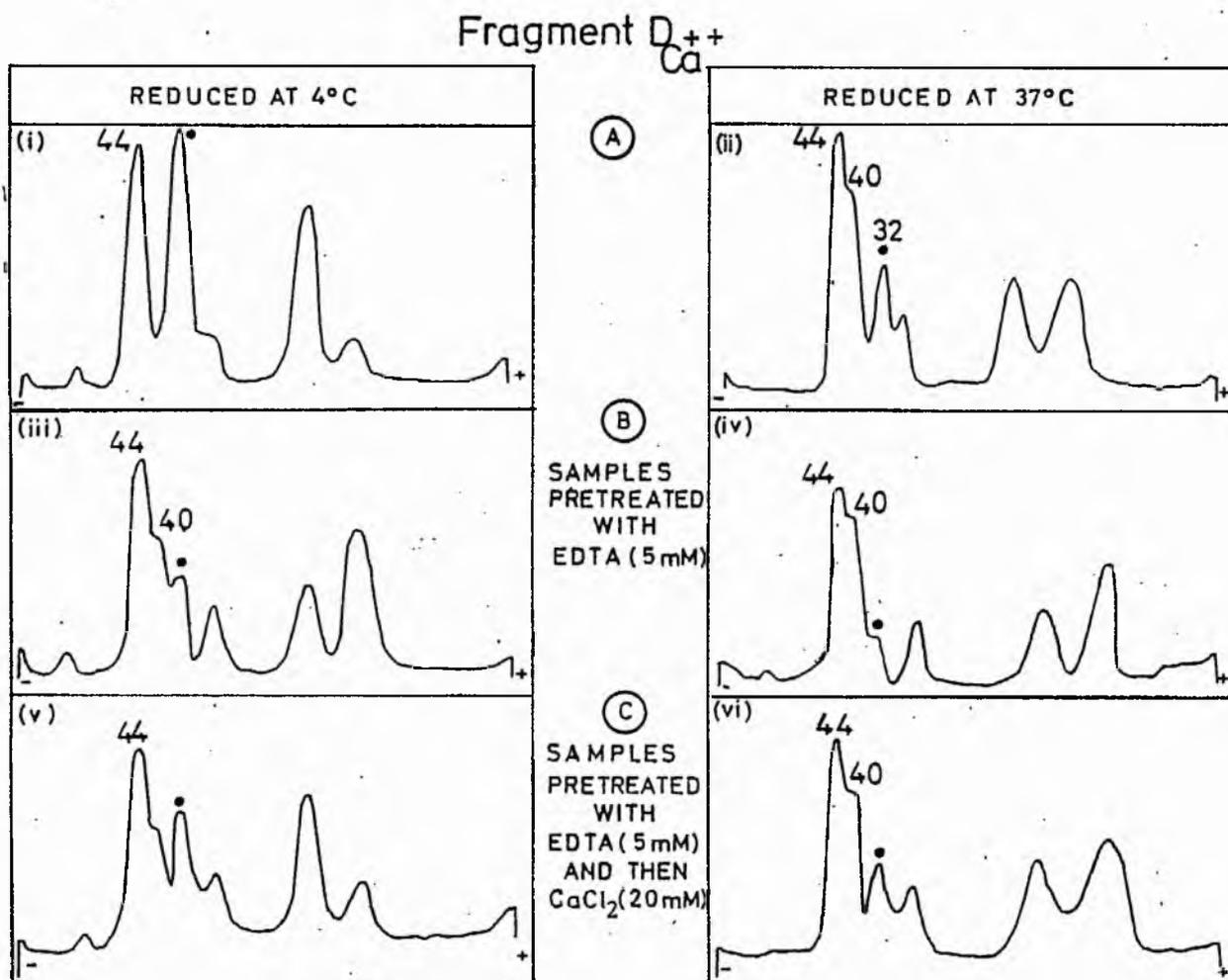
altered binding of SDS to the molecule. The subjection of fragment $D_{Ca^{2+}}$ to mild reducing conditions (i.e. low incubation temperature) may aid the preservation of this Ca^{2+} -induced conformational form of the δ chain which would, due to its anomalous SDS-binding, display an uncharacteristically high electrophoretic mobility. The δ chain would show its "true", lower electrophoretic mobility, following the disruption of the conformational constraint induced by bound Ca^{2+} either by raising the temperature during reduction or by the exposure of the molecule to the calcium chelating agent, EDTA.

Several aspects of this proposed model for the fragment $D_{Ca^{2+}}$ molecule are considered in greater detail in the Discussion of this Section.

6.3.3 The ability of fragment $D_{Ca^{2+}}$ to rebind Ca^{2+}

The nature of the binding of Ca^{2+} to fragment $D_{Ca^{2+}}$ was investigated by examining the electrophoretic pattern displayed by an EDTA-treated fragment $D_{Ca^{2+}}$ sample which was then incubated in the presence of an excess of Ca^{2+} . The model proposed above for the fragment $D_{Ca^{2+}}$ molecule infers that the extra, high mobility peak demonstrated upon electrophoresis of a sample of fragment $D_{Ca^{2+}}$ which has been reduced at $4^{\circ}C$, represents the constituent δ chain in a conformationally altered form as a consequence of

Fig. 6.5 The effect of EDTA and CaCl_2 on the reduced chain
electrophoretic pattern of fragment $\text{D}_{\text{Ca}^{++}}$



Fragment $\text{D}_{\text{Ca}^{++}}$ samples - A, B and C (B and C were pretreated as shown) were examined, following reduction at 4°C and 37°C, on 10% SDS polyacrylamide gels. The additional, higher mobility γ chain peak is indicated (●). Apparent mol. wts. ($\times 10^{-3}$) are shown.

bound Ca^{2+} . Therefore the "reappearance" of this high mobility δ chain component in an EDTA-treated fragment $\text{D}_{\text{Ca}^{2+}}$ sample after subsequent exposure to excess Ca^{2+} would infer that the binding of Ca^{2+} to fragment $\text{D}_{\text{Ca}^{2+}}$ is reversible.

The results of this investigation are presented in Fig. 6.5. An aliquot (sample A) was removed from a fibrinogen digest sample containing fragment $\text{D}_{\text{Ca}^{2+}}$. The remainder was incubated in the presence of 5 mM-EDTA for 4h and a sample (B) removed. To the residue (sample C) was added CaCl_2 (final concentration 20 mM). Each sample was examined on 10% SDS-polyacrylamide gels following reduction at 4°C and 37°C . Samples A and B produced the typical pattern described earlier for fragment $\text{D}_{\text{Ca}^{2+}}$. The "extra" form of the δ chain is marked (\bullet). The intensity of this peak was decreased either by raising the temperature of reduction or by the addition of EDTA (Sample A, gels (i) and (ii); Sample B, gels (iii) and (iv)). The results from sample C (gels (v) and (vi)) imply that this EDTA-induced effect may be reversed by the addition of Ca^{2+} i.e. that fragment $\text{D}_{\text{Ca}^{2+}}$ may indeed rebind Ca^{2+} . The levels of the high mobility form of the δ chain have been partially restored following the addition of excess Ca^{2+} . However this conclusion

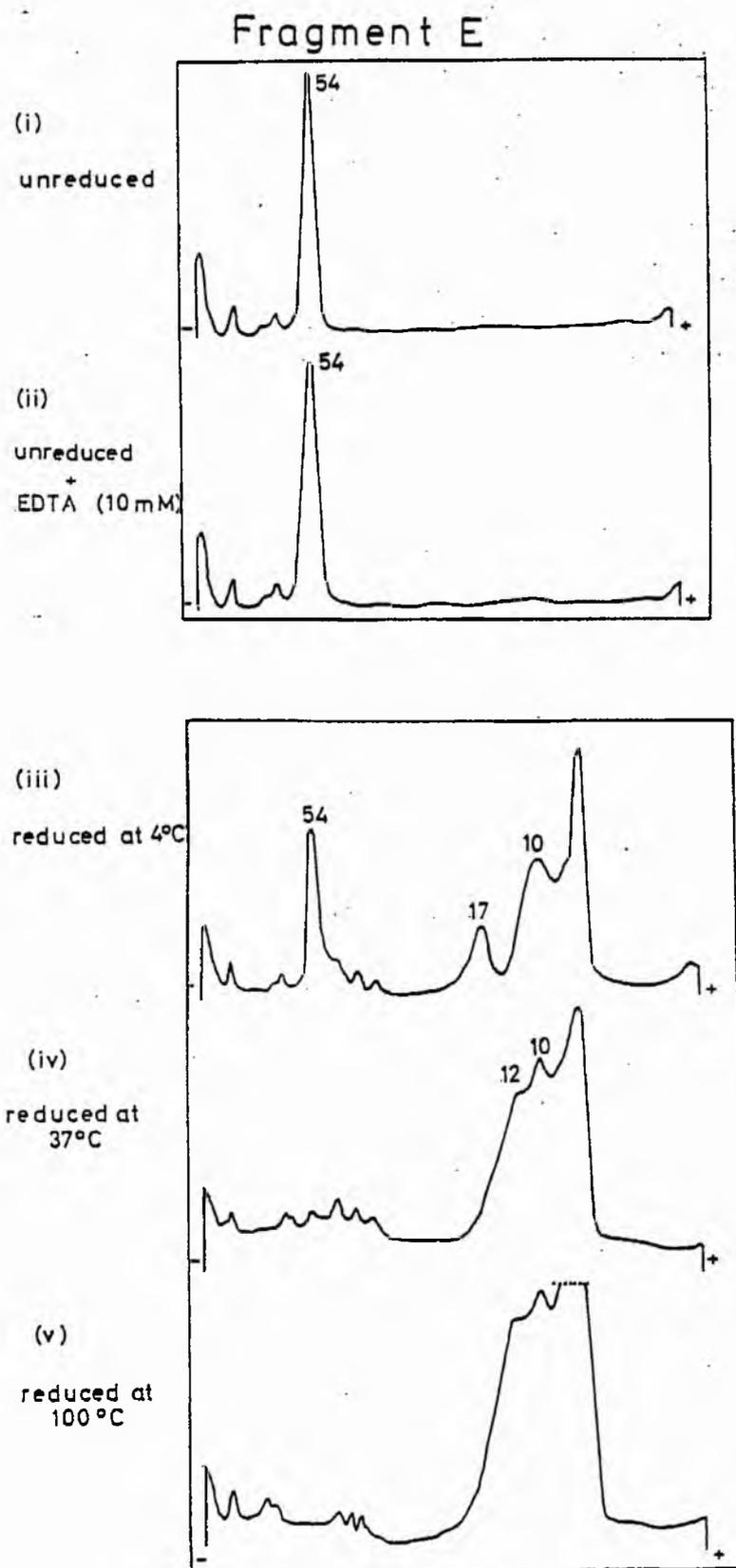
may be disputable. Inspection of Fig. 6.5 reveals that although the conversion of the high mobility form of the δ chain to the lower form was promoted either by raising the reduction temperature or by the addition of EDTA neither treatment alone promoted a complete transition, (Fig. 6.5 (ii) and (iii)). Nevertheless the combined effect of these treatments (gel(iv)) caused an almost complete conversion of the high to the low mobility peak and it seems probable therefore that part of the action of EDTA occurs during the reduction process at 37°C. Thus with reference to the proposal that fragment $D_{Ca^{2+}}$ is able to rebind Ca^{2+} , the addition of excess Ca^{2+} to an EDTA-treated fragment $D_{Ca^{2+}}$ sample may not promote the reassociation of Ca^{2+} with the molecule but more simply reduce the effective concentration of EDTA during the reduction incubation period and thereby preserve the high mobility form of the δ chain.

6.3.4 Experiments with fragment E

It must be borne in mind that the foregoing experiments have employed fibrinogen digest samples. Conditions of digestion were selected to ensure the maximal production of fragment D with minimal contamination from higher molecular weight digestion products. Nevertheless the possibility that the

Fig. 6.6 SDS-polyacrylamide gel electrophoresis of fragment E

Fragment E was examined (i) unreduced, (ii) unreduced but following the addition of EDTA and also following reduction at (iii) 4°C, (iv) 37°C and (v) 100°C. App. mol. wts ($\times 10^{-3}$) are shown. (10 % gels).



presence within the digest samples of lower molecular weight species, particularly fragment E, might complicate the interpretation of polyacrylamide gel electrophoresis results must be considered. To this end a pure fragment E preparation was examined by SDS-gel electrophoresis (unreduced) both before and after treatment with EDTA. In addition, samples were reduced at 4°C, 37°C and 100°C. In each case the concentration of fragment E was approximately four times that present in previous digest samples. The densitometric scans corresponding to each of these five gels are presented in Fig. 6.6.

The fragment E sample displayed three peaks upon reduction at 100°C of apparent molecular weights 12,000, 10,000 and less than 10,000. Values of 10,000, 9,000 and 7,000 have been reported for the three types of fragment E constituent chains (Kowalska-Loth et al., 1973). However the present molecular weight values are inaccurate. The polyacrylamide gels were purposefully overloaded, consequently the protein bands were broad and poorly resolved. Furthermore the calculated mobilities are outwith the range covered by the molecular weight standards. Be that as it may the most important conclusion deduced from Fig. 6.6 is that no significant peaks are evident within the gel mobility region appropriate to the fragment D_{Ca}²⁺ β and δ

chains in any of the reduced fragment E samples.

Thus it is unlikely that the extra protein band credited to a conformationally altered form of the fragment $D_{Ca^{2+}}$

δ chain might instead be attributable to some stage of the fragment E reduction process. A peak with an apparent molecular weight of 17,000 is obvious in the sample of fragment E reduced at 4°C (scan (iii)).

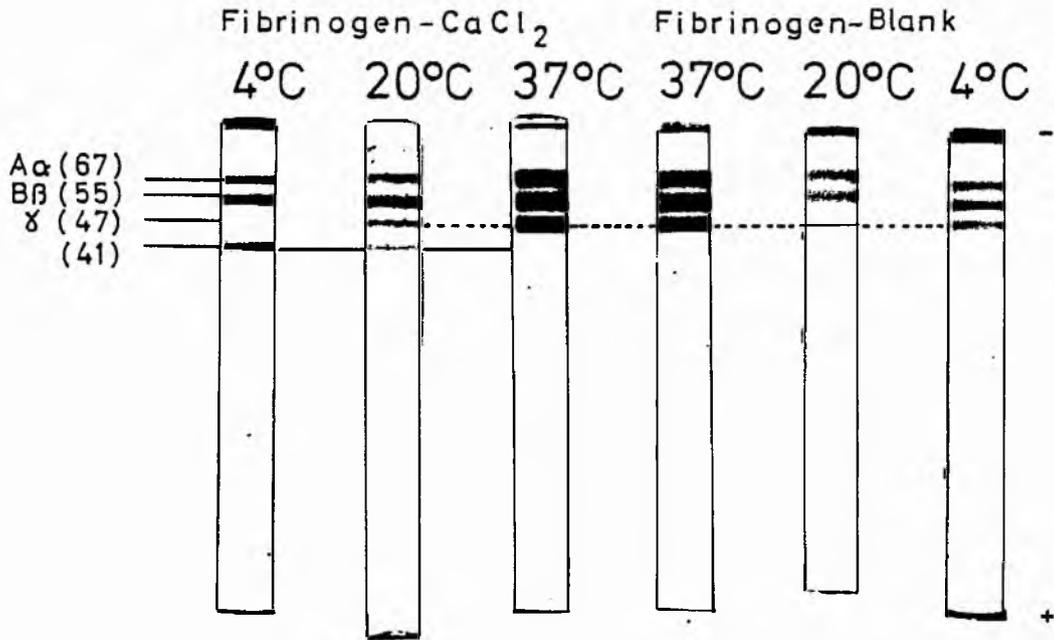
This peak may correspond to an incompletely reduced product of fragment E - a proposal consistent with the observed "disappearance" of this peak upon raising the temperature of reduction. Similar changes in the pattern of peaks within this high mobility gel region were noted during the analysis of fibrinogen digest samples which had been reduced at various temperatures (Section 6.2.1). No effect of EDTA on the mobility of the intact fragment E is apparent (scans (i) and (ii)).

It is worth stressing here that, for the reasons given above, the molecular weight value ascribed throughout this Section to the fragment $D \alpha$ chain cannot be quoted with confidence.

6.3.5 The subunit composition of fibrinogen- $CaCl_2$, fibrinogen-EDTA and fibrinogen-Blank

The possibility that the constituent δ chain of fibrinogen- $CaCl_2$ might also display a dual electrophoretic mobility was investigated as follows.

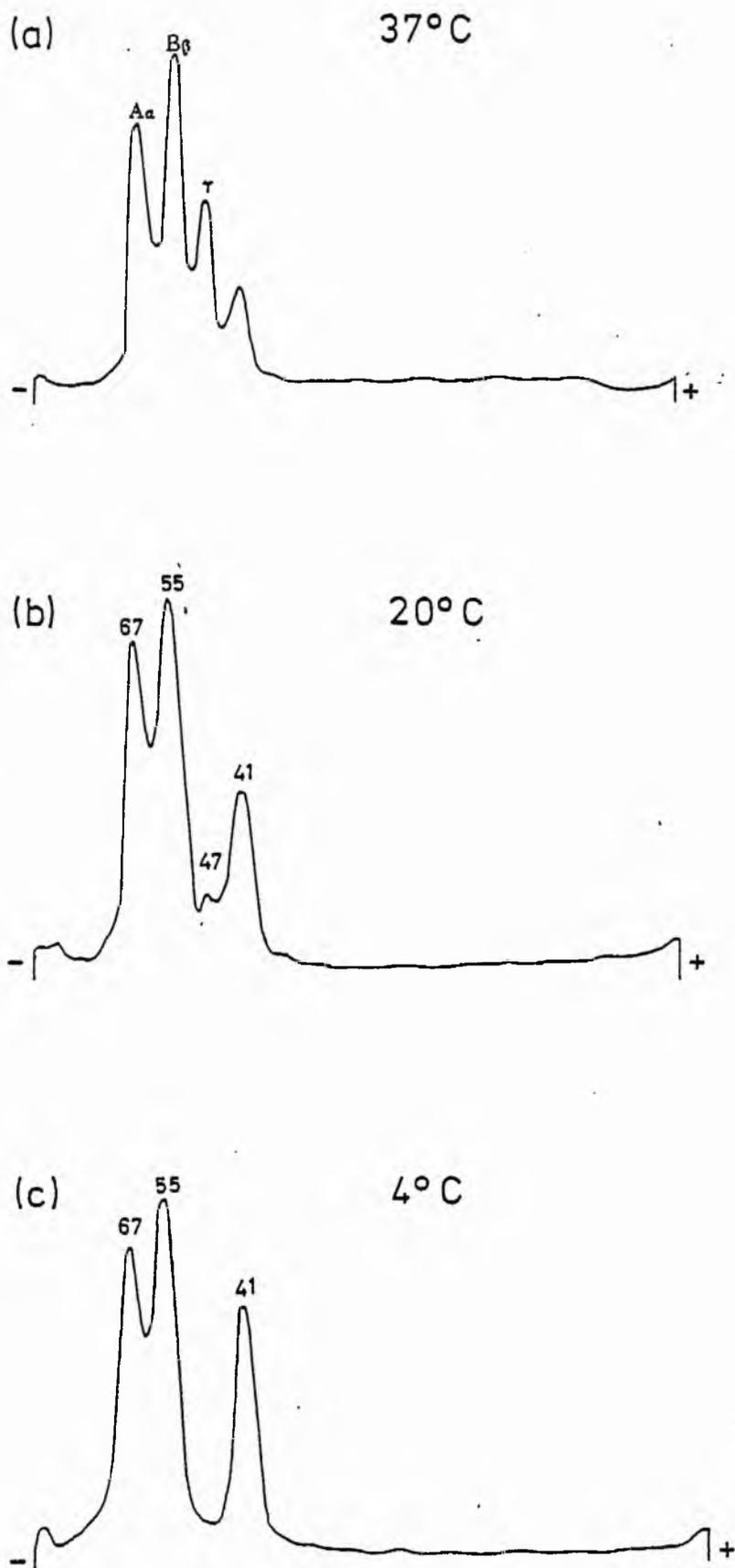
Fig. 6.7 SDS-gel electrophoresis of fibrinogen- CaCl_2 and
fibrinogen-Blank



Fibrinogen- CaCl_2 and fibrinogen-Blank solutions were reduced at one of three temperatures (4°C, 20°C or 37°C) and then examined by SDS-gel electrophoresis on 10% polyacrylamide gels. The apparent molecular weights ($\times 10^{-3}$) of the $A\alpha$, $B\beta$ and γ subunit chains are shown in brackets.

Fig. 6.8 SDS-polyacrylamide gel electrophoresis of reduced samples of fibrinogen-CaCl₂

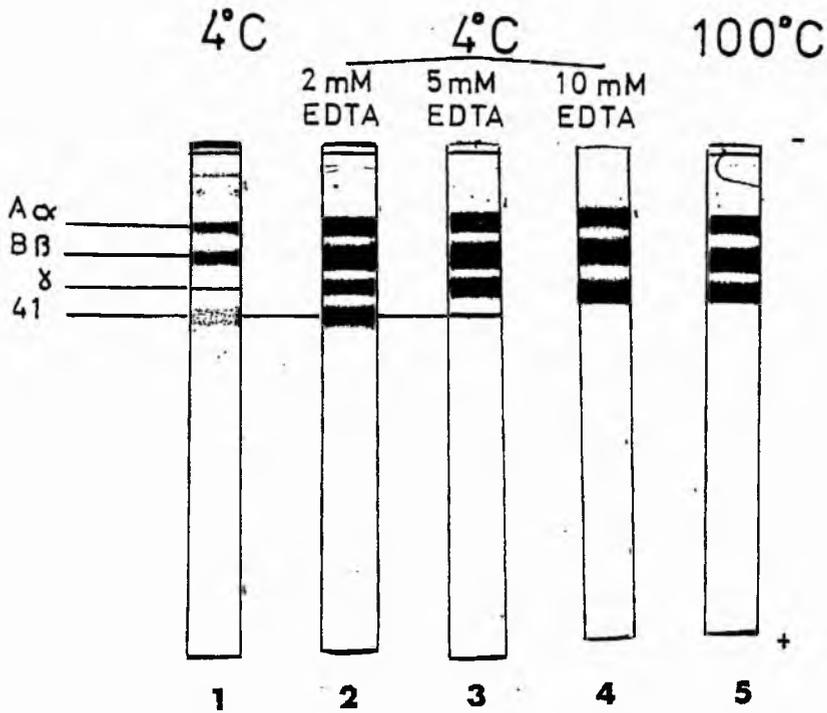
Fibrinogen-CaCl₂ was reduced at (a) 37°C (b) 20°C and (c) 4°C and then examined on 10% gels. The corresponding gel scans are shown. Apparent molecular weights ($\times 10^{-3}$) are indicated.



Fibrinogen-CaCl₂ and fibrinogen-Blank samples were reduced at 4°C, 20°C and 37°C and examined by SDS-gel electrophoresis on 10% polyacrylamide gels. The results are presented in Fig. 6.7. Each of the three reduced fibrinogen-Blank samples displays the characteristic three band distribution of A α , B β and δ subunit chains while the three fibrinogen-CaCl₂ samples contain four components. Three of these gel bands exhibit electrophoretic mobilities identical to those described for the fibrinogen-Blank sample while the extra fourth band has an electrophoretic mobility slightly greater than that of the fibrinogen δ chain. In addition the intensity of the δ chain peak in the fibrinogen-CaCl₂ samples is reduced in a temperature-related manner. These points are also illustrated by the three densitometer scans corresponding to the fibrinogen-CaCl₂ samples shown in Fig. 6.8. The apparent molecular weight of the high mobility component is 41,000.

The addition of EDTA prior to electrophoresis of the fibrinogen-CaCl₂ sample reduced at 4°C produced two effects. The intensity of the fourth, high mobility component decreased while that of the fibrinogen δ chain increased. These results are presented in Fig. 6.9 and suggest that the magnitude of the gel band transition from high to low mobility is related to the concentration of EDTA. In the presence of 10 mM-EDTA, the highest concentration employed, the reduced chain pattern of the

Fig. 6.9 The effect of EDTA on the reduced chain
pattern of fibrinogen-CaCl₂



Fibrinogen-CaCl₂ was reduced at 4°C and then examined by SDS-polyacrylamide gel electrophoresis without further treatment (gel 1) and after the addition of EDTA to a final concentration of 2 mM (gel 2), 5 mM (gel 3) and 10 mM (gel 4). A fifth sample of fibrinogen-CaCl₂ was also examined following reduction at 100°C (gel 5). The apparent molecular weight of the additional high mobility band is shown ($\times 10^{-3}$). (10% gels).

fibrinogen-CaCl₂ sample appears identical to that displayed by the same fibrinogen sample reduced at 100°C, and to the pattern obtained with the fibrinogen-Blank sample reduced at 4°C (Fig. 6.7).

Thus the mild reduction experiments have revealed similar properties for the δ constituent chains of fibrinogen-CaCl₂ and fragment D_{Ca²⁺}. The dual mobility displayed by the constituent δ chain of fibrinogen-CaCl₂ may therefore be attributable to the conformational restraint imposed by bound Ca²⁺.

However it is significant that the reduced chain pattern displayed by samples of fibrinogen-Blank gradually changed over a period of approximately one month from that shown in Fig. 6.7 to a pattern similar to that obtained with fibrinogen-CaCl₂ samples. The additional high mobility δ chain component being obvious upon reduction of a sample of fibrinogen-Blank at 4°C, 20°C or 37°C. A possible explanation of this observation is that the fibrinogen-Blank solution had been contaminated with Ca²⁺ and had therefore been converted to fibrinogen-CaCl₂. The preparation and widespread use of CaCl₂⁻ containing buffer solutions may have induced the necessary level of Ca²⁺ contamination of fibrinogen. This topic will be reconsidered in Division 2.

The three types of fibrinogen (fibrinogen-EDTA, -CaCl₂ and -Blank) were examined unreduced on 3% polyacrylamide gels. Identical results were obtained. The electrophoretic mobility of the intact fibrinogen

Fig. 6.10 a and b SDS-gel electrophoresis of fibrinogen after
various dialysis treatments

Fig. 6.10a

Densitometric scans of SDS-polyacrylamide gel electrophoresis separations of fibrinogen samples prepared according to the dialysis scheme of Fig. 6.10b. The additional higher mobility chain peak is indicated (●). (10% gels)

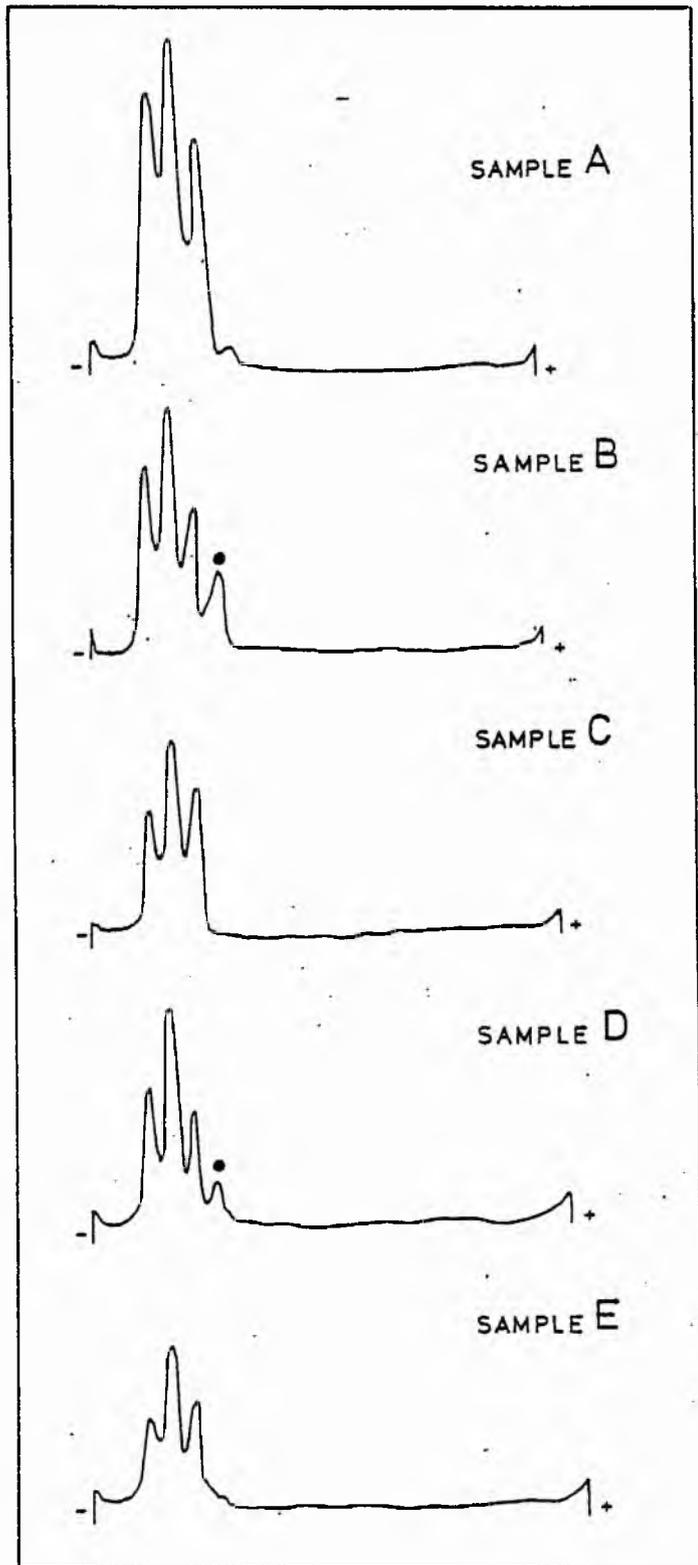
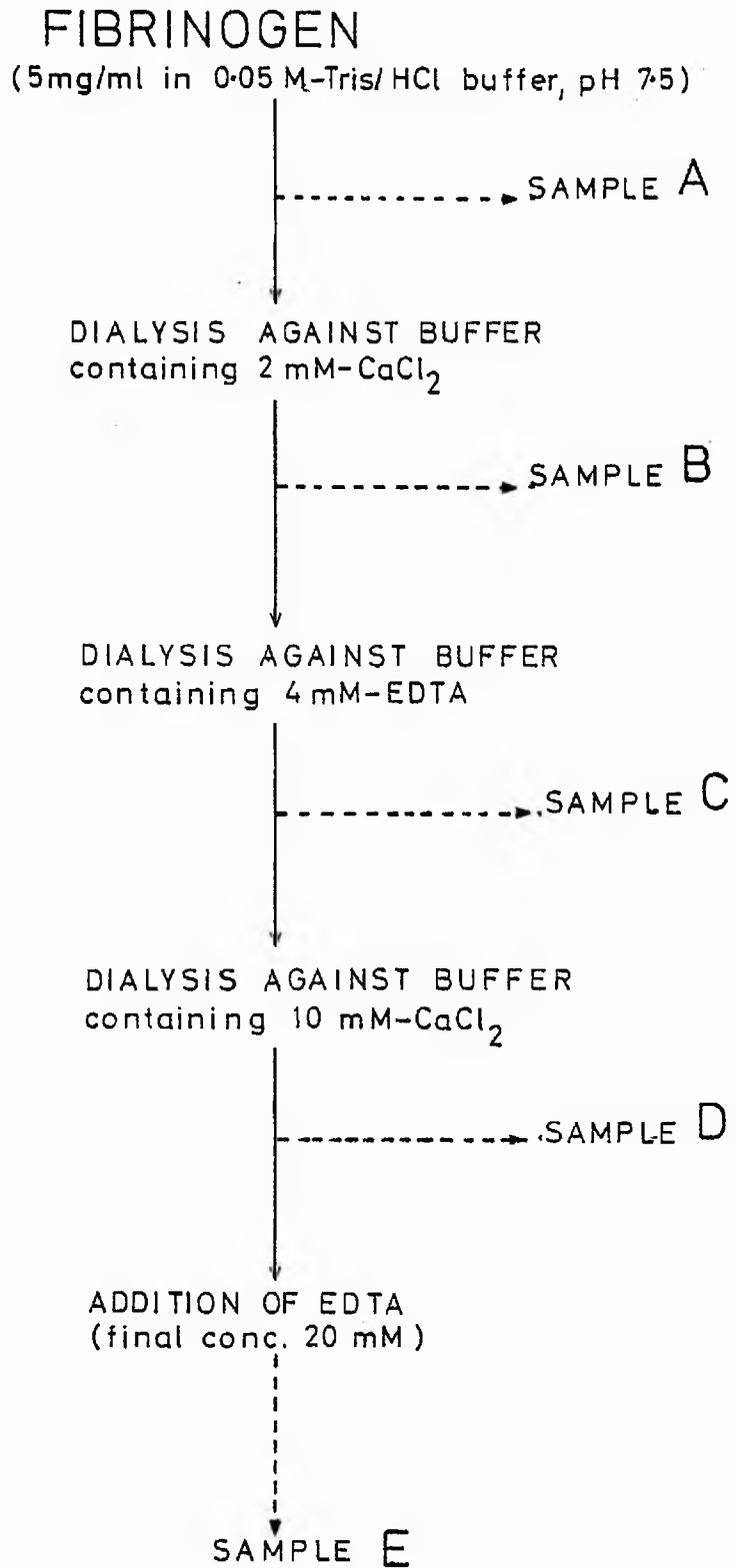


Fig. 6.10 b



Fibrinogen dialysis scheme corresponding to Fig. 6.10a

molecule does not appear to be affected by Ca^{2+} .

6.3.6 The removal and readdition of Ca^{2+} to fibrinogen

The following procedure was designed to investigate the ability of fibrinogen to rebind Ca^{2+} . An aliquot of a fibrinogen-Blank solution was subjected to a series of 24h dialysis treatments at 4°C as shown in Fig. 6.10b. Between each stage a sample was removed and, following its reduction at 37°C , examined by SDS-gel electrophoresis (10% gels). Densitometric scans corresponding to each of the samples (A-E) are shown in Fig. 6.10a. Each of the fibrinogen samples which had been exposed to an excess of Ca^{2+} exhibited the additional high mobility peak upon reduction (samples B and D). However subsequent dialysis against an EDTA-containing solution promoted the "disappearance" of the extra peak and the resulting peak pattern (samples C and E) reverted to that typical of the three fibrinogen $\text{A}\alpha$, $\text{B}\beta$ and γ chains. These results suggest that the " Ca^{2+} effect" upon the reduced chain pattern of fibrinogen is reversible. The apparent molecular weight difference between the third and fourth peaks is 6,000.

To summarise; the dual electrophoretic mobility displayed by the γ chain component of fragment $\text{D}_{\text{Ca}^{2+}}$ prepared from the parent molecule under mild reducing

conditions has also been demonstrated for the δ chain of fibrinogen- CaCl_2 . This additional component displayed a similar susceptibility to EDTA-treatment. However in the case of fibrinogen the effects of both EDTA and Ca^{2+} on the number and mobility of the fibrinogen constituent chains are readily reversible.

6.4 Discussion

A dual electrophoretic mobility has been demonstrated for the constituent δ chain of both fragment $\text{D}_{\text{Ca}^{2+}}$ and fibrinogen. In each case the additional, higher mobility, component could be converted to its higher molecular weight counterpart either by raising the temperature of incubation of the sample or by the addition of EDTA. These findings are in accordance with the model proposed in Section 5 for the fragment $\text{D}_{\text{Ca}^{2+}}$ molecule. The conformational change induced in the COOH-terminal region of the δ chain by bound Ca^{2+} may account for the properties displayed by both the intact molecule and the δ chain component i.e. the resistance to further attack by plasmin and also the EDTA-susceptible electrophoretic mobilities.

It must be conceded that by themselves the results presented from studies of the reduced chain pattern of various fibrinogen solutions do not demonstrate a

specific effect of Ca^{2+} within the structure of fibrinogen. The additional high mobility component of the reduced sample may more simply result from a non-specific action of CaCl_2 during the reduction process. However if this explanation is correct why was the effect restricted to the γ chain? The results described for fibrinogen are given credence by comparison with the studies employing fragment D. The fact that the result of the reduced chain analysis of fragment D_B was unaffected by the presence of Ca^{2+} is inconsistent with the proposal that Ca^{2+} had a non-specific action on the electrophoretic pattern. Furthermore the difference in apparent molecular weight between the two forms of γ chain for both fibrinogen and fragment $\text{D}_{\text{Ca}^{2+}}$ was 6,000-8,000. If the model of Ca^{2+} binding is correct then the site of binding of Ca^{2+} to the γ chain must be similar in both fragment $\text{D}_{\text{Ca}^{2+}}$ and fibrinogen. The digestion studies reported in Section 5 favoured the location of the Ca^{2+} -binding site towards the COOH-terminal region of the fragment $\text{D}_{\text{Ca}^{2+}}$ γ chain and these two proposals are consistent with the report that the COOH-terminus of the fibrinogen γ chain is preserved virtually intact during the fibrinogen plasmin-induced degradation process (Takagi & Doolittle, 1975).

The investigation of the nature of the binding of Ca^{2+} to fibrinogen suggested that it was reversible.

The evidence in the case of fragment $D_{Ca^{2+}}$ was inconclusive.

Finally the results reported with fragment E require corroboration. Various reduction experiments failed to reveal the presence of fragment E degradation products which might complicate the fragment D β and δ constituent chain electrophoretic pattern. However this fragment E preparation was isolated in Part A by digestion of fibrinogen in the presence of a phosphate buffer system. It is therefore unlikely that fragment E was prepared in the presence of Ca^{2+} . Later experiments to be described in Division 2 permitted the isolation of a fragment E prepared under identical conditions to those of fragment $D_{Ca^{2+}}$. The experiments with fragment E described in Section 6.3.4 were repeated and identical results obtained.

SECTION 7

DISCUSSION

The present results have confirmed the production of a single high molecular weight form of fragment D from fibrinogen in the presence of Ca^{2+} as reported by Haverkate & Timan (1977). Further substantiation of this claim has been provided by Purves et al. (1978). The difference in molecular weight between this fragment D, $\text{D}_{\text{Ca}^{2+}}$, and those fragments D_{B} prepared in the absence of Ca^{2+} has been attributed to the former containing a single, higher molecular weight form of its constituent γ chain. Haverkate & Timan (1977) demonstrated that fragment $\text{D}_{\text{Ca}^{2+}}$ could be converted to fragment D_{B} by the combined action of a chelating agent and plasmin - a finding corroborated in the present work. This transition occurred by progressive proteolysis of the constituent γ chain. Hitherto the heterogeneity of fragment D preparations has been attributed to the effect of the sequential attack of plasmin at the COOH-terminal end of the γ chain (e.g. Furlan et al., 1975). Thus the COOH-terminus of the γ chain is strongly implicated as the site of action of Ca^{2+} and it seems reasonable to suggest that Ca^{2+} binds to this region of the γ chain thereby inducing a plasmin-resistant conformation. This same phenomenon, it is proposed, may account for the anomalously high electrophoretic mobilities displayed by the fibrinogen degradation products, fragments $\text{D}_{\text{Ca}^{2+}}$ and Y, and by the

γ constituent chains of fragments $D_{Ca^{2+}}$ and fibrinogen. These mobilities were decreased by the addition of a chelating agent. The Ca^{2+} -induced effect on the conformation of the COOH-terminal region may also render the molecule less susceptible to the unfolding influence of SDS. Consequently, the fragment or chain containing bound Ca^{2+} would appear smaller and therefore migrate further.

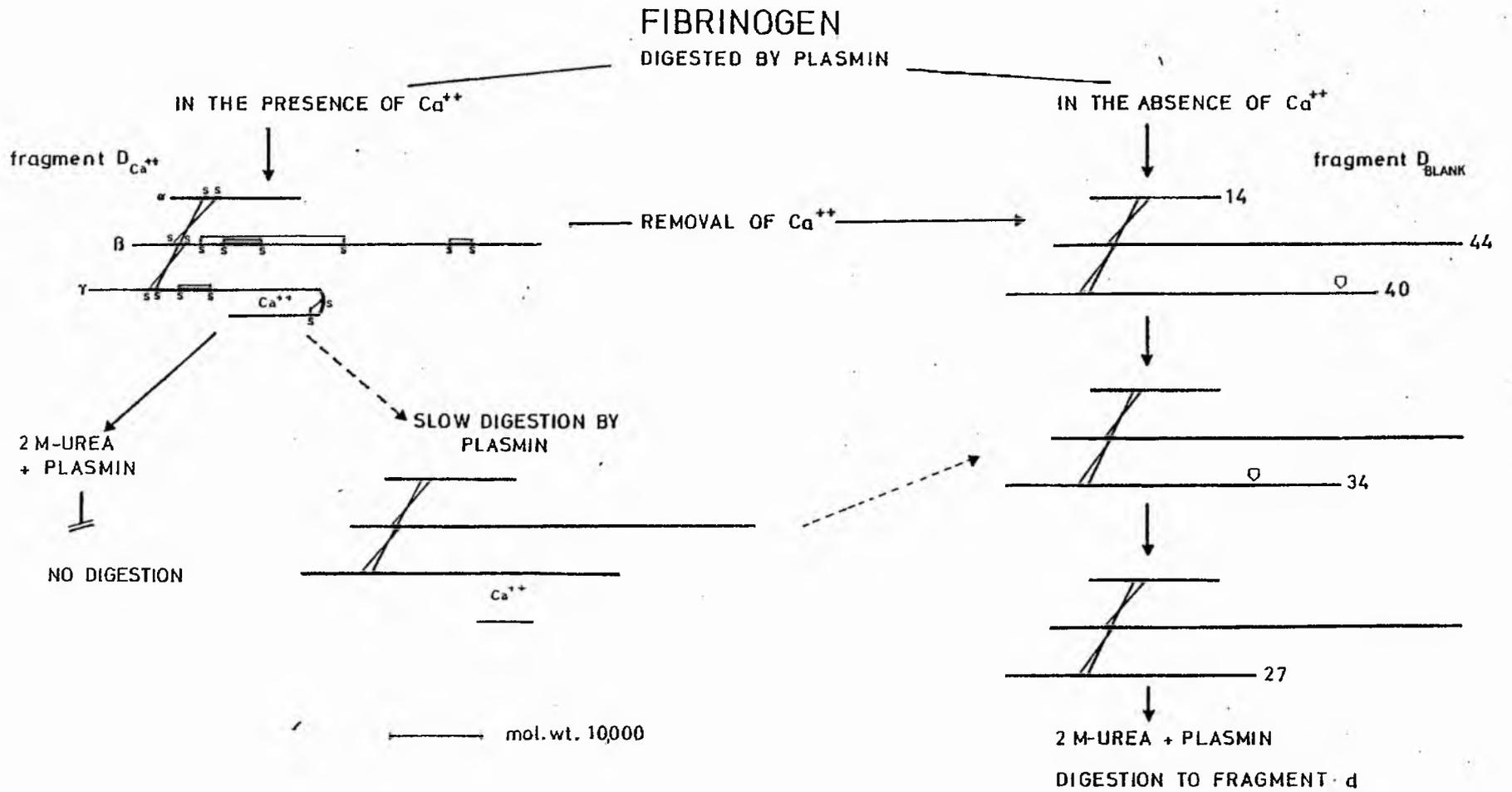
The above reasoning implies that a binding site for Ca^{2+} is located towards the COOH-terminus of the γ chain of fragment $D_{Ca^{2+}}$ and fibrinogen. This accords with recent reports by Lindsey et al. (1978) and Nieuwenhuizen et al. (1979) demonstrating the presence of three high affinity Ca^{2+} binding sites within human fibrinogen and, more significantly, the location of one of these sites within fragment $D_{Ca^{2+}}$. Both groups reported that neither fragment E nor a fragment D prepared in the absence of Ca^{2+} contained a Ca^{2+} binding site. Substantiation of the theory that the binding of Ca^{2+} induces an altered conformation of the COOH-terminal region of the γ chain is provided by the report of Haverkate & Timan (1977), later confirmed by Purves et al. (1978) in which the resistance of fragment D-dimer to digestion by plasmin was attributed, not to crosslinking as had been suggested by Ferguson et al. (1975), but to bound Ca^{2+} .

Two models for the binding of Ca^{2+} to fragment $D_{Ca^{2+}}$ have been proposed - not dissimilar to that described above. Van Ruijven-Vermeer et al. (1978) assigned a Ca^{2+}

Fig. 7.1 Schematic diagram of the effect of Ca^{++} on the digestion of fragment D

A proposed scheme for the digestion of fragment D prepared by plasminic degradation of fibrinogen both in the presence and absence of Ca^{++} is shown. The site of binding of Ca^{++} within the γ chain is indicated. Disulphide bonds are represented by the thinner lines. The positions are approximate but are based on the data of Henschen (1978) and Gardlund *et al.* (1977). Apparent molecular weights ($\times 10^3$) of the various subunit chains are shown. The proposed location of the bonds split by plasmin are indicated \square .

Fig. 7.1



binding site to a 13,000 molecular weight region at the COOH-terminus of the δ chain of fragment D while Purves *et al.* (1978a) suggested that Ca^{2+} "held down a δ chain loop" of about 100 residues from the COOH-terminal end on a stable α and β chain base.

In Fig. 7.1 a scheme is presented depicting the protective effect of Ca^{2+} during the degradation of fragment D by plasmin. The proposed arrangement of an intrachain Ca^{2+} -bridge within the δ chain of fragment $\text{D}_{\text{Ca}^{2+}}$ is shown. This molecular conformation corresponds to the high electrophoretic mobility form while the structure produced following the action of EDTA represents the low mobility form. No degradation of the constituent chains is envisaged during this transition however the low mobility form is rendered susceptible to the action of plasmin as a consequence.

Fig. 7.1 illustrates the composition of a third type of fragment $\text{D}_{\text{Ca}^{2+}}$; here limited degradation of the δ chain has occurred despite the presence of Ca^{2+} , however the COOH-terminal peptide produced is not released but remains attached to the rest of the fragment $\text{D}_{\text{Ca}^{2+}}$ molecule by the Ca^{2+} - bridge. Evidence of the existence of this fragment $\text{D}_{\text{Ca}^{2+}}$ was provided by studying the effect of EDTA on the electrophoretic mobility of fragment $\text{D}_{\text{Ca}^{2+}}$. A small component of the fragment $\text{D}_{\text{Ca}^{2+}}$ band did not exhibit the characteristic EDTA-induced mobility decrease and, instead,

displayed a mobility greater than either of the other fragment $D_{Ca^{2+}}$ species. Similarly reduction of fragment $D_{Ca^{2+}}$ produced a faint band (immediately below that of the γ chain) whose mobility was unaffected by EDTA. Each of these observations may be accounted for by this third representation of the fragment $D_{Ca^{2+}}$ molecule. Therefore fragment $D_{Ca^{2+}}$ may not be completely resistant to the action of plasmin and digestion of the molecule may occur; specifically within the Ca^{2+} -induced γ chain loop. This proposal is consistent with the finding that the resistance of fragment $D_{Ca^{2+}}$ to digestion by plasmin in the presence of 2 M-urea may not be as complete as suggested by Haverkate & Timan (1977).

It should be noted in passing that the scheme presented in Fig. 7.1 assumes that the degradation of fibrinogen in the absence of Ca^{2+} proceeds first to a third, higher molecular weight form of fragment D_B . Since only two lower molecular weight fragment D_B forms were detected it must be proposed that this initial form of fragment D_B is rapidly degraded by plasmin to the relatively plasmin-resistant lower molecular weight forms.

Several points arising from the foregoing results sections warrant further discussion. First, it must be stressed that the investigation of fragment D employed fibrinogen digest samples and, although steps were taken

to overcome any possible complication of the results by additional digest components, the possibility that the electrophoretic results were affected by such contaminants cannot be ruled out.

Secondly, it must be considered whether the observed anomalous electrophoretic mobilities are due, not to the consequence of the conformational restraint imposed on the molecule by bound Ca^{2+} , but to some non-specific effect of Ca^{2+} on the reduction process. The known ability of metal ions to catalyse the auto-oxidation of cysteine groups might instead attribute the additional γ chain band observed upon reduction of fragment $\text{D}_{\text{Ca}^{2+}}$ and fibrinogen- CaCl_2 to the oxidation of the two cysteine groups located within the COOH-terminal region of the γ chain of both molecules (Lottspeich & Henschen, 1977 and Takagi & Doolittle, 1975). However this situation also occurs in the β chain of both fragment D and fibrinogen (Henschen & Lottspeich, 1977). The β chain did not display a dual electrophoretic mobility. Furthermore the proposal that Ca^{2+} catalysed the formation of an intrachain disulphide bond (despite the presence of urea and 2-mercaptoethanol) is not reconcilable with the observed action of EDTA. This agent promoted a mobility decrease which, by the above reasoning, must have occurred as a consequence of the reduction of this disulphide bond; this situation is not

compatible with the known inhibitory action of EDTA upon the reduction of cystine residues (Jocelyn, 1972).

Another, not unrelated, but remote possibility accounting for the second higher mobility form of the δ chain is that Ca^{2+} alters the conformation by forming an intrachain stable complex with the cysteine residues following their exposure to 2-mercaptoethanol. This situation is however, unlikely to occur, Ca^{2+} binds so weakly to SH groups that they do not form a chelation complex (Jocelyn, 1972).

It must be conceded that each of the above objections to the model proposed for the fragment $D_{Ca^{2+}}$ molecule nevertheless accepts the basic premise that the δ chain of fragment $D_{Ca^{2+}}$ is an extended form of that produced in the absence of Ca^{2+} .

Finally it must be considered whether or not it is reasonable to propose that Ca^{2+} could maintain the altered conformation of the δ chain during electrophoresis in the presence of urea and SDS i.e. denaturing conditions. In this respect it is significant that the results presented from fibrinogen digestion studies performed in the presence of 4 M-urea (the concentration employed during electrophoresis) do indeed imply that Ca^{2+} may maintain a fibrinogen structure less susceptible to the degradative action of plasmin despite the high level of this denaturing agent.

To conclude, the evidence from fibrinogen digestion studies is consistent with the localisation of a Ca^{2+} binding site towards the COOH-terminal region of the fragment $\text{D}_{\text{Ca}^{2+}}$ δ chain. Since one molecule of fibrinogen gives rise to two molecules of fragment D, two of the three Ca^{2+} binding sites reported for the human fibrinogen molecule (Lindsey et al., 1978; Nieuwenhuizen et al., 1979) are thus accounted for.

PART B

DIVISION 2

SECTION 1

INTRODUCTION

The foregoing work, Division 1, described an investigation of the fragment D produced by plasmic digestion of fibrinogen in the presence of Ca^{2+} . A prerequisite for further characterisation studies was the development of a chromatographic procedure for the isolation of fragment $\text{D}_{\text{Ca}^{2+}}$ free from contamination by other fibrinogen digestion products and, in addition, under conditions compatible with the presence of free Ca^{2+} . This Division commences with a description of the work performed to achieve this aim. An account of the application of the separation procedure to the isolation of a fragment D_B suitable for comparative studies follows.

Although the terminal digestion products of fibrinogen, fragments D and E, differ in molecular weight by 20-40,000 no marked separation of them is achieved by gel filtration (Niléhn, 1967). Budzynski et al. (1967) suggested that fragments D and E may associate and several reports have supported the existence of a D:E complex as a discrete molecular entity (e.g. Plow & Edgington, 1972; Gaffney et al., 1975; Mihalyi & Towne, 1976). The immunochemical studies of Plow & Edgington (1973) suggest that the ratio of fragment D to E in the complex is 1:1 and Plow & Edgington (1972) have implicated the

involvement of both hydrophobic and hydrophilic forces in the maintenance of the structure.

This phenomenon of a D:E complex has instigated the development of a variety of chromatographic procedures to achieve the separation of fragments D and E. For example Mihalyi & Towne (1976) employed heat treatment to precipitate the D-fragments. Kemp et al. (1973) employed CM -cellulose and an ammonium formate buffer at pH 3.5. The method described in Part A utilised a sulphopropyl-Sephadex C-25 ion-exchange procedure with a sodium acetate buffer at pH 4.3. However the results of Marguerie et al. (1977) suggest that an acidic buffer system may be prejudicial to the binding of Ca^{2+} to fibrinogen. They reported that the binding of at least one Ca^{2+} is abolished at pH values below 6.5. Published DEAE-cellulose methods (Nussenzweig et al., 1961) are again incompatible with the presence of Ca^{2+} because they employ phosphate buffer systems.

Two alternative separation procedures are described for the isolation of fibrinogen fragment $\text{D}_{\text{Ca}^{2+}}$. The first utilises Sephadex G-200 gel filtration in the presence of the chaotropic agent KI the second, DEAE-cellulose. In each case the buffer system which was employed throughout Division 1 viz. Tris/HCl was used.

SECTION 2
GENERAL METHODS

Eluates from the various chromatographic procedures described in this section were routinely monitored at 280nm (Pye Unicam SP 500 spectrophotometer) for protein concentration. Those fractions exhibiting significant absorbances were examined using the techniques of SDS-polyacrylamide gel electrophoresis (5% and 10% gels) and in certain instances, immunoelectrophoresis. These methods were described in Section 3.2.1 of Division 1, Part B and Section 2.2.4 of Part A respectively.

SECTION 3

THE ISOLATION OF FRAGMENT D_{Ca²⁺} BY GEL FILTRATION

3.1 Introduction

This procedure for the isolation of fragment D_{Ca²⁺} from a fibrinogen digest is based upon the method advocated by Matthias & Hocke (1976). They reported that the separation of fragments D and E could be achieved by a single gel filtration procedure if the chaotropic substance, KI, was incorporated into the buffer system. A Tris/HCl buffer at pH 7.4 was employed - a system compatible with the presence of free Ca²⁺. This method offered one additional benefit; it was a single step isolation procedure. The foregoing results had suggested that fragment D_{Ca²⁺} may undergo a conformational change upon storage. Therefore it was important to accomplish the separation in as short a time as possible.

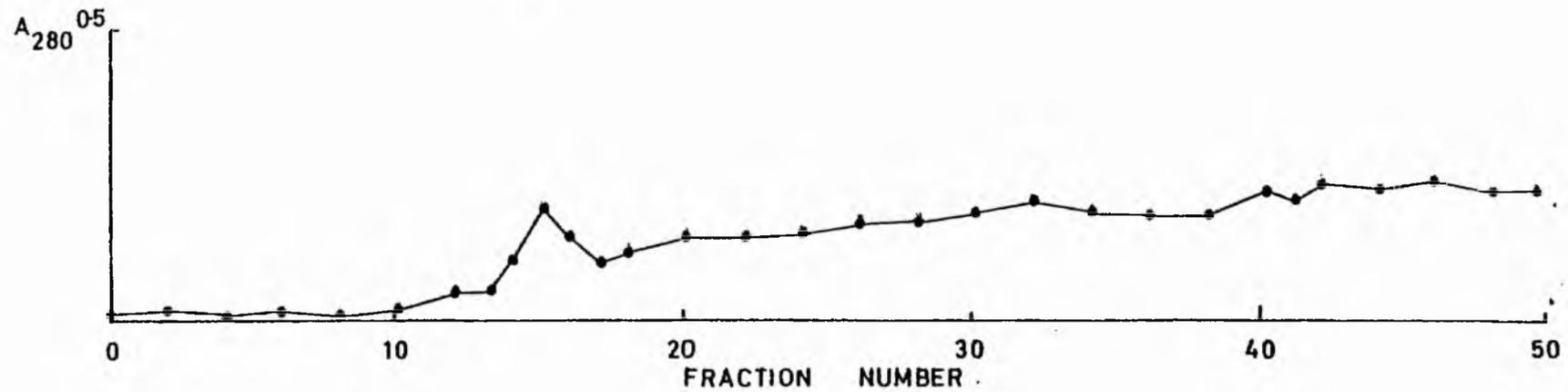
The method of Matthias & Hocke was modified slightly. Trisodium citrate was omitted from the buffer while calcium chloride was added.

3.2 Methods

3.2.1 Digestion of fibrinogen

Fibrinogen (10mg/ml) was dialysed against 0.05 M-Tris/HCl buffer, pH 7.4, containing 2 mM-CaCl₂ for 18h

Fig. 3.1 Sephadex G-200 chromatography of a fibrinogen digest



A plasmic digest of fibrinogen was applied to a column (2.5 x 30cm) of Sephadex G-200 (Superfine grade) and then eluted at a flow rate of 9ml/h with 0.025 M-Tris/HCl buffer, pH 7.4 containing 1.0 M-KI, 2 mM-CaCl₂ and 100 KIU.-Trasylol/ml. Fractions: 30 min.

at 4°C and then digested by the addition of plasmin (0.17 CA units/mg fibrinogen). Following the inclusion of a suitable aliquot of 80 mM-CaCl₂ to maintain the concentration of CaCl₂ at 2 mM, digestion was allowed to proceed at 37°C for 1h 30min when Trasylol (240 K.I.U./CA unit) was added.

3.2.2 Gel filtration of the fibrinogen digest

The digest was applied to a column (2.5 x 30cm) of Sephadex G-200 (Superfine grade). The column was equilibrated and the digest eluted using the following buffer; 0.025 M-Tris/HCl buffer pH 7.4 containing 1.0 M-KI, 2 mM-CaCl₂ and 100 K.I.U. Trasylol/ml. The column flow rate was 9ml/h and 30min fractions were collected.

3.3 Results

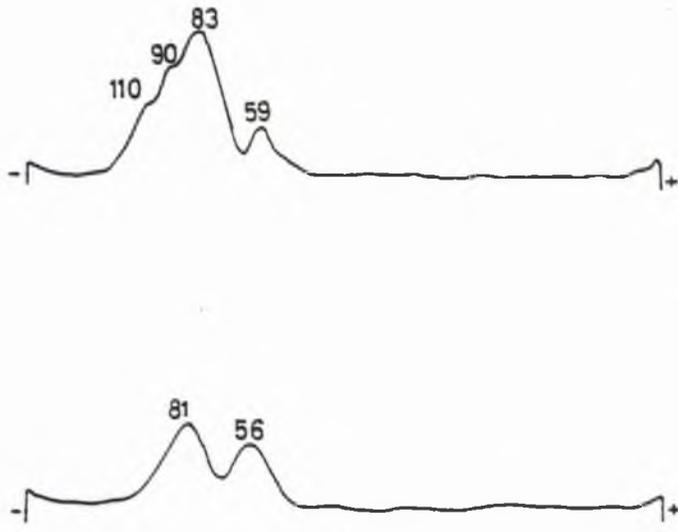
3.3.1 Gel filtration of the fibrinogen digest

Fig. 3.1 demonstrates the elution profile of the fibrinogen digest from a column of Sephadex G-200. A single peak is obvious, an observation contrary to the results reported by Matthias & Hocke (1976) who described the resolution of two peaks corresponding to fragments D and E. The results from analysis of the eluted fractions are presented in Fig. 3.2. SDS-polyacrylamide gel electrophoresis of samples (unreduced) removed from fractions 14 and 15 (Fig. 3.2 A)

Fig. 3.2 Analysis of the Sephadex G-200
column products

Fractions prepared by gel filtration of a plasmic digest of fibrinogen (Fig. 3.1) were examined by the techniques of A, SDS-polyacrylamide gel electrophoresis and B, immunoelectrophoresis.

Fig. 3.2

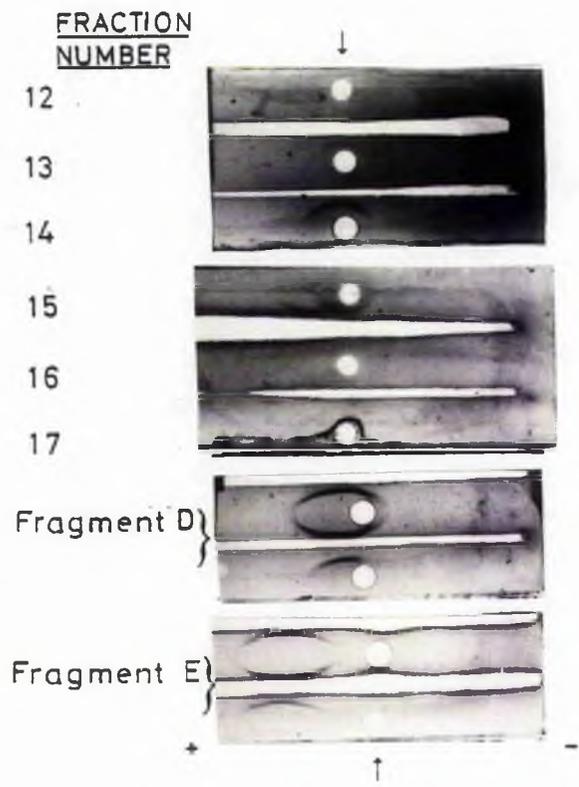


A

Densitometric tracings from SDS-polyacrylamide gel electrophoresis of fractions 14 and 15 obtained by Sephadex G-200 gel filtration of a fibrinogen digest. (5% gels). Estimated molecular weights ($\times 10^{-3}$) are shown.

B

Immuno-electrophoresis of Sephadex G-200 column fractions 12-17. The cathode is on the right. Anti-human fibrinogen antiserum was placed in the troughs. (Samples of fibrinogen fragments D and E are included for comparison. The origin is indicated by the arrows.



suggests that both fractions contain fragments D and E. This proposal is confirmed by the immunoelectrophoretic results presented in Fig.3.2 B. The method has failed to resolve fragments D and E. In addition fragment D appears to be heterogeneous, three molecular weight forms being evident. Furthermore the dialysis step required prior to electrophoresis, necessitated by the presence of 1.0 M-KI, negated the time-saving advantage obtained by the use of a single chromatographic procedure.

3.4 Discussion

The method of Matthias & Hocke (1976) for the separation of fragments D and E employs the chaotropic agent KI to disrupt the assumed hydrophobic attachment between these two terminal fibrinogen digestion products. The present small scale study failed to confirm the efficiency of this method. A single protein-containing peak was eluted from the Sephadex G-200 column and although fragment D was located predominantly in the fractions corresponding to the initial part of this peak, all fractions were contaminated with fragment E. Matthias & Hocke however, obtained highly purified fragments D and E according to immunoelectrophoretic and gel electrophoretic analysis.

This method was not investigated further. It is

possible, although speculative, that the failure to achieve the separation of fragments D and E could be ascribed to the reported stabilising action of Ca^{2+} on the structure of fibrinogen (Marguerie, 1977). The forces involved in the association of fragments D and E to form a complex may also be important to the maintenance of the conformation of the intact fibrinogen molecule (Plow et al., 1977). Thus the fibrinogen stabilising action of Ca^{2+} may also have prevented the disruption of the D:E complex by the chaotropic agent KI.

SECTION 4

THE ISOLATION OF FRAGMENT D_{Ca²⁺} BY
ION-EXCHANGE CHROMATOGRAPHY.

4.1 Introduction

This Section describes the application of the technique of ion-exchange chromatography employing DEAE-cellulose to achieve the isolation of fragment D_{Ca²⁺} .

The two terminal plasmic digestion products of fibrinogen, fragments D and E exhibit different isoelectric points. Arneson (1974) reported the isoelectric point of fragment E as 4.9 and that of fragment D as 6.05-6.45. By analogy with the results reported for fibrinogen by Godal (1960a) the influence of a Ca²⁺ bound to fragment D would be expected to shift the isoelectric point of the molecule in the alkaline direction. Thus the magnitude of the difference in the isoelectric points of fragments D and E may be even greater than that reported by Arneson. This reasoning instigated the development of a method to effect the separation of fragments D_{Ca²⁺} and E by ion-exchange chromatography. Three procedures involving DEAE-cellulose and a Tris/HCl buffer system at pH 7.5 were investigated. It was envisaged that this method in which the more electronegative fragment E would be bound to the DEAE-cellulose while the fragment D would be

eluted immediately after the void volume, offered several advantages. Fragment $D_{Ca^{2+}}$ would be isolated relatively quickly under conditions identical to those employed in its production from fibrinogen. Furthermore since no binding of fragment $D_{Ca^{2+}}$ to an insoluble support would be required, conditions promoting a possible detrimental effect on the binding of Ca^{2+} to fragment $D_{Ca^{2+}}$ would thereby be avoided.

Initially the results from the DEAE-cellulose chromatography of a fibrinogen digest employing a buffer system selected for its similarity of pH, NaCl and $CaCl_2$ concentrations to those of plasma will be presented. The effect on the efficiency of the separation procedure of (i) altering the ionic strength of the buffer and (ii) the addition of urea to the buffer will then be described.

The results presented in this Section represent the collation of a long term study to achieve the isolation of a pure preparation of fragment $D_{Ca^{2+}}$. At the outset it was hoped that if the digestion of fibrinogen by plasmin progressed completely to the terminal digestion products, fragments D and E, a single chromatographic procedure employing DEAE-cellulose would achieve their separation and thereby yield a fragment $D_{Ca^{2+}}$ free from both higher and lower molecular weight contaminants. However two further purification

steps were necessary to achieve this goal.

Firstly an investigation of the fate of plasminogen employed to digest fibrinogen on a column of DEAE-cellulose revealed that the fractions containing fragment $D_{Ca^{2+}}$ were contaminated with plasmin(ogen). This necessitated the use of an affinity chromatography technique to remove this contaminant.

Secondly the fragment $D_{Ca^{2+}}$ preparation thus isolated, contained, in addition, both higher and lower molecular weight species. Their subsequent removal by gel filtration will be described.

4.2 Methods

4.2.1 General

Three variations of a Tris/HCl buffer system were employed to fractionate the fibrinogen digestion products fragments $D_{Ca^{2+}}$ and E by DEAE-cellulose ion-exchange chromatography.

Within each investigation the buffer solution was used;

- a) as a medium for the preparatory dialysis and subsequent digestion of fibrinogen
- b) to equilibrate the DEAE-cellulose column
- c) following the addition of Trasylol (100 K.I.U./ml) to perform the first stage of the elution of the fibrinogen digest sample from the DEAE-cellulose column

- d) following the addition of NaCl (final concentration 0.3 M) to complete the elution procedure and
- e) to equilibrate columns employed in secondary separation procedures.

The three buffer solutions employed were;

- (A) 0.1 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂ and 0.1 M-NaCl
- (B) 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂
- (C) 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂ and 2 M-urea.

However in the case of the buffer solution C, the preliminary dialysis of fibrinogen and the initial hour of digestion were performed in the absence of 2 M-urea.

4.2.2 Digestion of fibrinogen

The conditions of fibrinogen digestion were based upon those employed in Division 1; the ratio of plasmin to fibrinogen being sufficient to achieve the almost complete conversion of fibrinogen, fragments X and Y to fragments D and E. Fibrinogen was prepared for digestion by dialysis against the appropriate buffer at 4°C for 18h. During this period a slight white precipitate formed. This was removed by centrifugation

on a bench centrifuge and the protein concentration of the remaining supernatant estimated spectrophotometrically. The fibrinogen was then digested by the addition of plasmin (0.17 CA units/mg fibrinogen). Following the addition of a suitable volume of a 0.4 M-CaCl₂ solution (to maintain the CaCl₂ concentration at 2 mM) the digest solution was incubated at 37°C for 1h 15min. Digestion was terminated by the addition of Trasylol (240 K.I.U./CA unit).

4.2.3 The separation of the fibrinogen digest on DEAE-cellulose

A column (2.5 x 30cm) of DEAE-cellulose was prepared as recommended by the manufacturers. The equilibration and elution of the column was controlled by a peristaltic pump. The fibrinogen digest sample was applied to the top of the column and then eluted in the first instance with the Trasylol-containing buffer and secondly with buffer containing NaCl (concentrations as defined above, Section 4.2.1).

4.2.4 The elution of plasmin(ogen) from DEAE-cellulose

Plasminogen (4.4 CA units) was activated by incubation at 37°C in the presence of streptokinase (80 units/CA unit). After an incubation period of

30min, Trasylol (110 K.I.U./CA unit) was added and the enzyme/inhibitor solution applied to a column (1.0 x 5cm) of DEAE-cellulose. The latter had been equilibrated and was subsequently eluted with buffer system (B) (Section 4.2.1) as described in Section 4.2.3.

4.2.5 Lysine-Sepharose 4B purification of fragment D-containing fractions eluted from the DEAE-cellulose column

The fragment D-containing fractions eluted from the DEAE-cellulose column were pooled and applied to a column of lysine-Sepharose 4B which had been equilibrated using the appropriate standard buffer solution (Section 4.2.1). The column was eluted (i) with this standard buffer, (ii) with this buffer containing 0.5 M-NaCl (iii) with this buffer containing 0.2 M-6-amino-n-hexanoic acid and finally (iv) with this buffer containing 0.2 M-6-amino-n-hexanoic acid and 0.5 M-NaCl. Those fractions comprising the initial (fragment D-containing) elution were pooled and after a sample was removed, they were freeze dried. Those elutions promoted by buffer solutions (ii) - (iv) were separately pooled, dialysed and freeze dried.

4.2.6 Gel filtration of the fragment D-containing sample

Sephadex G-200 (Superfine grade) was packed into a

column and then equilibrated with the appropriate buffer solution. The reconstituted fragment D-containing sample (5ml) was mixed with 20 μ l of bromophenol blue tracking dye (0.05% (w/v) in water) and then applied to the Sephadex gel surface. The column was eluted with equilibration buffer and fractions collected at appropriate time intervals.

4.3 Results

4.3.1 Plasmic digestion of fibrinogen and subsequent separation by DEAE-cellulose chromatography

The consideration of the results from the chromatographic column separations of a fibrinogen digest employing in turn each of the three buffer systems A, B and C will be facilitated by referring to them as -A (NaCl), -B (blank) and -C (urea).

The elution patterns obtained following the application of a plasmic digest of fibrinogen to a column of DEAE-cellulose are shown in Fig. 4.1 for digest-A (NaCl), Fig. 4.2 for digest-B (blank) and Fig. 4.3 for digest-C (urea). Aliquots removed from each of the digests prior to their application to the column, and from the protein-containing fractions subsequently eluted, were examined (unreduced) by SDS-polyacrylamide gel electrophoresis (5% gels). A photograph of each gel is inset into the appropriate

Fig. 4.1 DEAE-cellulose chromatography of a plasmic digest of fibrinogen

Digest - A (NaCl).

A plasmic digest of fibrinogen was applied to a DEAE-cellulose column and eluted with 0.1 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂, 100 K.I.U./ml-Trasylo^l and 0.1 M-NaCl. At ^A ₀ elution was continued with 0.1 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂ and 0.3 M-NaCl. Polyacrylamide gel electrophoretograms of the fibrinogen digest sample, the fibrinogen digest sample after storage for 48h at 4°C and of eluted fractions (unreduced) are shown, (5% gels). Apparent molecular weights (x 10⁻³) are indicated. Column dimensions: 2.5 x 30cm. Flow rate: 30ml/h. Fractions: 7.5min.

Fig. 4.1

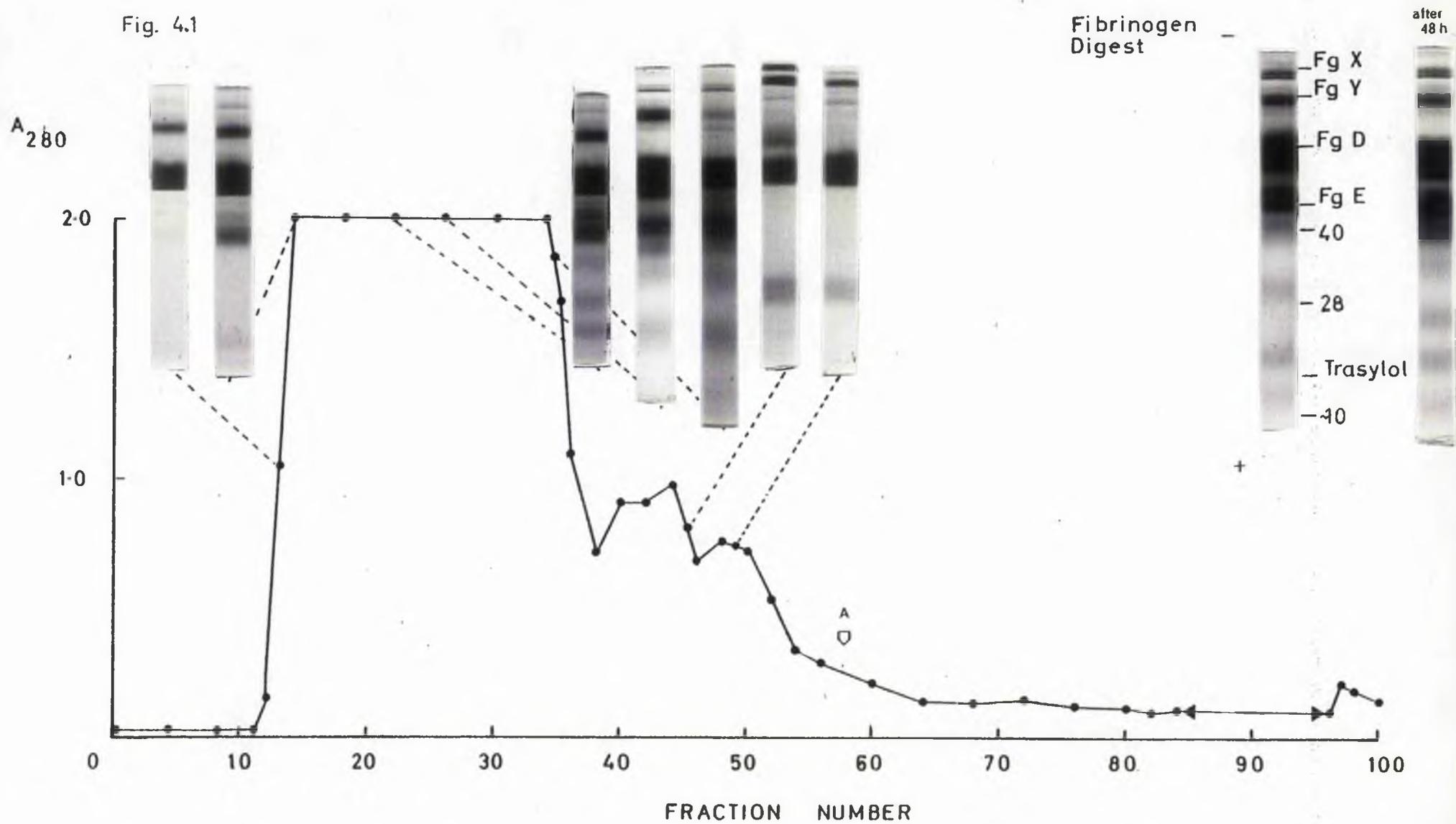


Fig. 4.2 DEAE-cellulose chromatography of a plasmic digest of fibrinogen

Digest - B (blank)

A plasmic digest of fibrinogen was applied to a DEAE-cellulose column and eluted with 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂ and 100 K.I.U./ml-Trasylo1. At ^A \square elution was continued with 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂ and 0.3 M-NaCl. Polyacrylamide gel electrophoretograms of the fibrinogen digest sample and of eluted fractions (unreduced) are shown, (5% gels). Apparent molecular weights ($\times 10^{-3}$) are indicated. Column dimensions: 2.5 x 30cm. Flow rate: 20ml/h. Fractions: 15min. Fractions were pooled as indicated by the horizontal bar for further separation procedures.

Fig. 4.2

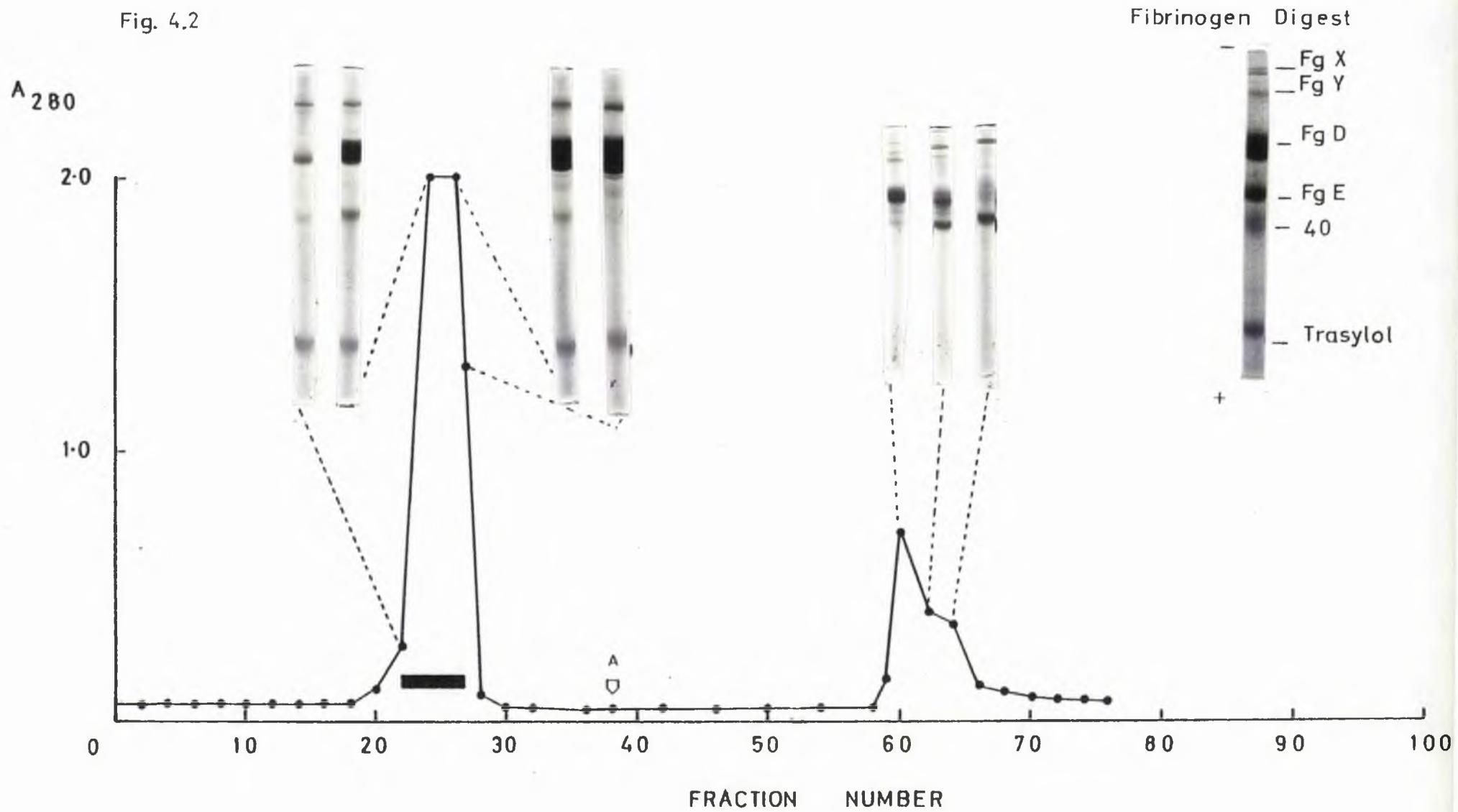
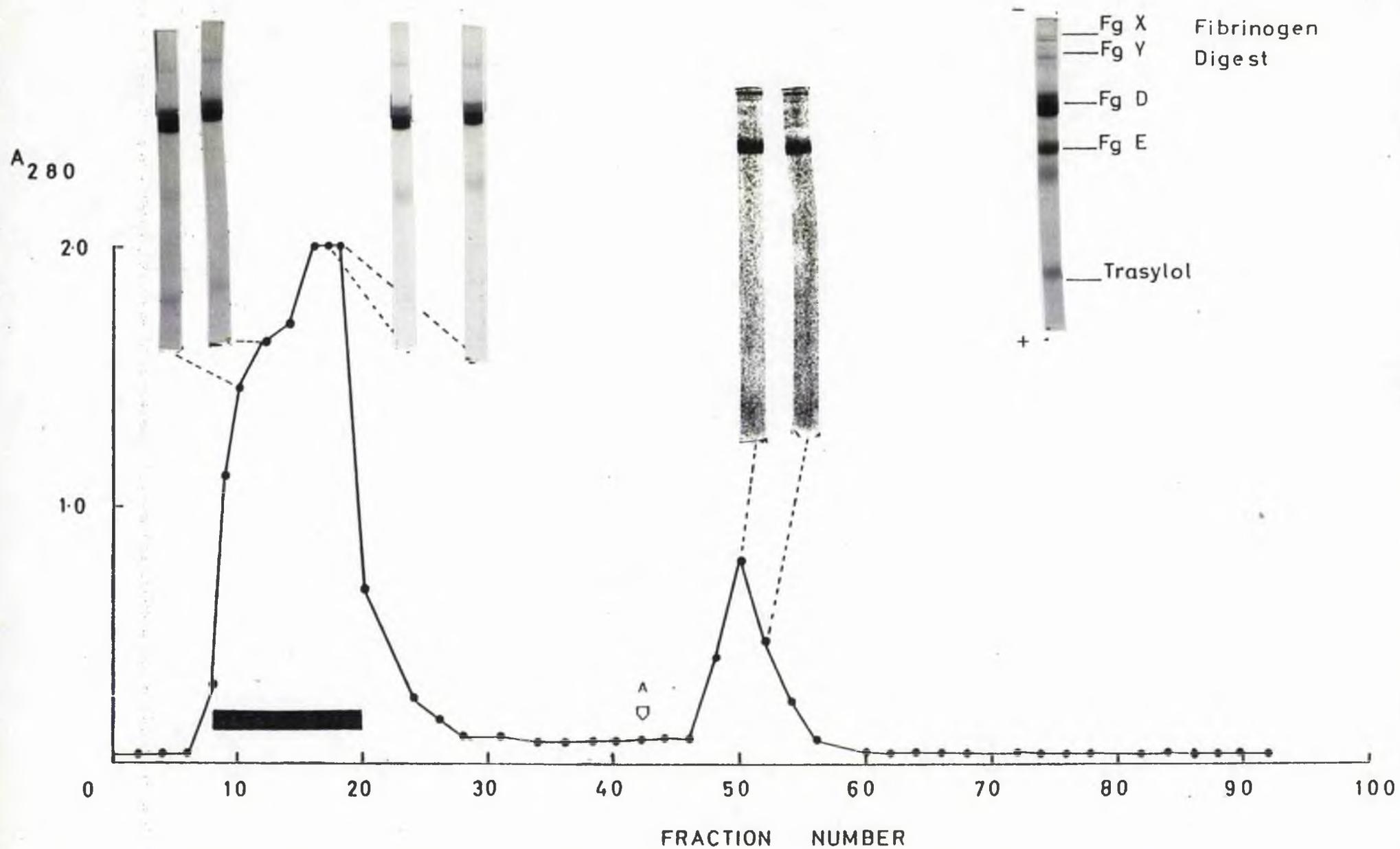


Fig. 4.3 DEAE-cellulose chromatography of a plasmic digest of fibrinogen
Digest - C (urea)

A plasmic digest of fibrinogen was applied to a DEAE-cellulose column and eluted with 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂, 100 K.I.U./ml-Trasylo1 and 2 M-urea. At ^A □ elution was continued with 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂, 2 M-urea and 0.3 M-NaCl. Polyacrylamide gel electrophoretograms of the fibrinogen digest sample and of eluted fractions (unreduced) are shown, (5% gels except those corresponding to fractions 50 and 52 which are 10% gels). Apparent molecular weights (x 10⁻³) are indicated. Column dimensions: 2.5 x 30cm. Flow rate: 30ml/h. Fractions: 10min. Fractions were pooled as indicated by the horizontal bar for further chromatography.

Fig. 4.3



elution diagram.

A comparison of the photographs relating to each of the three fibrinogen digest samples applied to the DEAE-cellulose columns reveals that while digest-B (blank) and -C (urea) appear similar and contain predominantly fibrinogen digestion products, fragments D (assumed to be $D_{Ca^{2+}}$) and E, digest-A (NaCl) contains significant levels of higher molecular weight digestion intermediates. All three digest samples display bands corresponding to apparent molecular weights of 40,000 and 13,000. The latter species may be Trasylol while the 40,000 molecular weight band may represent (i) a degradation product of fragment D (which however, seems unlikely due to the presence of the fragment $D_{Ca^{2+}}$ stabilising agent, Ca^{2+}) or (ii) the 40,000 molecular weight fibrinogen $A\alpha$ chain remnant proposed by Furlan & Beck (1972) and by Mills & Karpatkin (1972) to result from the initial attack of plasmin on fibrinogen, or finally (iii) a contaminant introduced by the streptokinase-plasmin solution. The relatively overloaded gel corresponding to the digest-A (NaCl) displays, in addition, two faint bands of apparent molecular weights 28,000 and approximately 10,000. An aliquot of digest-A (NaCl) was stored at 4°C for 48h and then re-examined by SDS-gel electrophoresis. A photograph of the resulting gel is shown in Fig. 4.1. The band of apparent molecular weight 40,000 appears more

intense. An additional band is obvious between it and the band corresponding to Trasylol. This apparent degradation occurred in the digest sample despite the presence of Trasylol and therefore may not be of enzymic origin. Alternatively the enzyme inhibitor complex of Trasylol and plasmin may be less stable in buffer solution A.

The foregoing studies presented in Division 1 attributed the decrease in electrophoretic mobility induced by EDTA as being a characteristic of fragment $D_{Ca^{2+}}$. Therefore to test if fragment $D_{Ca^{2+}}$ had indeed been prepared in each of the digests A, B and C the effect of EDTA on the electrophoretic pattern produced by each digest sample was investigated. In each case a decrease in the mobility of fragments Y and D was detected while the mobilities of all lower molecular weight digestion products were unaffected. Thus in each of the three fibrinogen digest preparations it appears that fragment $D_{Ca^{2+}}$ has indeed been produced.

Inspection of the DEAE-cellulose column elution profiles corresponding to each of the three digest samples reveals further points of dissimilarity. The elution profiles relating to the fractionation of digest-B (blank) and -C (urea) both display a major initial peak followed by the high salt-induced elution

of a minor peak. However in the case of the digest-A (NaCl) sample (Fig. 4.1) one single broad elution peak is obvious, no significant elution being promoted by the high salt-containing buffer.

The polyacrylamide gel electrophoresis results relating to the fractions eluted from this latter column reveal that virtually no separation of fragments D and E has occurred. In addition each fraction is contaminated by both lower and higher molecular weight species.

The comparable analysis of the fractions prepared by chromatography of digest-B (blank) (Fig.4.2) reveals that a partial separation of the various components of the digest has been achieved. Fragment $D_{Ca^{2+}}$ was predominantly eluted in the fractions comprising the initial peak while the elution of fragment E was induced by the high salt-containing buffer. In addition this buffer promoted the elution of some fragment $D_{Ca^{2+}}$ and fragments X and Y. Fragment Y along with Trasylol and the 40,000 molecular weight digest component were also present in the initial fragment $D_{Ca^{2+}}$ -containing fractions. Thus despite the presence of higher and lower molecular weight species fragment $D_{Ca^{2+}}$ has been prepared apparently free from significant contamination by fragment E. (A faint band of mobility corresponding to that of fragment E is obvious in fractions 26 and 27.) The ability of EDTA to induce its

characteristic affect on the electrophoretic mobility of fragment $D_{Ca^{2+}}$ was again monitored at this stage of the isolation procedure. The results implied that the fragment D located in the initial fractions eluted from the column did indeed conform to the behaviour of a fragment $D_{Ca^{2+}}$.

The SDS-gel analysis of the fractions prepared by DEAE-cellulose chromatography of digest-C (urea) (Fig. 4.3) depicts a similar pattern of elution to that described above for digest-B (blank). However in the presence of urea a more efficient separation of fragments $D_{Ca^{2+}}$ and E has been achieved. No contamination of the fragment $D_{Ca^{2+}}$ -containing fractions by fragment E is apparent. The characteristic biphasic appearance of the fragment $D_{Ca^{2+}}$ band reported in the previous Division is obvious in the gels corresponding to fractions 10-20. The typical electrophoretic mobility decrease displayed by fragment $D_{Ca^{2+}}$ upon exposure to EDTA was again observed.

To summarise, only buffer solutions-B (blank) and -C (urea) have promoted the separation of fibrinogen digestion fragments $D_{Ca^{2+}}$ and E by DEAE-cellulose chromatography. However, in each case the fragment $D_{Ca^{2+}}$ - containing fractions were contaminated by both lower and higher molecular weight species. Therefore further purification procedures to achieve a pure fragment $D_{Ca^{2+}}$ preparation are required.

4.3.2 Further treatment of the Digest-A (NaCl)
sample

DEAE-cellulose chromatography was unsuccessful in isolating fragment $D_{Ca^{2+}}$ from digest-A (NaCl). It was considered that the failure of the ion-exchange procedure might be attributed to the relatively high ionic strength of this buffer solution compared with that of B and C. Accordingly further separation techniques were preceded by the dialysis of the pooled fractions eluted from the DEAE-cellulose column against 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM- $CaCl_2$. However this treatment did not facilitate the resolution of the various digest components. Subjection to three column procedures viz. Sephadex G-200 gel filtration, DEAE-cellulose chromatography and finally gel filtration on Sephadex G-100 failed to produce a relatively pure fragment $D_{Ca^{2+}}$ sample. It can be concluded that the anomalous behaviour of the components of digest-A (NaCl) may not be attributable to some influence of the buffer on the efficiency of the chromatographic separation but instead, to an effect of the buffer on the process of fibrinogen digestion. Thus the molecular properties of the fragments $D_{Ca^{2+}}$ and E produced may differ and, as a consequence, this may affect their respective abilities to bind to each other and to DEAE-cellulose.

No further results relating to digest-A (NaCl)

will be presented.

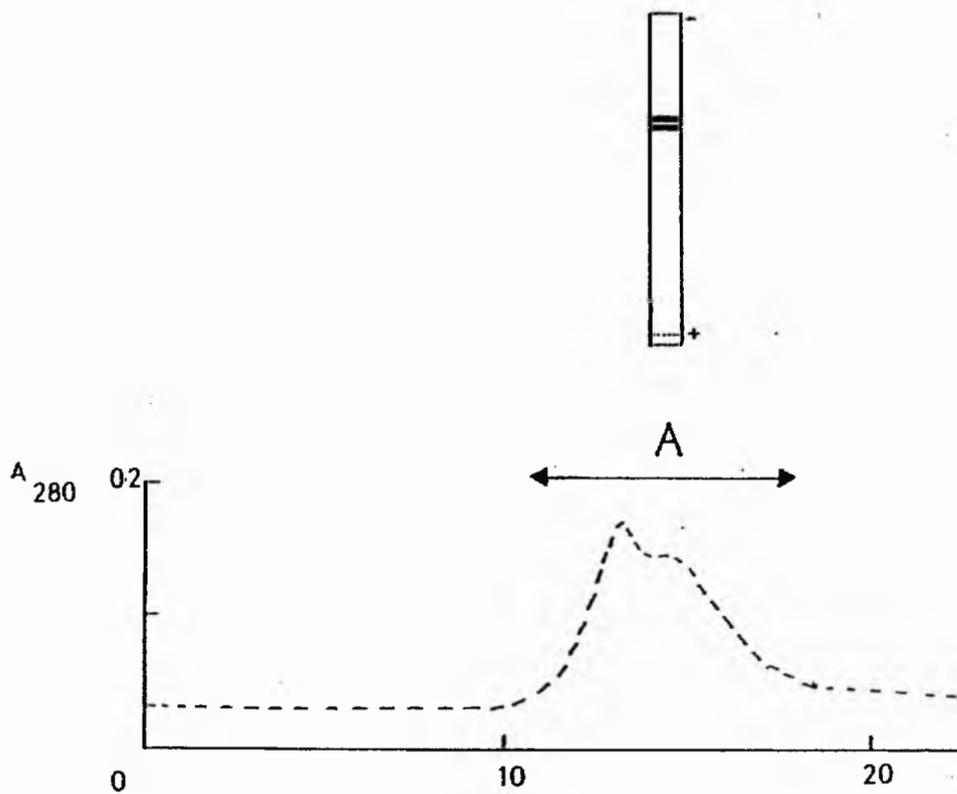
4.3.3 The elution behaviour of streptokinase, plasmin(ogen) and Trasylol from DEAE-cellulose

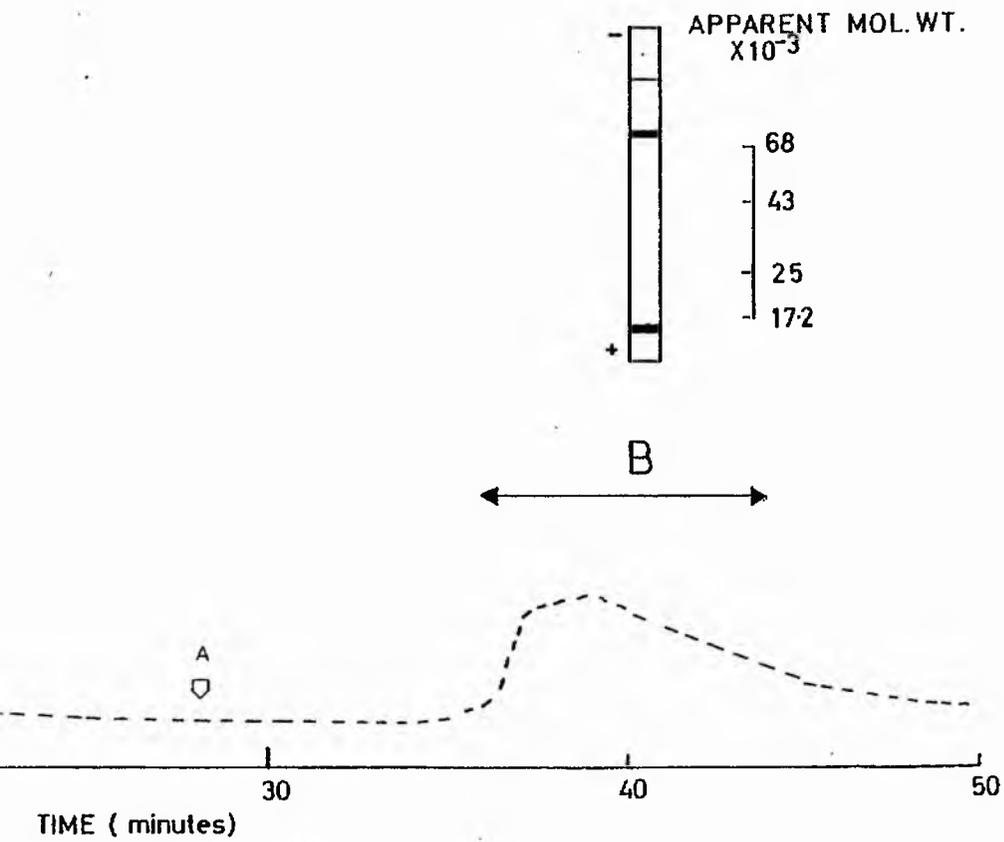
The application of a sample of streptokinase, plasmin(ogen) and Trasylol to a column of DEAE-cellulose produced the elution pattern shown in Fig. 4.4. Two peaks are obvious. Those fractions corresponding to the initial peak (A) and to the second high salt-eluted peak (B) were pooled as shown and aliquots were examined (unreduced) by SDS-gel electrophoresis. The resulting gel band patterns are shown diagrammatically. Analysis of peak A revealed two proteins of apparent molecular weights 74,000 and 69,000. A faint band corresponding to an approximate molecular weight of 11,000 is also obvious. A sample of peak B produced three bands of apparent molecular weights 115,000, 73,000 and 14,500. Isoelectric points of 6.0 to 8.5 have been reported for the various forms of plasminogen (Summ-aria et al., 1973), the higher values being characteristic of the lower molecular weight forms. The electro-phoretic behaviour of plasmin(ogen) streptokinase and Trasylol was investigated in Part A, Section 6.3.1. A comparison with these results suggests that both peaks A and B may contain plasmin(ogen) and a high mobility species which may represent Trasylol or a

Fig. 4.4 DEAE-cellulose chromatography of streptokinase-activated plasminogen and Trasylol

A sample containing plasminogen, streptokinase and Trasylol was applied to a DEAE-cellulose column and eluted with 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂. At ^A ∇ elution was continued with the same buffer containing 0.3 M-NaCl. Fractions corresponding to peaks A and B were pooled as indicated by the arrows and examined (unreduced) on 5% polyacrylamide gels. The corresponding gels are shown in diagrammatic form. Column dimensions: 1.0 x 5cm. Flow rate: 40ml/h.

Fig. 4.4





peptide released during the streptokinase activation of plasminogen. In addition the gel corresponding to peak B displays a higher molecular weight component which may be a contaminant introduced by the streptokinase preparation.

These results have important implications for the procedure employed to isolate fragment $D_{Ca^{2+}}$. Firstly, the fragment $D_{Ca^{2+}}$ fractions prepared by DEAE-cellulose chromatography of a plasmic digest of fibrinogen will be contaminated with plasmin(ogen). Part of the plasmin(ogen) sample was eluted, like fragment $D_{Ca^{2+}}$, by the equilibration buffer of the DEAE-cellulose column. Furthermore the level of plasmin(ogen) contamination of fragment $D_{Ca^{2+}}$ -containing column fractions will not be revealed by SDS-gel analysis. The SDS-gel patterns of samples corresponding to the fragment $D_{Ca^{2+}}$ -containing DEAE-column fractions and to the streptokinase/plasmin(ogen)/Trasylol-containing fractions appear similar. Thus the designation of the various SDS-gel bands observed upon electrophoresis of the products of the foregoing DEAE-cellulose column separations of the three fibrinogen digests must be viewed with caution.

Plasmin(ogen) present in the fragment $D_{Ca^{2+}}$ preparation is a serious contaminant. Its presence would invalidate the results of proposed characterisation studies with fragment $D_{Ca^{2+}}$. Furthermore

the degradation of fragment $D_{Ca^{2+}}$ must be avoided. Haverkate & Timan (1977) proposed that fragment $D_{Ca^{2+}}$ was completely resistant to further digestion by plasmin - a proposal not totally substantiated by the studies described in Division 1. Thus the requirement for a single high molecular weight form of fragment $D_{Ca^{2+}}$ necessitates the removal of contaminating plasmin(ogen).

4.3.4 The removal of contaminating plasmin(ogen) from fragment $D_{Ca^{2+}}$ preparations by lysine-Sepharose 4B affinity chromatography

The lysine-Sepharose 4B affinity chromatography method employed in Division 1, Section 4.2.1 to remove contaminating plasminogen from fibrinogen was similarly applied to purify fragment $D_{Ca^{2+}}$ preparations. The results obtained with samples isolated from digest-B (blank) will be presented to illustrate the process. The fragment $D_{Ca^{2+}}$ - containing fractions eluted from the DEAE-cellulose column were pooled and applied immediately to a column of equilibrated lysine-Sepharose 4B. The corresponding elution pattern is presented in Fig. 4.5(i). The results from SDS-gel electrophoresis of the fragment $D_{Ca^{2+}}$ preparation before and after its passage through the column are shown by gels (a) and (b). The densitometer scans corresponding to these gels suggested that as a

Fig. 4.5 Lysine-Sepharose 4B chromatography
of a fragment D_{Ca}²⁺ preparation

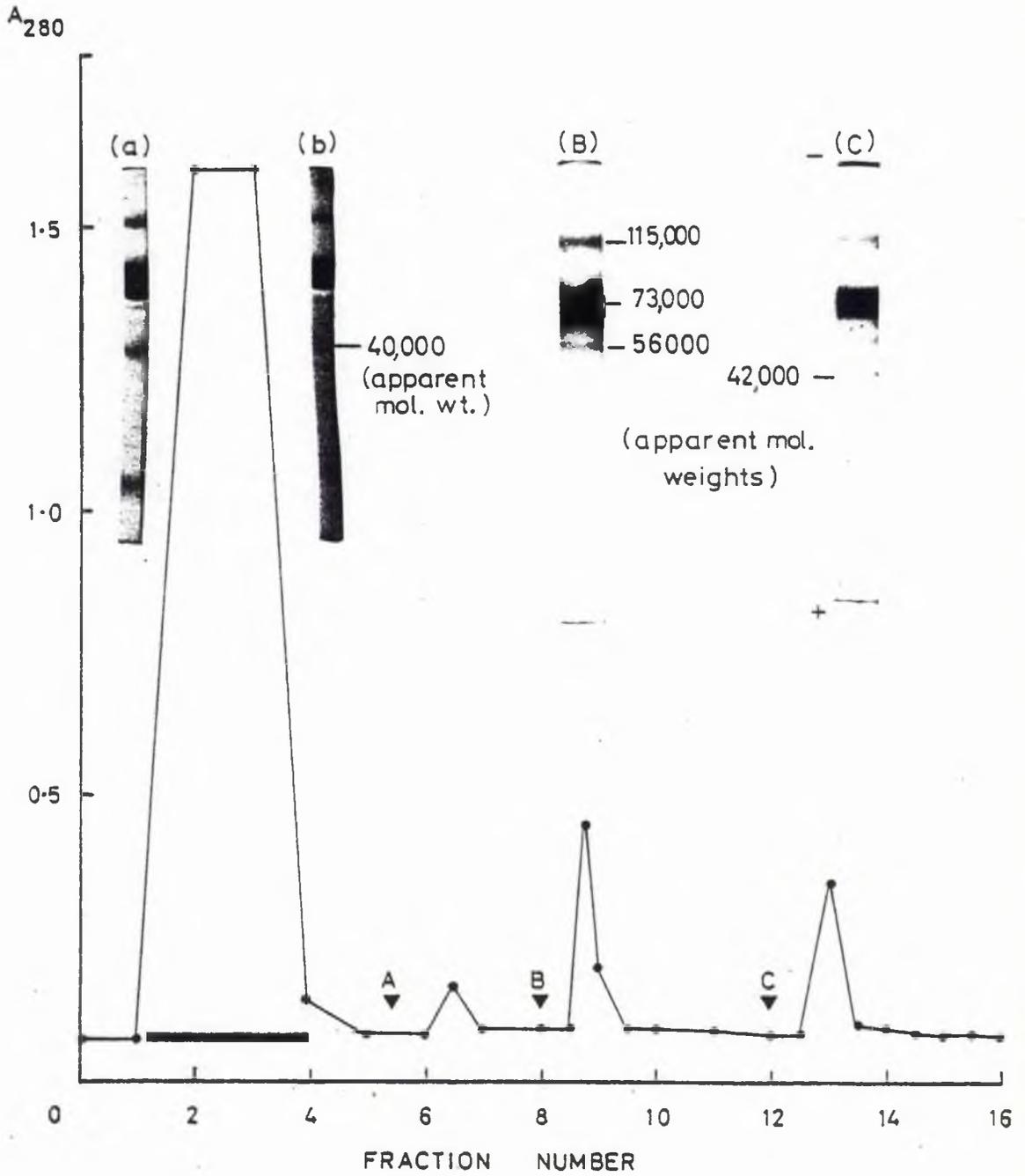
Digest - B (blank)

The removal of contaminating plasmin(ogen) from a fragment D_{Ca}²⁺ preparation:

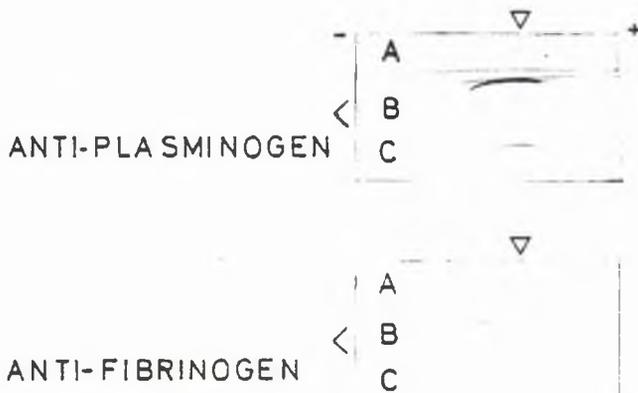
- (i) Lysine-Sepharose 4B chromatography. Column dimensions: 1.0 x 10cm. Flow rate 45ml/h. Elution procedure: 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂ followed by A, 0.5 M-NaCl in the same buffer, B, 0.2 M-6-amino-n-hexanoic acid in distilled water and C, 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂, 0.5 M-NaCl and 0.2 M-6-amino-n-hexanoic acid. Polyacrylamide gel electrophoretograms of (a) the sample applied to the gel, (b) the sample eluted from the gel, (B) the material eluted by solution B and (C) the material eluted by buffer C are shown (5% gels). All samples were examined unreduced. Fractions (10min) were pooled as indicated by the horizontal bar for further chromatography.
- (ii) Immuno-electrophoresis of the material eluted from the lysine-Sepharose 4B column by solutions A, B and C against anti-human plasminogen antiserum and anti-human fibrinogen antiserum. The cathode is on the left and the origin is indicated by the arrows.

Fig. 4.5

(i)



(ii)



consequence of the lysine-Sepharose 4B column treatment, the amount of the 40,000 molecular weight band had decreased but no change in the magnitude of the most intense band of mobility appropriate to both fragment $D_{Ca^{2+}}$ and plasmin(ogen), was discernible. Three further fractions were eluted from the column. Fraction A, eluted by a NaCl-containing buffer, produced no band pattern upon SDS-gel analysis, and also failed to react with anti-human fibrinogen antiserum and anti-human plasminogen antiserum. Fraction B, eluted by a 6-amino-n-hexanoic acid solution and fraction C eluted by a buffer containing both NaCl and 6-amino-n-hexanoic acid were examined, unreduced, on 5% gels. Both gels (Fig. 4.5 (i)) appear similar. Each gel contains bands of apparent molecular weights 115,000, 73,000 and 56,000. Gel C contains, in addition, a band of 42,000 molecular weight. Both samples B and C reacted against anti-human fibrinogen and anti-human plasminogen antisera (Fig. 4.5 (ii)). Thus both plasminogen-like and fibrinogen-like proteins were removed from the fragment $D_{Ca^{2+}}$ preparation by the lysine-Sepharose 4B purification procedure. It must be concluded that the yield of fragment $D_{Ca^{2+}}$ may have been reduced as a consequence of this purification process. A similar conclusion has been reached elsewhere in this work where

lysine-Sepharose 4B chromatography has been employed, (e.g. Part A, Section 4.3.2).

Those fractions eluted directly from the lysine-Sepharose 4B gel and assumed to contain fragment $D_{Ca^{2+}}$ free from contaminating plasmin(ogen) were pooled and concentrated prior to the final column procedure, gel filtration. An aliquot removed from this concentrate did not react against anti-human plasminogen antiserum while a broad precipitate was formed with anti-human fibrinogen antiserum. Thus, within the limits of sensitivity of the immunoelectrophoretic technique, the lysine-Sepharose 4B column procedure accomplished the removal of contaminating plasmin(ogen) from fragment $D_{Ca^{2+}}$ preparations.

4.3.5 Gel filtration of fragment $D_{Ca^{2+}}$ - containing preparations

The fragment $D_{Ca^{2+}}$ preparations prepared and purified as described above were subjected finally to Sephadex G-200 gel filtration. The elution profiles corresponding to each of the fragment $D_{Ca^{2+}}$ preparations -B (blank) and -C (urea) are shown in Figs. 4.6 and 4.7 respectively. Polyacrylamide gel electrophoresis results (5% gels) of the sample applied to the column and of aliquots removed from eluted fractions (unreduced) are shown.

Fig. 4.6

Fractionation of a Fragment $D_{Ca^{2+}}$ preparation on Sephadex G-200

Digest - B (blank).

The fragment $D_{Ca^{2+}}$ preparation was applied to a column of Sephadex G-200. The column was eluted with 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM- $CaCl_2$. Polyacrylamide gel electrophoretograms of the fragment $D_{Ca^{2+}}$ - containing sample applied to the column, eluted fractions and of pooled eluted fractions (indicated by the horizontal bars) are shown, (5% gels). All samples were examined unreduced. Column dimensions: 2.5 x 30 cm. Flow rate: 10 ml/h. Fractions: 30 min.

Fig. 4.6

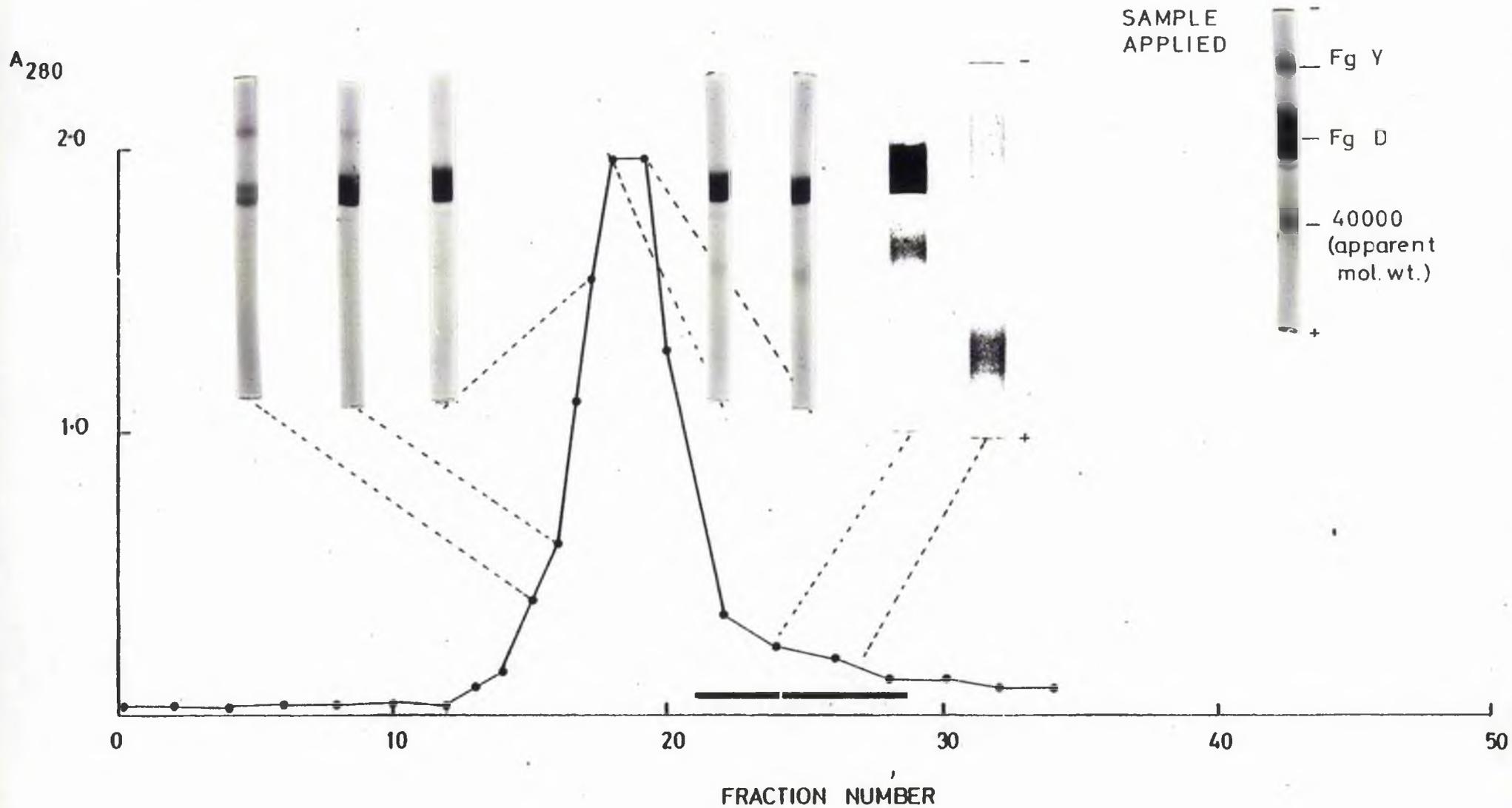


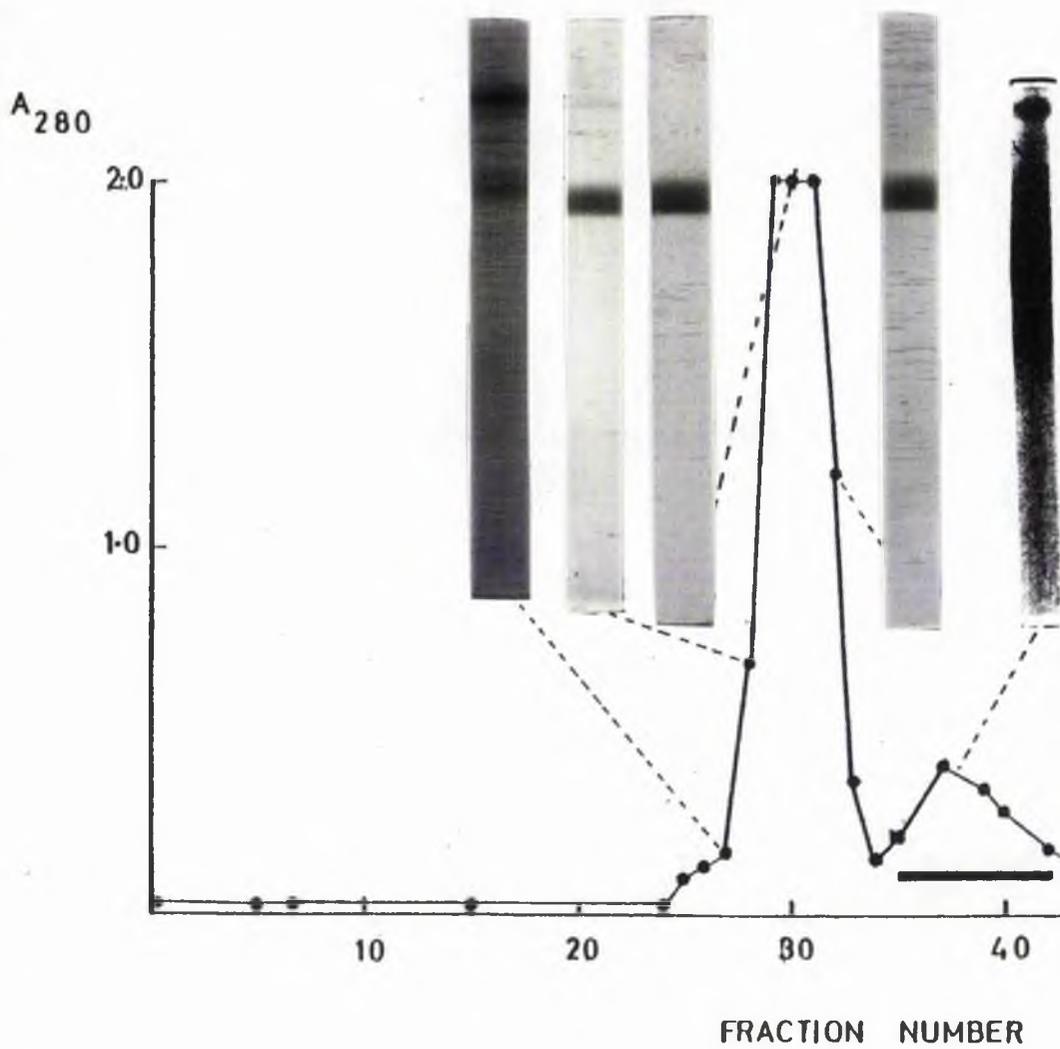
Fig. 4.7

Fractionation of a fragment D_{Ca}^{2+} preparation on Sephadex G-200

Digest - G (urea).

The fragment D_{Ca}^{2+} preparation was applied to a column of Sephadex G-200. The column was eluted with 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM- $CaCl_2$ and 2 M-urea. Polyacrylamide gel electrophoretograms of the fragment D_{Ca}^{2+} - containing sample applied to the column, eluted fractions and of pooled eluted fractions (indicated by the horizontal bars) are shown, (5% gels). All samples were examined unreduced. Column dimensions: 2.5 x 34cm. Flow rate: 3.5ml/h. Fractions: 1h.

Fig. 4.7

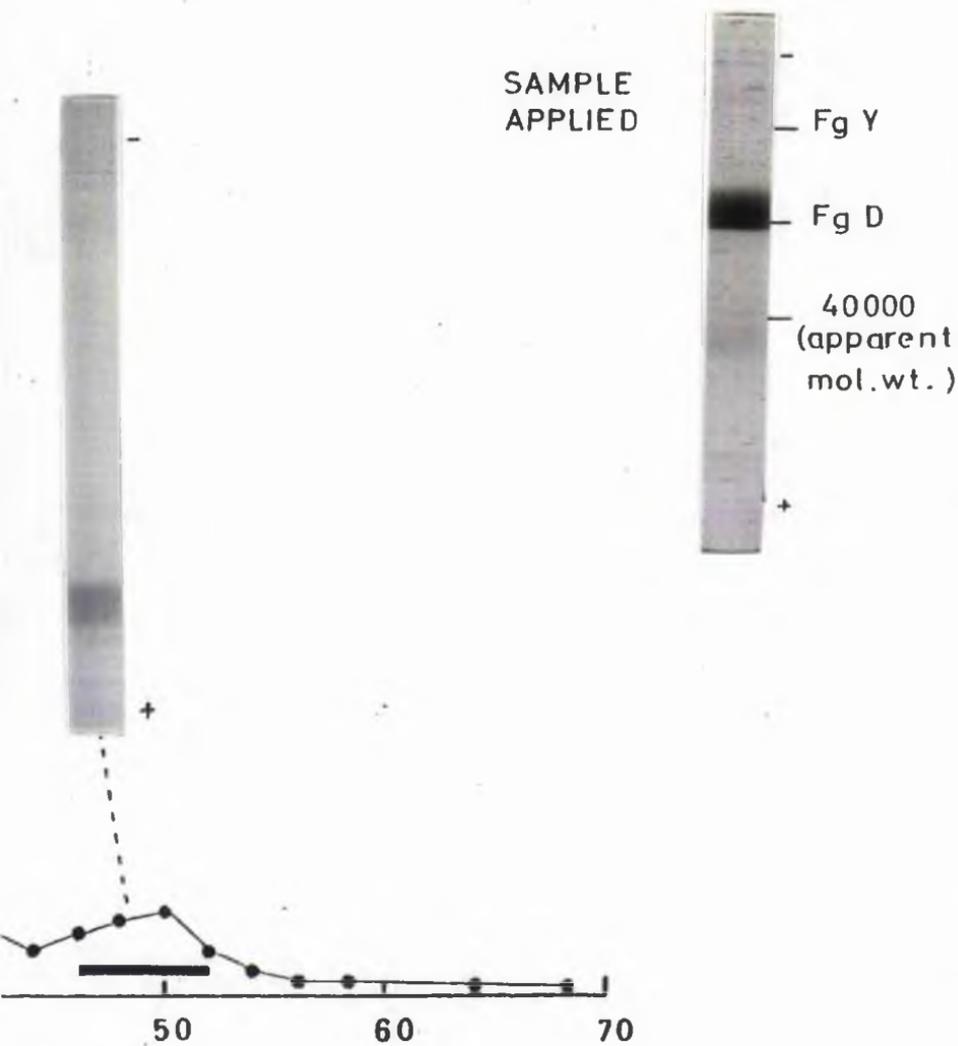


SAMPLE
APPLIED

Fg Y

Fg D

40000
(apparent
mol.wt.)



The sample of fragment $D_{Ca^{2+}}$ -B (blank) applied to the Sephadex G-200 column displays three components; one of mobility appropriate to fragment Y another to fragment D and a third to a species of apparent molecular weight 40,000. The Sephadex G-200 column elution profile displays a broad peak and SDS-gel analysis of the eluted fractions suggests that the initial fractions contain fragment $D_{Ca^{2+}}$ and fragment Y. Those fractions eluted in the middle portion of this major peak apparently contain pure fragment $D_{Ca^{2+}}$ while the latter fractions contain increasing amounts of the 40,000 molecular weight component. A species of similar molecular weight to this latter band was detected in the original digest sample which, it was proposed, could have arisen from the initial attack of plasmin on the A α chain of fibrinogen. If the two 40,000 molecular weight species are in fact identical then the continued contamination of fragment $D_{Ca^{2+}}$ by this species throughout the various stages of the purification of the fibrinogen digest may be of significance since it is interesting to reflect that both fragment $D_{Ca^{2+}}$ (Haverkate & Timan, 1977) and this region of the A α chain of fibrinogen (Marguerie, 1977) have been implicated as sites in the binding of Ca^{2+} to fibrinogen.

The electrophoretic examination of those pooled and concentrated fractions eluted from the Sephadex G-200 column immediately before the marker dye revealed a

broad, faint band of apparent molecular weight 13,500 - a value similar to that of Trasylol. A faint band of similar mobility was obvious in the sample applied to the column (although not apparent in the corresponding gel photograph).

The analogous elution profile obtained with the sample of fragment $D_{Ca^{2+}}-C$ (urea), shown in Fig. 4.7 implies a greater degree of resolution of the sample components applied to the column. Three distinct peaks are obvious. This suggestion is confirmed by the results of SDS-gel analysis of the various eluted fractions. A greater yield of apparently pure fragment $D_{Ca^{2+}}-$ containing fractions has been achieved. This phenomenon may be due in part to the presence of the chaotropic agent urea, in the buffer solution. The elution of fragment Y was restricted to the initial shoulder of the major fragment $D_{Ca^{2+}}-$ containing peak while the 40,000 and 13,000 molecular weight components were eluted in the second and third, minor peaks.

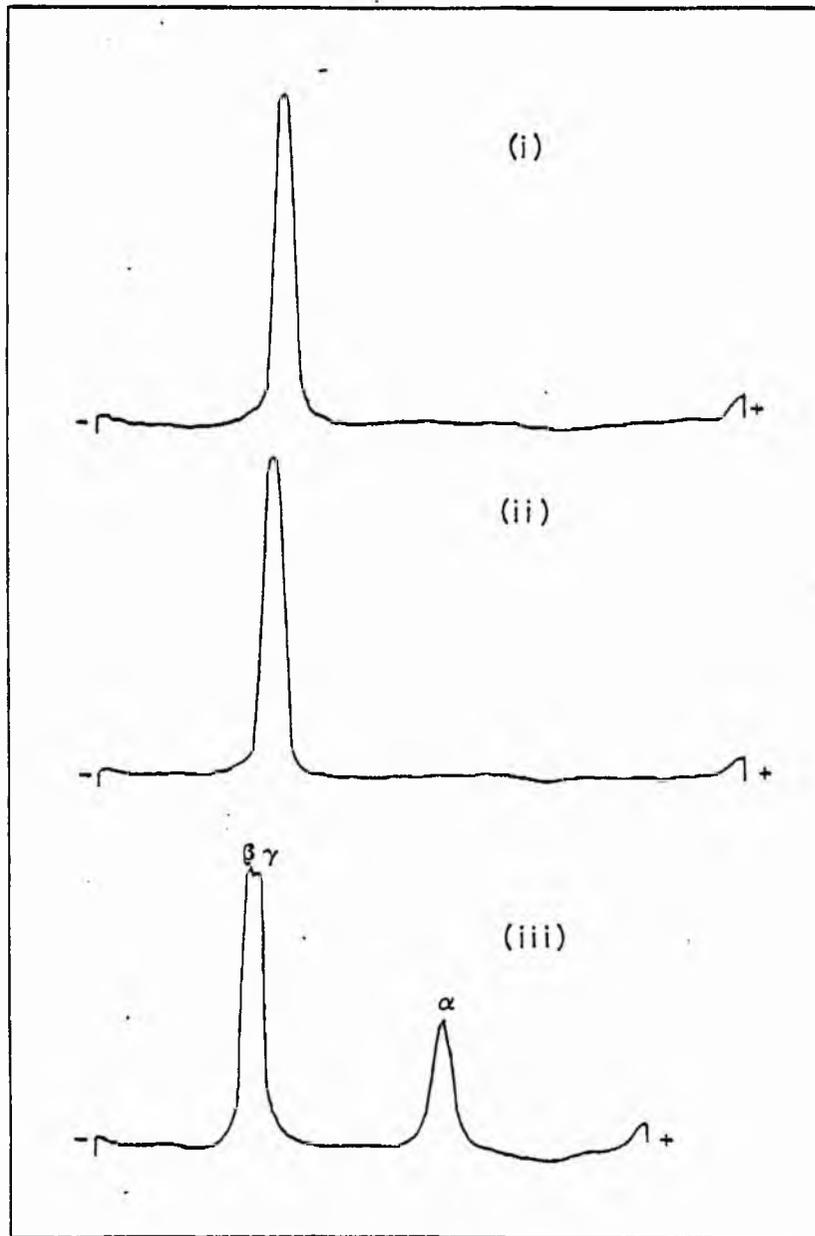
Previous work, (Division 1) described several characteristics of the fragment $D_{Ca^{2+}}$ molecule. It is composed of an α chain (MW 14,000) a β chain (MW 44,000) and a δ chain (MW 40,000). The electrophoretic mobility of the fragment $D_{Ca^{2+}}$ molecule decreases upon the addition of EDTA. The ability of the fragment $D_{Ca^{2+}}$, isolated in each of the above gel

Fig. 4.8 SDS - Polyacrylamide gel electrophoresis
of fragment D_{Ca2+}

Digest - B (blank)

Fragment D_{Ca2+} prepared by digestion of fibrinogen and then isolated by DEAE-cellulose chromatography and gel filtration on Sephadex G-200 in the presence of 2 mM-CaCl₂ was examined on 5% polyacrylamide gels (i) unreduced and (ii) unreduced but following its incubation in the presence of 10 mM-EDTA for 10 min at 20°C. The reduced chain composition of fragment D_{Ca2+} was examined on a 10% polyacrylamide gel - (iii). The densitometer scans corresponding to each of these gels are shown.

Fig. 4.8



filtration procedures, to exhibit these characteristics was investigated.

Fig. 4.8 illustrates the polyacrylamide gel patterns obtained by examination of the fragment $D_{Ca^{2+}}$ eluted in fraction 18 of the Sephadex G-200 column -B (blank) (i) unreduced (ii) unreduced following the addition of EDTA, (5% gels) and (iii) reduced (10% gel). EDTA caused the characteristic decrease in the mobility of the fragment $D_{Ca^{2+}}$ band. The gel corresponding to the reduced fragment $D_{Ca^{2+}}$ sample was overloaded. A very broad poorly resolved peak is obvious in (iii) corresponding to a molecular weight of 42,000 and probably contains the β and δ chain remnants of fragment $D_{Ca^{2+}}$ while only one other peak of apparent molecular weight 14,000 is obvious. This must, by inference, represent the D α chain remnant (although the mobility is outwith the range covered by SDS-gel protein molecular weight standards). Thus the fragment D isolated from fibrinogen digest -B (blank) does indeed exhibit characteristics displayed by fragment $D_{Ca^{2+}}$.

The results from similar studies performed with fragment $D_{Ca^{2+}}$ samples isolated from digest-C (urea) are shown in rather more detail in Figs. 4.9 and 4.10. The densitometer scans corresponding to two fractions eluted from the Sephadex G-200 column (28 and 32)

Fig. 4.9 SDS - Polyacrylamide gel electrophoresis of fragment D_{Ca}2+

Digest - C (urea)

Fragment D_{Ca}2+ prepared by digestion of fibrinogen by plasmin and isolated by DEAE-cellulose chromatography and gel filtration on Sephadex G-200 in the presence of 2M-urea and 2mM-CaCl₂ was examined on 5% polyacrylamide gels (i) unreduced, (ii) unreduced but following its incubation at 4°C for 48h and (iii) unreduced but following its incubation in the presence of 10mM-EDTA for 10 min at 20°C. The corresponding densitometric scans are shown for two fragment D_{Ca}2+ - containing fractions eluted from the Sephadex G-200 column viz. fractions 28 and 32.

Fig. 4.9

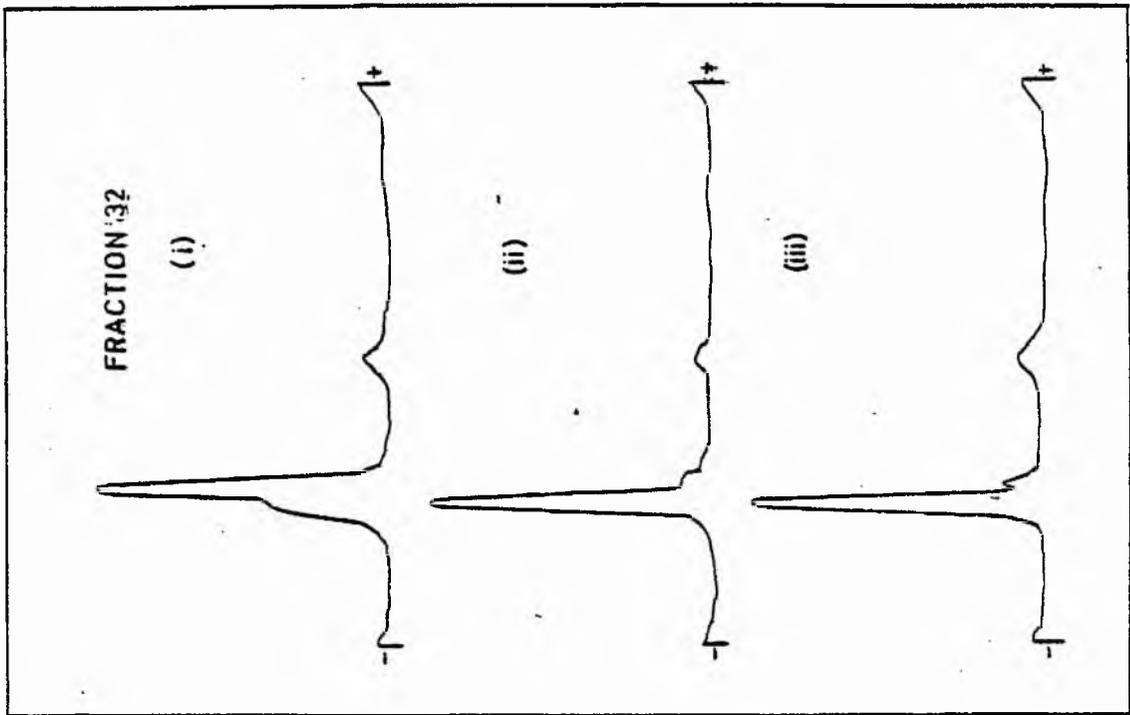
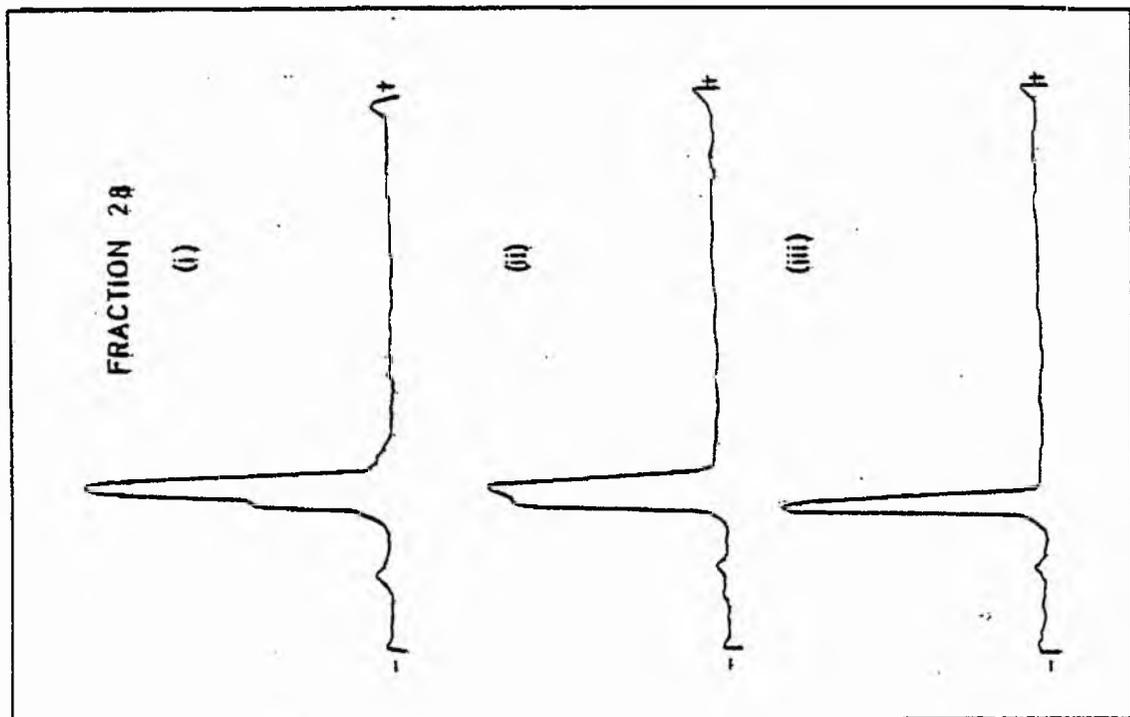
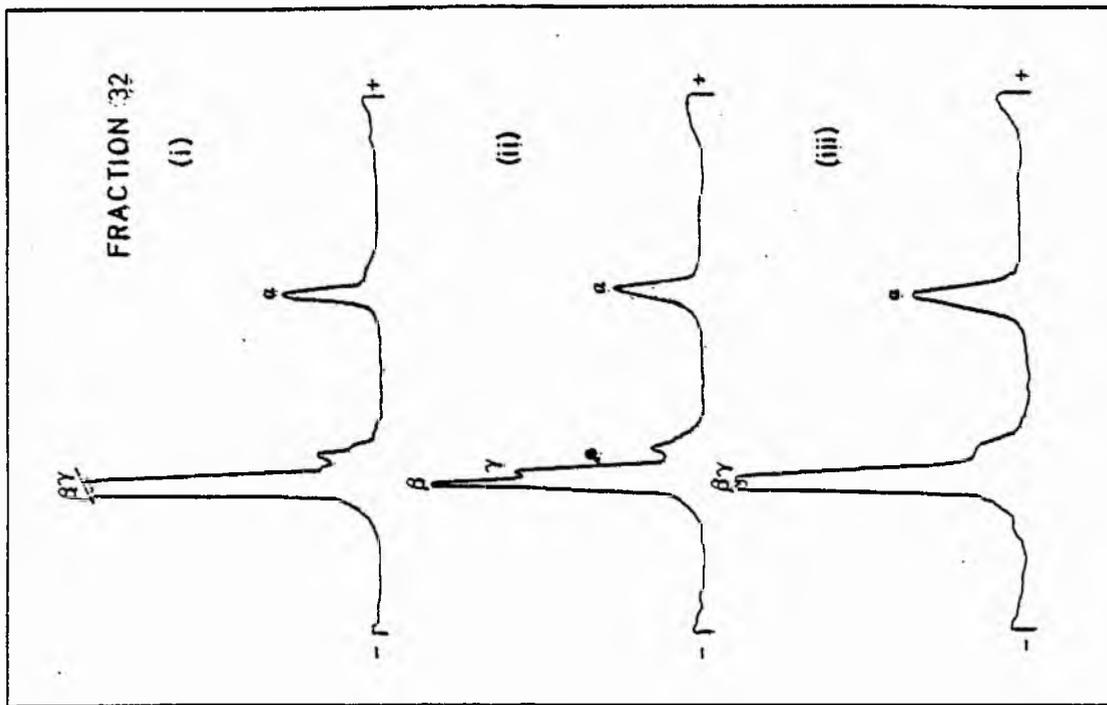
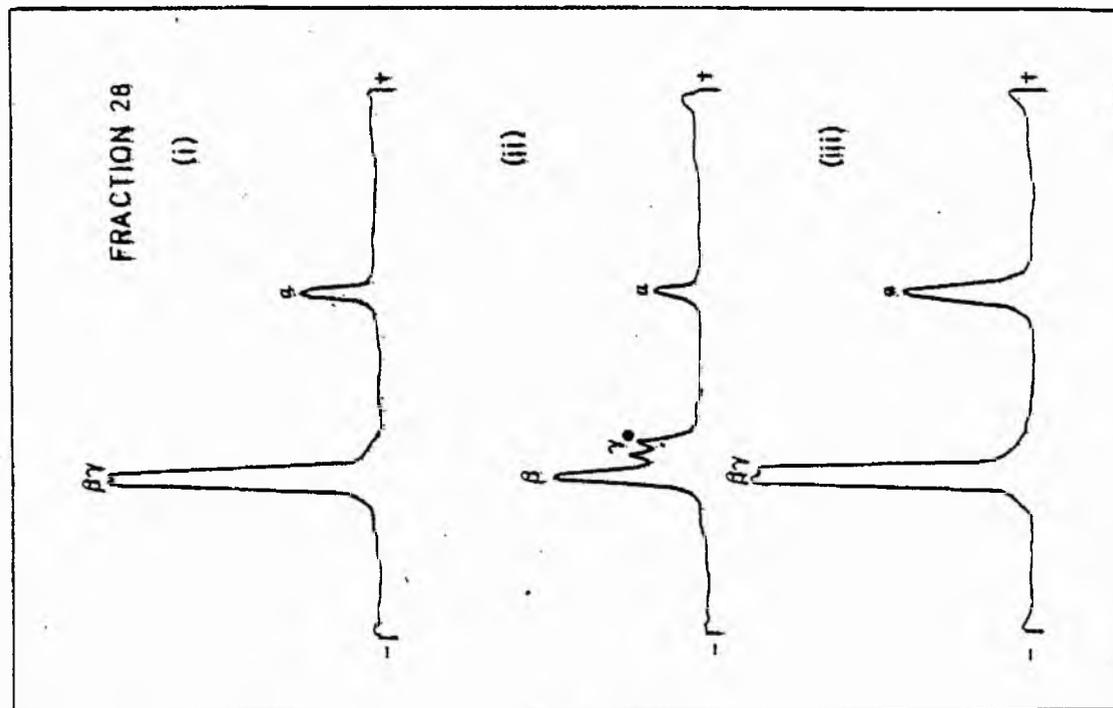


Fig. 4.10 SDS - Polyacrylamide gel electrophoresis of reduced fragment D_{Ca}²⁺

Digest - C (urea)

Fragment D_{Ca}²⁺ prepared as described in fig. (4.9) in the presence of 2M-urea and 2mM-CaCl₂ was examined on 10% polyacrylamide gels (i) after its reduction at 100°C for 5 minutes in the presence of 1.5% (V/V) 2-mercaptoethanol (ii) after its reduction at 4°C for 30 minutes in the presence of 1% (V/V) 2-mercaptoethanol and (iii) as for (ii) but made 10 mM with respect to EDTA after reduction. The corresponding densitometric scans are shown for two fragment D_{Ca}²⁺-containing fractions eluted from the Sephadex G-200 column viz. fractions 28 and 32.

Fig. 4.10



following their examination unreduced on 5% gels are shown in Fig. 4.9 (i). Both samples exhibit a biphasic peak within the mobility range expected for fragment $D_{Ca^{2+}}$. A single peak of mobility equal to the lower mobility component of this biphasic peak was produced by the addition of EDTA to the sample prior to electrophoresis, Fig. 4.9 (iii). A similar, but less complete, transition was induced in the fragment D peak mobility pattern by simply incubating the sample at 4°C for 48h (Fig. 4.9 (ii)). This treatment was more effective with the later column fractions and indeed the final fractions contained the low mobility form of fragment $D_{Ca^{2+}}$ exclusively without the incubation treatment being necessary. This observation may be attributed to the presence of 2 M-urea in the column buffer. The mobility change induced in fragment $D_{Ca^{2+}}$ by exposure to EDTA or to denaturing conditions has been proposed (Division 1) to represent a conformational change in the fragment $D_{Ca^{2+}}$ molecule as a consequence of the removal of the restraint imposed by bound Ca^{2+} . Thus the isolation of fragment $D_{Ca^{2+}}$ in a buffer containing the denaturing agent urea may account for the apparent instability of fragment $D_{Ca^{2+}}$.

In Division 1 it was also reported that the δ chain constituent of fragment $D_{Ca^{2+}}$ exhibited a higher electrophoretic mobility upon reduction at 4°C. The

intensity of this higher mobility component was decreased either by raising the temperature of reduction or by the subsequent addition of EDTA. These treatments induced a parallel increase in the intensity of the δ chain peak immediately below that of the β chain - the resulting peak pattern being identical to that obtained by reduction at 100°C. The peak patterns obtained upon reduction of fragment $D_{Ca^{2+}}$ samples (removed from fractions 28 and 32) at 100°C are shown in Fig. 4.10 (i). Each sample displays the characteristic β , δ and α chain pattern of fragment $D_{Ca^{2+}}$. However, upon reduction at 4°C the higher mobility form of the δ chain is obvious (scan (ii) (marked•)) with both fragment $D_{Ca^{2+}}$ samples. The addition of EDTA to these samples prior to electrophoresis produced the effect on the peak pattern illustrated by scan (iii). In each case the higher mobility form of the D- δ chain is no longer apparent. This change is accompanied by an intensification of the low mobility D- δ chain peak.

In each of the densitometer scans corresponding to the reduced chain pattern of the fragment $D_{Ca^{2+}}$ isolated in fraction 32 a shoulder is obvious below the main δ chain peak. This minor peak displays an apparent molecular weight of 34,000 - a value similar to that attributed in Division 1 to a δ

chain constituent of a fragment $D_{Ca^{2+}}$ which had undergone limited plasmic degradation. This finding is consistent with the demonstration in Fig. 4.9 (ii) and (iii) that a component of the unreduced fragment $D_{Ca^{2+}}$ peak did not exhibit the characteristic EDTA-induced mobility decrease. Therefore a degraded form of fragment $D_{Ca^{2+}}$ may be present in the preparation isolated from the Sephadex G-200 column. This proposal concurs with the suggestion that the fragment $D_{Ca^{2+}}$ isolated in buffer -C (urea) may be less stable.

4.4 Discussion

The purpose of the foregoing procedures was the development of a method for the large scale production of a pure sample of fragment $D_{Ca^{2+}}$ as a prerequisite for proposed characterisation studies. The first technique employed was that of DEAE-cellulose ion-exchange chromatography and the results of three methods each employing a different buffer system were described. In each case a fragment D exhibiting properties identical to those of fragment $D_{Ca^{2+}}$ was produced. However an unsatisfactory separation of fragment $D_{Ca^{2+}}$ from fragment E was achieved when fibrinogen was digested and chromatographed in a buffer containing 0.1 M-Tris and 0.1 M-NaCl (buffer -A (NaCl)). The possibility that this

effect may have been attributable to the relatively high ionic strength of the buffer was rejected in favour of the proposal that the plasmic digestion of fibrinogen in the presence of buffer A produced structurally modified forms of either or both of fragments D and E. As a consequence the affinity of these molecules for each other and for the ion-exchanger may have been altered. This reasoning was prompted by finding that dialysis of the digest sample to reduce both the level of Tris and NaCl did not increase the affinity of fragment E for the ion-exchanger. The effect of buffer A in relation to fragment E was not reversible. Of the three buffer systems studied, the composition of buffer -A (NaCl) most closely resembles that of plasma. Thus the higher affinity of fragment E for fragment D than for the ion-exchanger may be an illustration of the forces of attraction between the D and E domains of fibrinogen which, according to Plow et al. (1977), are important in the maintenance of fibrinogen conformation in vivo.

The failure of this procedure to provide a preparation of pure fragment $D_{Ca^{2+}}$ necessitated the modification of the buffer system. Two alterations were made to its composition. In the first instance the ionic strength of the buffer was decreased by reducing both the concentration of Tris and by omitting NaCl (buffer -B (blank)). Secondly, buffer system C

was prepared by adding urea to buffer B. The use of 2 M-urea was inspired by the work of Haverkate & Timan (1977) who reported that fragment $D_{Ca^{2+}}$ is resistant to further proteolysis by plasmin even in the presence of 2 M-urea. Thus it was proposed to exploit the chaotropic properties of urea to dissociate the fragment D:E complex with, in theory, no resulting detrimental effect on fragment $D_{Ca^{2+}}$.

The composition of the fibrinogen digest samples prepared in each of the buffer systems was similar but a greater degree of resolution of fragment $D_{Ca^{2+}}$ from other digest components was obvious with buffer system -C (urea) in each of the column procedures. The fragment $D_{Ca^{2+}}$ isolated with buffer system -C (urea) and with -B (blank) displayed characteristics which were described in Division 1 as being typical of fragment $D_{Ca^{2+}}$.

The final Division (3) of Part B will describe the results of studies designed to contrast the properties of a fragment D prepared both in the presence and the absence of Ca^{2+} . This study necessitated the isolation of a pure sample of each type of fragment D. In the case of fragment $D_{Ca^{2+}}$ the sample prepared in the presence of buffer system B was selected. The reason for this choice was twofold; firstly the present results suggest that the fragment $D_{Ca^{2+}}$ produced in the presence of 2 M-urea (i.e. buffer -C (urea)) is

unstable even at 4°C. After storage at 4°C for 48h fragment $D_{Ca^{2+}}$ exhibited a decreased electrophoretic mobility - a transition which has been hitherto ascribed to a change in the molecular conformation of fragment $D_{Ca^{2+}}$. Evidence consistent with limited degradation of the constituent δ chain having occurred was also presented. Secondly buffer system -C (urea) could not be applied to the isolation of a fragment D_B , the second requirement for comparative studies. This fragment D is susceptible to further degradation by plasmin in the presence of urea. Thus the requirement of proposed characterisation studies necessitating the production of each type of fragment D under identical conditions favoured the selection of buffer system -B (blank) for the large scale production of fragment $D_{Ca^{2+}}$.

SECTION 5

THE ISOLATION AND PURIFICATION OF
FRAGMENT D_B

5.1 Introduction

The ensuing experiments describe the preparation of fragment D_B i.e. fragment D prepared in the absence of added CaCl₂. The purpose of this work was to achieve a homogeneous, pure preparation of fragment D_B suitable for proposed comparative studies.

In the first instance the separation method employed was identical to that developed in the preceding experiments for the isolation of fragment D_{Ca²⁺} but 2 mM-CaCl₂ was omitted from all buffer solutions. However when results indicated failure to isolate fragment D_B the isolation procedure was modified by the addition of EDTA to all buffer solutions. In accordance with the nomenclature rules previously established (Division 1, Section 2) this fragment D will be referred to as fragment D_{EDTA}.

Finally the isolation of yet another fragment D sample i.e. fragment Ca²⁺D_{EDTA} which was prepared by limited plasmic digestion of EDTA-treated fragment D_{Ca²⁺} will be described.

5.2 Methods

5.2.1 Isolation of fragment D_B

Those procedures described in Sections 4.2.2-4.2.6

for the preparation of fragment $D_{Ca^{2+}}$ viz. (i) preparation and digestion of fibrinogen (ii) ion-exchange chromatography employing DEAE-cellulose (iii) lysine-Sepharose 4B affinity chromatography and finally (iv) gel filtration on Sephadex G-200 were applied in the isolation of (a) fragment D_B and (b) fragment D_{EDTA} . One important modification was made in each case; in (a) the buffer employed was 0.05 M-Tris/HCl, pH 7.5 and in (b) 0.05 M-Tris/HCl, pH 7.5 containing 5 mM-EDTA.

All column elutions were accomplished by additions, where appropriate, to the above standard buffer solutions as previously detailed (Section 4.2.1).

5.2.2 The preparation of fragment $Ca^{2+}D_{EDTA}$ by plasmic digestion of fragment $D_{Ca^{2+}}$.

Fragment $Ca^{2+}D_{EDTA}$ was prepared by limited plasmic digestion of fragment $D_{Ca^{2+}}$ which had been exposed to EDTA. Fragment $D_{Ca^{2+}}$ (10mg) isolated in Section 4 employing 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM- $CaCl_2$ was dialysed against this buffer containing 5 mM-EDTA in place of 2 mM- $CaCl_2$. Following the addition of plasmin (0.07 CA units/mg of fragment $D_{Ca^{2+}}$) digestion was allowed to proceed at 37°C for 40min; Trasylol was then added (250 K.I.U./CA unit plasmin).

5.2.3 The isolation of fragment $Ca^{2+}D_{EDTA}$

The fragment D digest solution prepared above was

Fig. 5.1 DEAE-cellulose chromatography of a plasmic digest
of fibrinogen containing fragment D_B

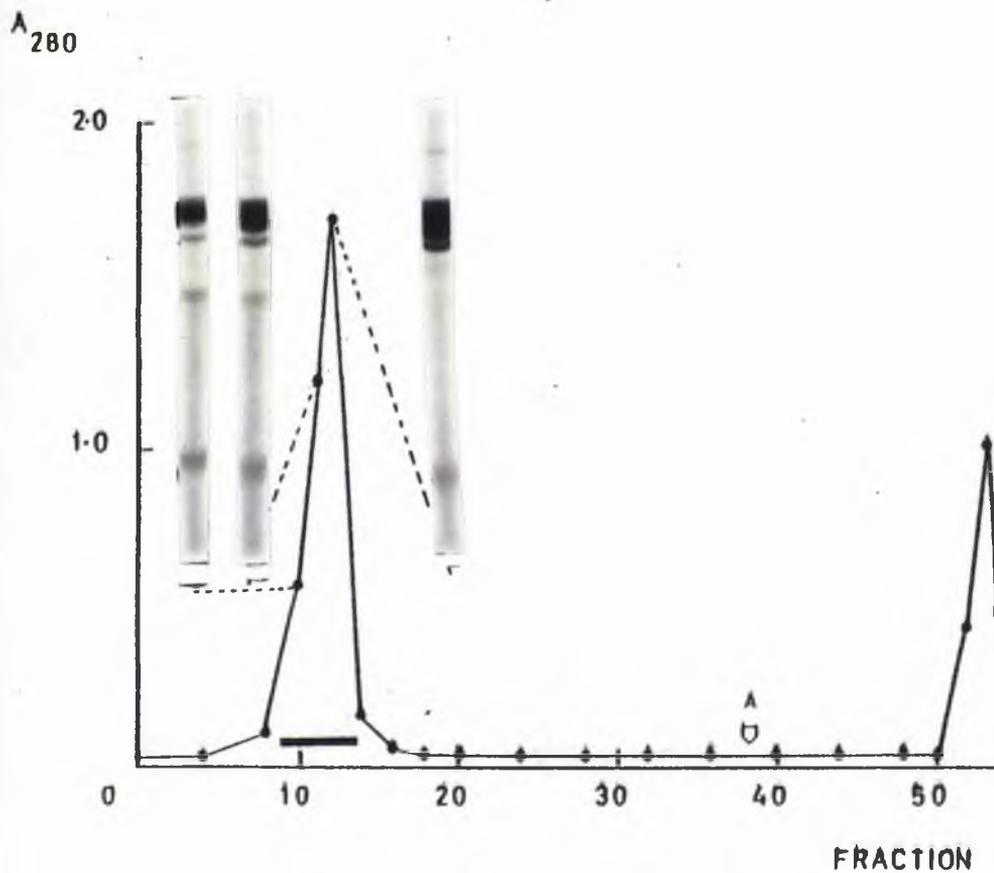
A plasmic digest of fibrinogen was applied to a DEAE-cellulose column and eluted with 0.05 M-Tris/HCl buffer pH 7.5 containing 100 K.I.U./ml-Trasylol.

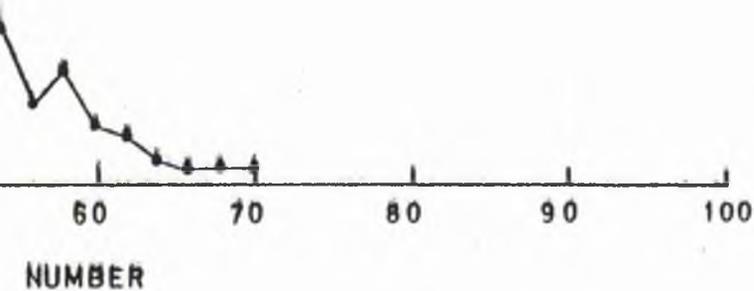
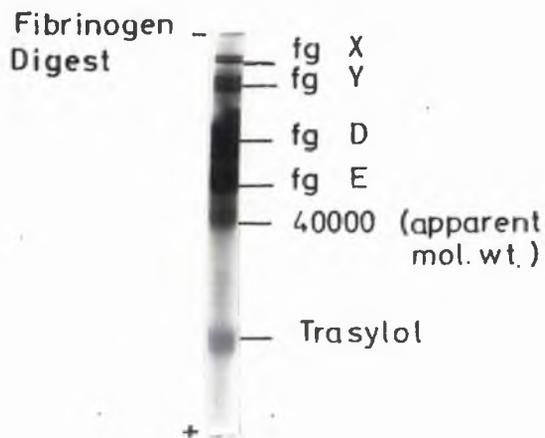
At ^A _Q elution was continued with 0.05 M-Tris/HCl buffer, pH 7.5 containing 0.3 M-NaCl. Polyacrylamide gel electrophoretograms of the fibrinogen digest sample and of eluted fractions (unreduced) are shown (5% gels).

Column dimensions 2.5 x 30cm. Flow rate: 40ml/h. Fractions: 10min.

Fractions were pooled as indicated by the horizontal bar for further chromatography.

Fig. 5-1





applied immediately to a column (2.5 x 5cm) of lysine-Sephrose 4B equilibrated with 0.05 M-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA. The sample was eluted with (a) equilibration buffer and (b) equilibration buffer containing 0.5 M-NaCl and 0.2 M-6-amino-n-hexanoic acid. The eluted fractions were each concentrated by freeze drying.

The reconstituted (fragment $\text{Ca}^{2+}\text{D}_{\text{EDTA}}$ - containing) sample corresponding to elution (a) was applied to a column (2.5 x 30cm) containing Sephadex G-200 which was equilibrated and then eluted with 0.05 M-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA.

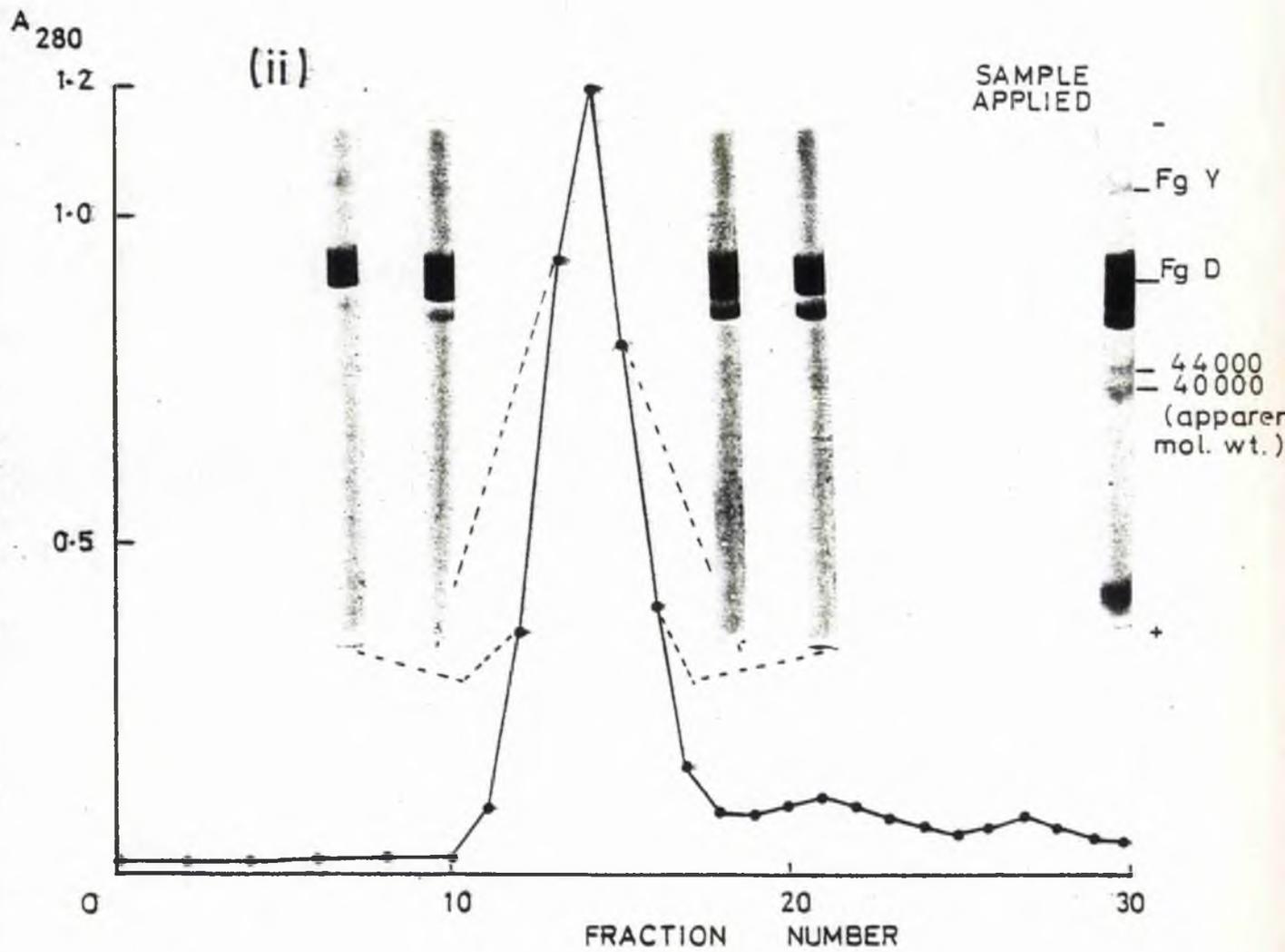
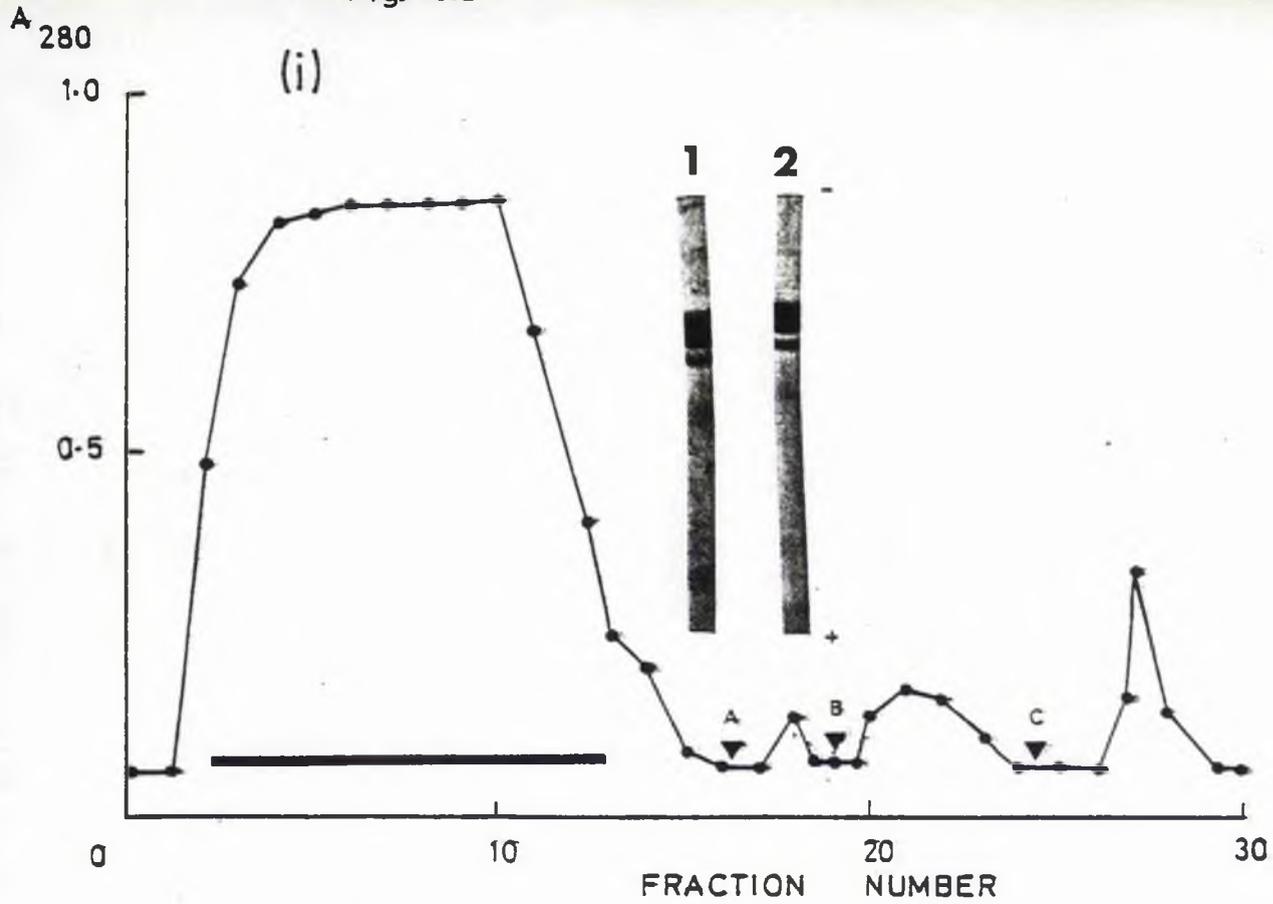
5.3 Results

5.3.1 The preparation of fragment D_{B}

Fragment D_{B} was prepared by plasmic digestion of fibrinogen in the absence of added CaCl_2 . DEAE-cellulose chromatography resolved the digest into two peaks - the second was eluted following the application of a high salt-containing buffer (Fig. 5.1). The results of SDS-polyacrylamide gel electrophoresis of the digest sample applied to the column and of aliquots removed from eluted fractions are shown and suggest that those fractions comprising the initial peak contain, in addition to fragment D_{B} , species of apparent molecular weights 120,000 (possible fragment Y), 40,000 and

Fig. 5.2 The isolation of fragment D_B

- (i) Contaminating plasmin(ogen) was removed from a preparation of fragment D_B by affinity chromatography on lysine-Sepharose 4B. Column dimensions: 1.0 x 10cm. Flow rate: 40ml/h. Elution procedure: 0.05 M-Tris/HCl buffer, pH 7.5, followed by A, 0.5 M-NaCl in the same buffer; B, 0.2 M-6-amino-n-hexanoic acid in distilled water and C, 0.05 M-Tris/HCl buffer, pH 7.5, containing 0.5 M-NaCl and 0.2 M-6-amino-n-hexanoic acid. 6ml fractions were collected. Polyacrylamide gel electrophoretograms of (1) the sample applied to the column and (2) the pooled sample eluted from the column are shown (unreduced, 5% gels). Fractions were pooled as indicated by the horizontal bar for further chromatography.
- (ii) The pooled fragment D_B-containing fractions eluted from the above column were fractionated by Sephadex G-200 gel filtration. The column was eluted with 0.05 M-Tris/HCl buffer, pH 7.5. Polyacrylamide gel electrophoretograms of the fragment D_B-containing sample applied to the column and of eluted fractions are shown, (unreduced, 5% gels). Column dimensions: 2.5 x 34cm. Flow rate 10ml/h. Fractions: 30min.



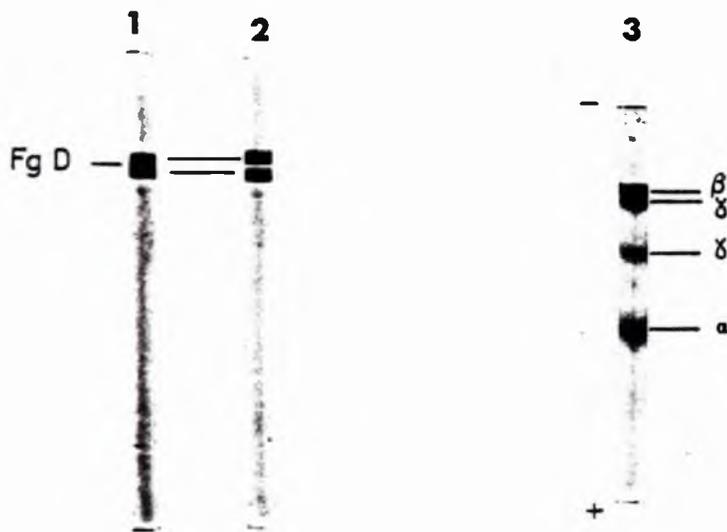
13,500 (Trasylol). This pattern of elution is very similar to that obtained during the isolation of fragment $D_{Ca^{2+}}$. However, it is noteworthy that in the present case the SDS-gel electrophoresis results reveal an obvious splitting of the fragment D_B band. The higher mobility component is more prevalent in the later fractions. The presence of this lower molecular weight form of fragment D_B implies that the absence of added Ca^{2+} has favoured the production from fibrinogen of a fragment D with an increased susceptibility to further degradation.

Those fractions comprising the initial (fragment D_B - containing) peak eluted above, were pooled and applied immediately to a column of lysine-Sepharose 4B to remove residual contaminating plasmin(ogen). The corresponding elution profile is shown in Fig. 5.2 (i). The results of SDS-gel analysis of the sample of fragment D_B (unreduced) (1) before and (2) after its passage through the gel are shown and do not reveal significant changes in the composition of the sample as a consequence of the column treatment. However material was eluted from the lysine-Sepharose 4B gel by solutions B (containing 6-amino-n-hexanoic acid) and C (containing both NaCl and 6-amino-n-hexanoic acid). The composition of each of these eluted fractions was not investigated.

The fragment D_B -containing fractions were concentrated prior to the final isolation step i.e.

Fig. 5.3

SDS-Polyacrylamide gel electrophoresis of fragment D_B



Fragment D_B prepared by digestion of fibrinogen by plasmin in 0.05 M-Tris/HCl buffer, pH 7.5, and isolated by DEAE-cellulose chromatography and gel filtration on Sephadex G-20 was examined **1** unreduced, **2** unreduced but following its incubation in the presence of 10 mM-EDTA (5% gels), and **3** reduced (100 °C, 5 min), (10% gel).

gel filtration. No reaction of this concentrate against anti-human plasminogen antiserum was detected by immunoelectrophoresis which, within the limits of this technique, implies that residual contaminating plasmin(ogen) was successfully removed from the fragment D_B preparation by the lysine-Sepharose 4B column procedure.

The elution profile corresponding to Sephadex G-200 gel filtration of the fragment D_B preparation is shown in Fig. 5.2 (ii). The results of SDS-gel electrophoresis of the sample applied to the column and of aliquots removed from eluted fractions (unreduced) are shown. A splitting of the fragment D_B is again obvious which, it has been suggested above, may reflect the omission of the fragment D stabilising agent Ca^{2+} from the buffer solutions. In addition the band of apparent molecular weight 40,000 is also split.

In the previous Section of work two tests were performed to confirm that the isolated fragment D was in fact $D_{Ca^{2+}}$. The results obtained with the present fragment D (D_B) are shown in Fig. 5.3. Pretreatment of fragment D_B with EDTA, contrary to expectation, was not without effect, instead, approximately one half of the fragment D_B sample displayed the characteristic electrophoretic mobility decrease hitherto ascribed to fragment $D_{Ca^{2+}}$ (gels 1 and 2). Upon reduction at $100^\circ C$ two species of δ chain were produced (gel 3) one of

which displays an apparent molecular weight of 40,000, a value attributed to the δ chain component of fragment $D_{Ca^{2+}}$, while the other has an apparent molecular weight of 27,000. This value is identical to that revealed by analysis of a fragment D prepared in the absence of added Ca^{2+} and described in Division 1. Thus this preparation appears to consist of both fragments $D_{Ca^{2+}}$ and D_B . At this point it became necessary to consider the possibility that the equipment which had been exposed to Ca^{2+} -containing buffer solutions, despite routine cleansing, had in fact retained sufficient Ca^{2+} to influence the course of fibrinogen digestion. A value of $9.0 \times 10^{-6}M$ has been reported for the dissociation constant of the Ca^{2+} -binding site of human fibrinogen fragment $D_{Ca^{2+}}$ (Nieuwenhuizen et al. 1979). Thus the assumption that a solution containing no "added $CaCl_2$ " is in fact " Ca^{2+} -free" may have been invalidated. This problem has been referred to earlier (Division 1, Section 6.3.5).

5.3.2 The preparation of fragment D_{EDTA}

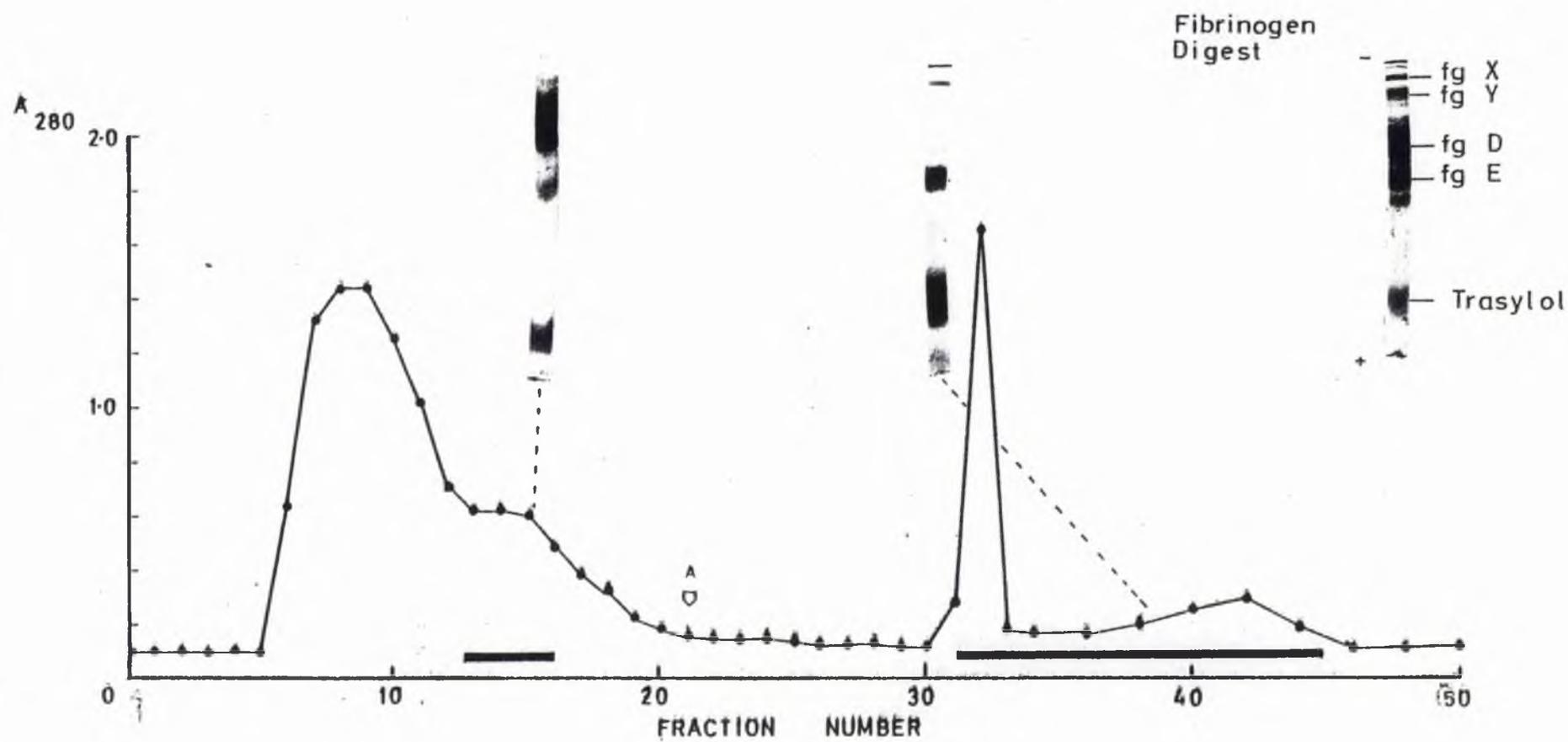
The method of preparing fragment D_{EDTA} was identical to that described for fragment D_B except that 5 mM-EDTA was present in all buffer solutions. The fibrinogen digest sample was applied to a column of DEAE-cellulose and the corresponding elution pattern

Fig. 5.4

DEAE - cellulose chromatography of a plasmic digest of fibrinogen
containing fragment D_{EDTA}

A plasmic digest of fibrinogen was applied to a DEAE-cellulose column and eluted with 0.05 M-Tris/HCl buffer, pH 7.5 containing 100 KIU/ml - Trasylol and 5 mM-EDTA. At \square^A elution was continued with 0.05 M-Tris/HCl buffer, pH 7.5, containing 0.3 M-NaCl. Polyacrylamide gel electrophoretograms of the fibrinogen digest sample and of eluted fractions (unreduced) are shown (5% gels). Column dimensions: 2.5 x 30 cm. Flow rate: 30 ml/h. Fractions: 10 min. Fractions were pooled as indicated by the horizontal bar.

Fig. 5.4

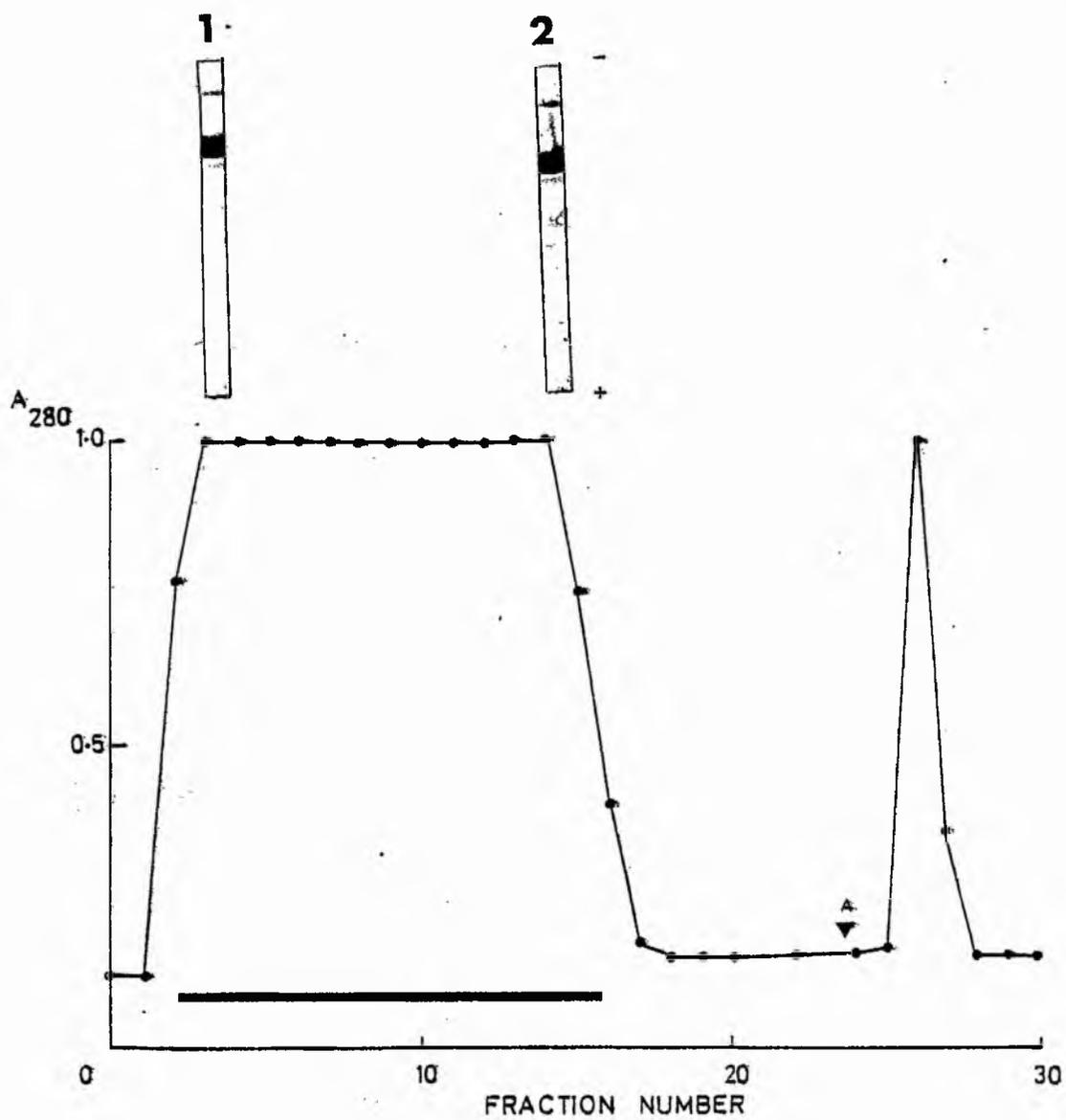


is shown in Fig. 5.4. The results of SDS-gel analysis (5% gels) of the unreduced sample applied to the column and of two pools of eluted fractions are shown. The digest sample displays a broad band of mobility appropriate to that of fragment D. A faint band is obvious between this band and that of fragment E and suggests that degradation of fragment D_{EDTA} had occurred. This is consistent with the concept that EDTA has removed a fragment D_{Ca²⁺} stabilising agent - namely Ca²⁺. A species of electrophoretic mobility appropriate to fragment E was eluted by the high salt-containing buffer. The fractions corresponding to the major portion of the initial peak were not examined by SDS-electrophoresis but were pooled and applied immediately to a column of lysine-Sepharose 4B to effect the removal of contaminating plasmin(ogen). The elution pattern is shown in Fig. 5.5. SDS-gel analysis of the sample before (gel 1) and after (gel 2) this purification process fails to reveal evidence of the removal of any component of the fragment D_{EDTA} sample. Neither the sample before nor after purification reacted against anti-human plasminogen antiserum. Nevertheless evidence consistent with the removal of contaminating plasmin(ogen) from the fragment D_{EDTA} sample was provided by the significant elution from the lysine-Sepharose 4B column induced by the application of a buffer containing NaCl and 6-amino-n-hexanoic acid. This material reacted

Fig. 5.5 Lysine-Sepharose 4B chromatography
of a fragment D_{EDTA} preparation

Contaminating plasmin(ogen) was removed from a preparation of fragment D_{EDTA} by affinity chromatography on lysine-Sepharose 4B. Column dimensions: 1.0 x 10cm. Flow rate: 40ml/h. Elution procedure: 0.05 M-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA followed by A, 0.5 M-NaCl and 0.2 M-6-amino-n-hexanoic acid in the same buffer. 6ml fractions were collected. Polyacrylamide gel electrophoretograms of (1) the sample applied to the column and (2) the pooled sample eluted from the column are shown (unreduced, 5% gels). Fractions were pooled as indicated by the horizontal bar for further chromatography.

Fig. 5.5

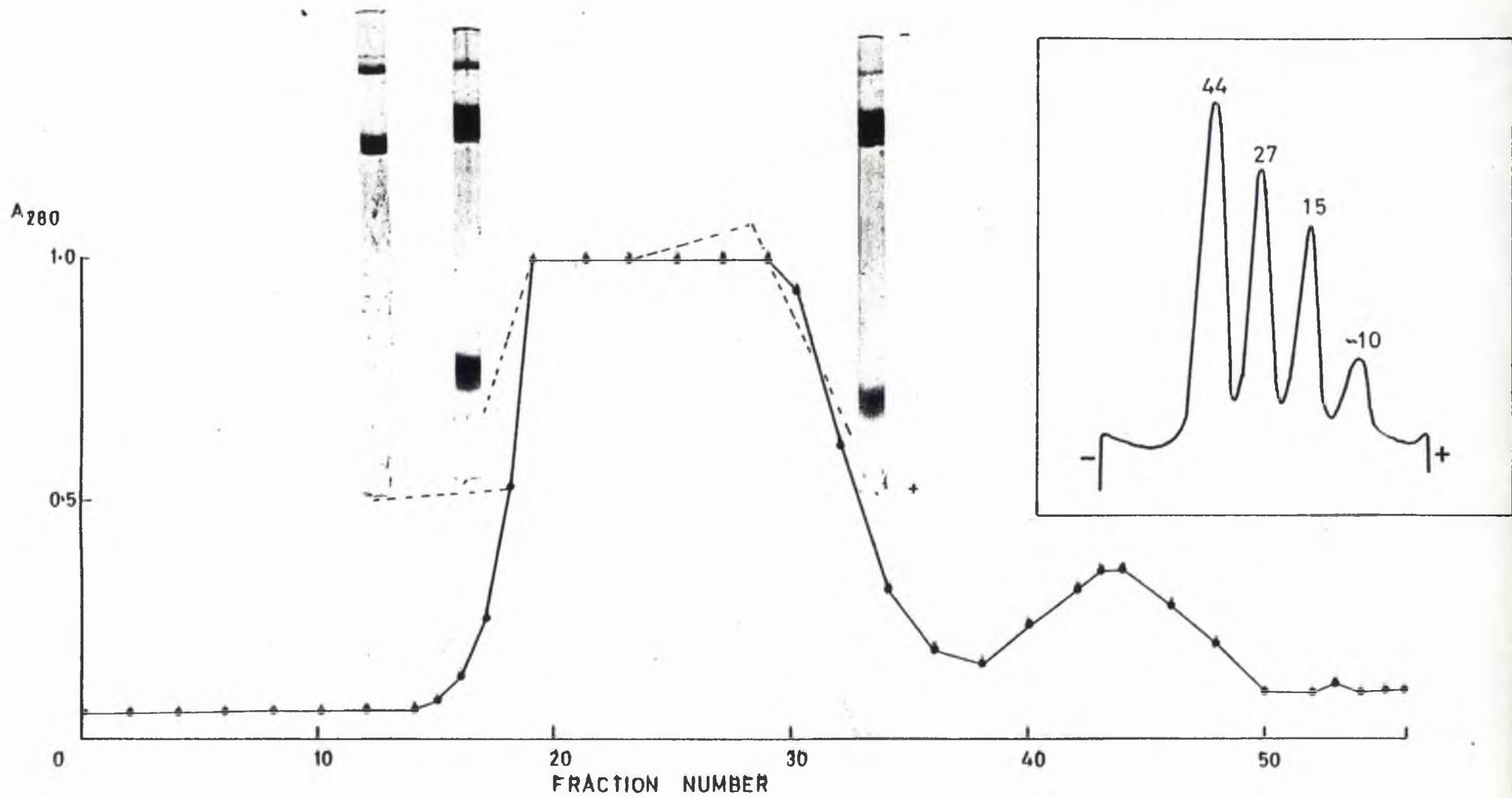


against anti-human plasminogen antiserum. A reaction against anti-human fibrinogen antiserum was also noted. This coelution of a fibrinogen-like species with plasmin(ogen) and the consequent detrimental effect on the yield of fragment D has been noted previously. Gel filtration of the purified fragment D_{EDTA} preparation (which produced a strong reaction against anti-human fibrinogen antiserum) on Sephadex G-200 gave the elution profile shown in Fig. 5.6. A broad fragment D_{EDTA} -containing peak and a less intense peak eluted immediately before the bromophenol blue marker dye, are obvious. Several points relating to this figure are worthy of comment. It will be noted that SDS-gel analysis of the eluted fractions has revealed that the fragment D_{EDTA} samples are contaminated by a high mobility band. This band was not apparent in the fragment D_{EDTA} preparation examined at the time of lysine-Sepharose 4B purification (Fig. 5.5). Furthermore a similar high mobility band was revealed by SDS-gel analysis of the pooled eluant comprising fractions 38-50. The molecular weight of this band could not be estimated with accuracy, being outwith the range covered by the protein molecular weight standards, but a possible, although speculative, explanation for each of these observations is as follows. The fragment D_{EDTA} molecule may be unstable. During gel filtration a chain fragment is released thereby producing the high mobility band obvious in the SDS-gels

Fig. 5.6 Fractionation of a fragment D_{EDTA} preparation on Sephadex G-200

A fragment D_{EDTA} preparation freed from contaminating plasmin(ogen) by lysine-Sepharose 4B chromatography was subjected to gel filtration on Sephadex G-200. The column was eluted with 0.05 M-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA. Polyacrylamide electrophoretograms of eluted fractions are shown, (unreduced, 5% gels). Column dimensions: 2.5 x 35cm. Flow rate: 9ml/h. Fractions: 30min. Inset: densitometer scan of a reduced sample of fragment D_{EDTA} examined by SDS-gel electrophoresis (10% gel). Apparent molecular weights ($\times 10^{-3}$) are shown.

Fig. 5.6



and the additional low molecular weight material identified by gel filtration. Information relating to the source of this released polypeptide is provided by the SDS-electrophoresis result shown inset into Fig. 5.6. This densitometric scan of a reduced sample of fragment D_{EDTA} shows four component peaks of apparent molecular weights 44,000, 27,000, 15,000 and approximately 10,000. Previous studies infer that the first three peaks represent the β , δ and α constituent chains of fragment D_{EDTA} respectively. The fourth component may represent both the low molecular weight material evident in the unreduced sample and the small peak apparent in the gel filtration elution profile. The reduced chain composition of fragment D_{EDTA} differs in one respect from that of a fragment $D_{Ca^{2+}}$ - the molecular weight of the constituent δ chain. It can therefore be proposed that the low molecular weight material detected during the isolation of fragment D_{EDTA} originated from the breakdown of a higher molecular weight form of the constituent δ chain. (The studies described in Division 1 suggest that this latter chain remnant has a molecular weight of 34,000.) Therefore the peptide detected during gel filtration may have a molecular weight of 34,000 minus 27,000 i.e. 7,000.

To summarise, a fragment D_{EDTA} has been isolated in a similar manner to that employed for the isolation of

fragment $D_{Ca^{2+}}$. The form of fragment D_{EDTA} containing a δ chain remnant of molecular weight 34,000, described in Division 1, may be unstable since only the lower molecular weight form of the δ chain (27,000) was detected in the latter stages of the isolation procedure. However several observations are consistent with the proposal that a polypeptide fragment of approximate molecular weight 10,000 was released during the isolation of fragment D_{EDTA} by the breakdown of the constituent δ chain from a molecular weight of 34,000 to 27,000.

5.3.3 The preparation of fragment $Ca^{2+}D_{EDTA}$

Fragment $Ca^{2+}D_{EDTA}$ was prepared by limited plasmin digestion of EDTA-treated fragment $D_{Ca^{2+}}$. The digest was purified, as before, by lysine-Sepharose 4B chromatography and then the eluted sample was fractionated by gel filtration on Sephadex G-200. Each of the elution profiles is shown in Fig. 5.7 (i) and (ii). Polyacrylamide gel analysis results of (1) the fragment $D_{Ca^{2+}}$ sample prior to its dialysis against EDTA, (2) after dialysis and (3) after digestion by plasmin are shown in Fig. 5.8 (i). The dialysis against EDTA precipitated a decrease in the electrophoretic mobility of fragment $D_{Ca^{2+}}$. This change supports the theory that the removal of Ca^{2+} by its chelation, favours a more extended conformation of the fragment $D_{Ca^{2+}}$ molecule. Subsequent exposure to plasmin promoted a splitting of

Fig. 5.7

The preparation of fragment $\text{Ca}^{2+}\text{D}_{\text{EDTA}}$

- (i) Contaminating plasminogen was removed from a preparation of fragment $\text{Ca}^{2+}\text{D}_{\text{EDTA}}$ by affinity chromatography on lysine-Sepharose 4B. Column dimensions: 2.5 x 5cm. Flow rate: 30ml/h. 3ml fractions were collected. Elution procedure: 0.05 M-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA followed by A, 0.5 M-NaCl and 0.2 M-6-amino-n-hexanoic acid in the same buffer. Fractions were pooled as indicated by the horizontal bars.
- (ii) The pooled fragment $\text{Ca}^{2+}\text{D}_{\text{EDTA}}$ -containing fractions eluted from the above column were fractionated by Sephadex G-200 gel filtration. The column was eluted with 0.05 M-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA. Polyacrylamide gel electrophoretograms of fractions eluted from the column are shown, (unreduced, 5% gels).

Fig. 5.7

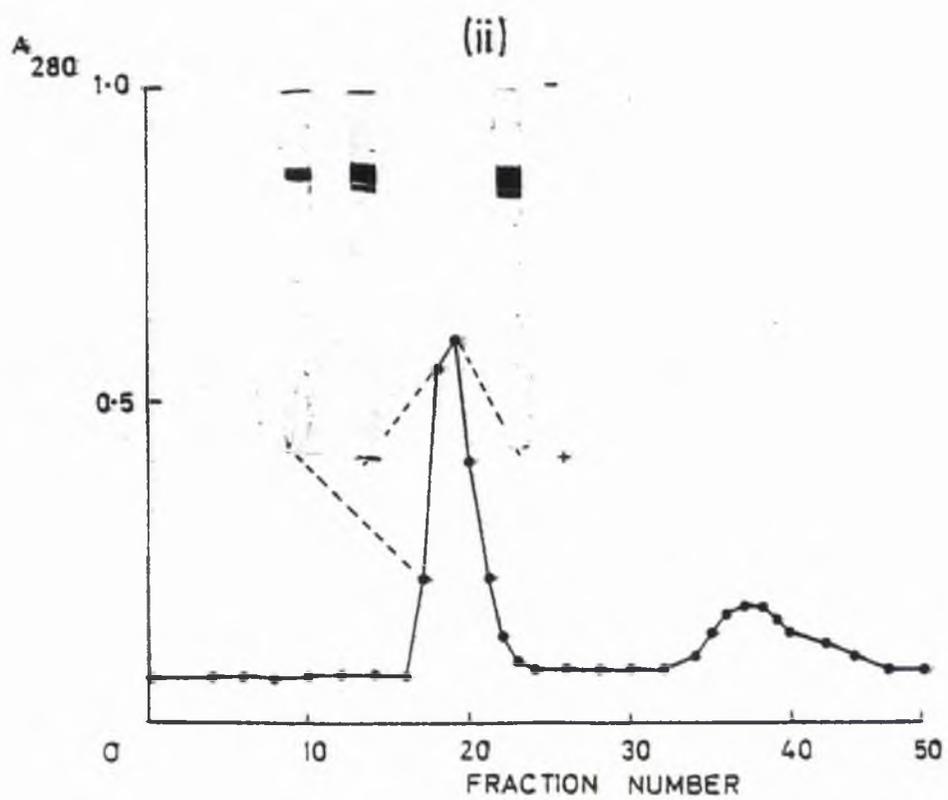
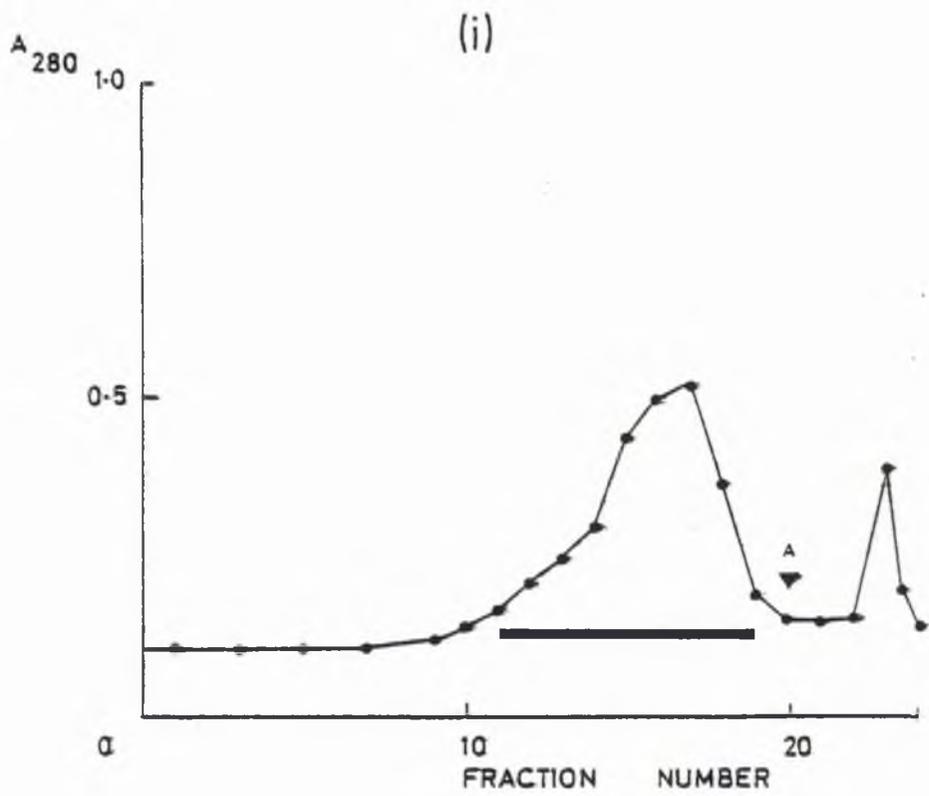
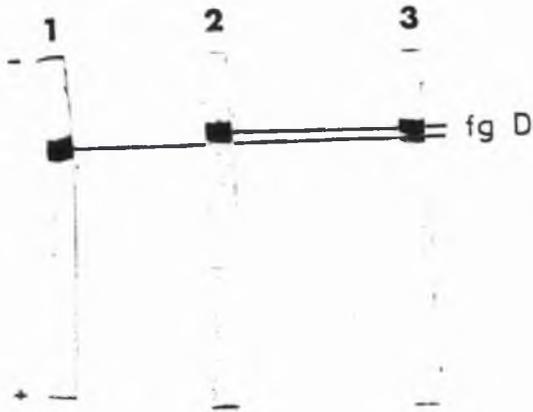


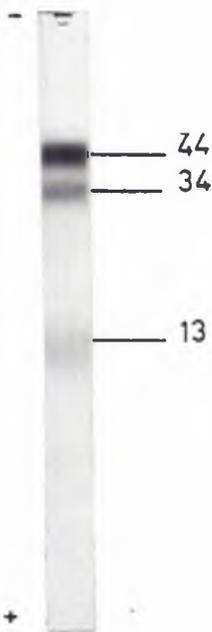
Fig. 5.8 SDS polyacrylamide gel electrophoresis of

fragment D_{Ca}^{++}

(i)



(ii)



Fragment D_{Ca}^{++} was examined by SDS-gel electrophoresis;

- (i) unreduced on 5% gels (**1** prior to dialysis against EDTA, **2** after dialysis and **3** after digestion by plasmin)
- (ii) reduced on 10% gel after treatment with EDTA and plasmin. Apparent mol wts ($\times 10^{-3}$) are shown.

the fragment D band (gel 3).

As in previous experiments the lysine-Sepharose 4B purification procedure removed a species from the fragment D digest which reacted against anti-fibrinogen and anti-plasminogen antisera. Thus the removal of plasmin(ogen) from the digest was accomplished with the loss of some of the fragment $\text{Ca}^{2+}\text{D}_{\text{EDTA}}$ sample. However no reaction of the concentrated fragment $\text{Ca}^{2+}\text{D}_{\text{EDTA}}$ sample eluted from the lysine-Sepharose 4B column against anti-plasminogen antisera was detected. This suggests that the removal of plasmin(ogen) had been successfully accomplished. The elution profile corresponding to the Sephadex G-200 gel filtration of the fragment $\text{Ca}^{2+}\text{D}_{\text{EDTA}}$ sample is composed of two peaks: a major one and a minor peak eluted immediately before the bromophenol blue marker dye. The results of polyacrylamide gel analysis of aliquots removed from fractions comprising the major peak are shown and a slight splitting of the fragment D band is obvious. The comparable reduced chain analysis (Fig. 5.9 (ii)) infers a chain composition for the fragment $\text{Ca}^{2+}\text{D}_{\text{EDTA}}$ molecule as follows: β -MW 44,000; δ -MW 34,000 and α -MW 13,000. The molecular weight of the protein comprising the peak eluted immediately before the bromophenol blue dye cannot be predicted accurately by polyacrylamide gel electrophoresis but a figure of approximately 10,000 was calculated. Fragment $\text{Ca}^{2+}\text{D}_{\text{EDTA}}$ is apparently

identical to fragment $D_{Ca^{2+}}$ except that the latter fragment contains a constituent δ chain of higher molecular weight. By inference therefore the production of fragment $Ca^{2+}D_{EDTA}$ involved the attack of plasmin on the δ chain component of fragment $D_{Ca^{2+}}$ with the resultant release of a polypeptide chain of molecular weight 40,000 minus 34,000 = 6,000. This released polypeptide may be responsible for the production of the minor protein-containing peak eluted from the Sephadex G-200 column. This proposal accords with the hypothesis put forward to explain the presence of a similar peak detected during the gel filtration of a sample containing fragment D_{EDTA} . However in the latter case the polypeptide fragment was assumed to represent the difference between two fragment D_{EDTA} δ chain constituents of molecular weights 34,000 and 27,000 and to arise from the breakdown of the higher to the lower molecular weight form during the gel fractionation process. This reasoning would explain the co-elution of the low molecular weight polypeptide, and the parent fragment D_{EDTA} molecule from the column. In this case however, the polypeptide may have arisen from the prior enzymic degradation of the δ chain constituent of fragment $D_{Ca^{2+}}$ from a molecular weight of 40,000 to 34,000 and would therefore not be expected to co-elute with the fragment D molecule during gel filtration.

5.4 Discussion

The results from three procedures for the isolation of fragment D from a plasmic digest of fibrinogen in the absence of added Ca^{2+} have been presented.

The first of these methods isolated a fragment D preparation which was composed, to a significant extent, of fragment $\text{D}_{\text{Ca}^{2+}}$. This occurrence was attributed to the degradation of fibrinogen in the presence of "contaminating" Ca^{2+} introduced by the buffer solution. Accordingly the method was modified by the addition of EDTA to all buffer solutions employed during the second procedure for the isolation of fragment D, which was therefore designated fragment D_{EDTA} . Apart from this alteration, fragment D_{EDTA} was isolated under identical conditions to those employed with fragment $\text{D}_{\text{Ca}^{2+}}$. The examination of the reduced chain composition of fragment D_{EDTA} revealed that it contained a lower molecular weight form of the constituent γ chain than did fragment $\text{D}_{\text{Ca}^{2+}}$. This result supports the view that the digestion of fibrinogen in the absence of Ca^{2+} produces a fragment D which is susceptible to the degradative action of plasmin, more particularly at the constituent γ chain.

Finally the preparation of fragment $\text{Ca}^{2+}\text{D}_{\text{EDTA}}$ was described. This fragment D was prepared by plasmic degradation of fragment $\text{D}_{\text{Ca}^{2+}}$ which had been exposed to

EDTA to effect the removal of stabilising Ca^{2+} . The analysis of the reduced chain composition of this fragment $\text{Ca}^{2+}\text{D}_{\text{EDTA}}$ molecule suggested that it had been produced as a consequence of the removal of a 6,000 molecular weight peptide from the constituent δ chain of fragment $\text{D}_{\text{Ca}^{2+}}$.

The purpose of the work described in this Section was to procure the isolation of a pure sample of a fragment D, prepared in the absence of added Ca^{2+} , suitable for comparative studies with fragment $\text{D}_{\text{Ca}^{2+}}$. The fragment D preparation selected for these studies was that of fragment D_{EDTA} , a choice governed by the fact that this fragment D was prepared under identical conditions to those employed in the isolation of fragment $\text{D}_{\text{Ca}^{2+}}$. This condition was not satisfied by the sample of fragment $\text{Ca}^{2+}\text{D}_{\text{EDTA}}$ which was instead produced from fragment $\text{D}_{\text{Ca}^{2+}}$ by further plasmic digestion.

SECTION 6

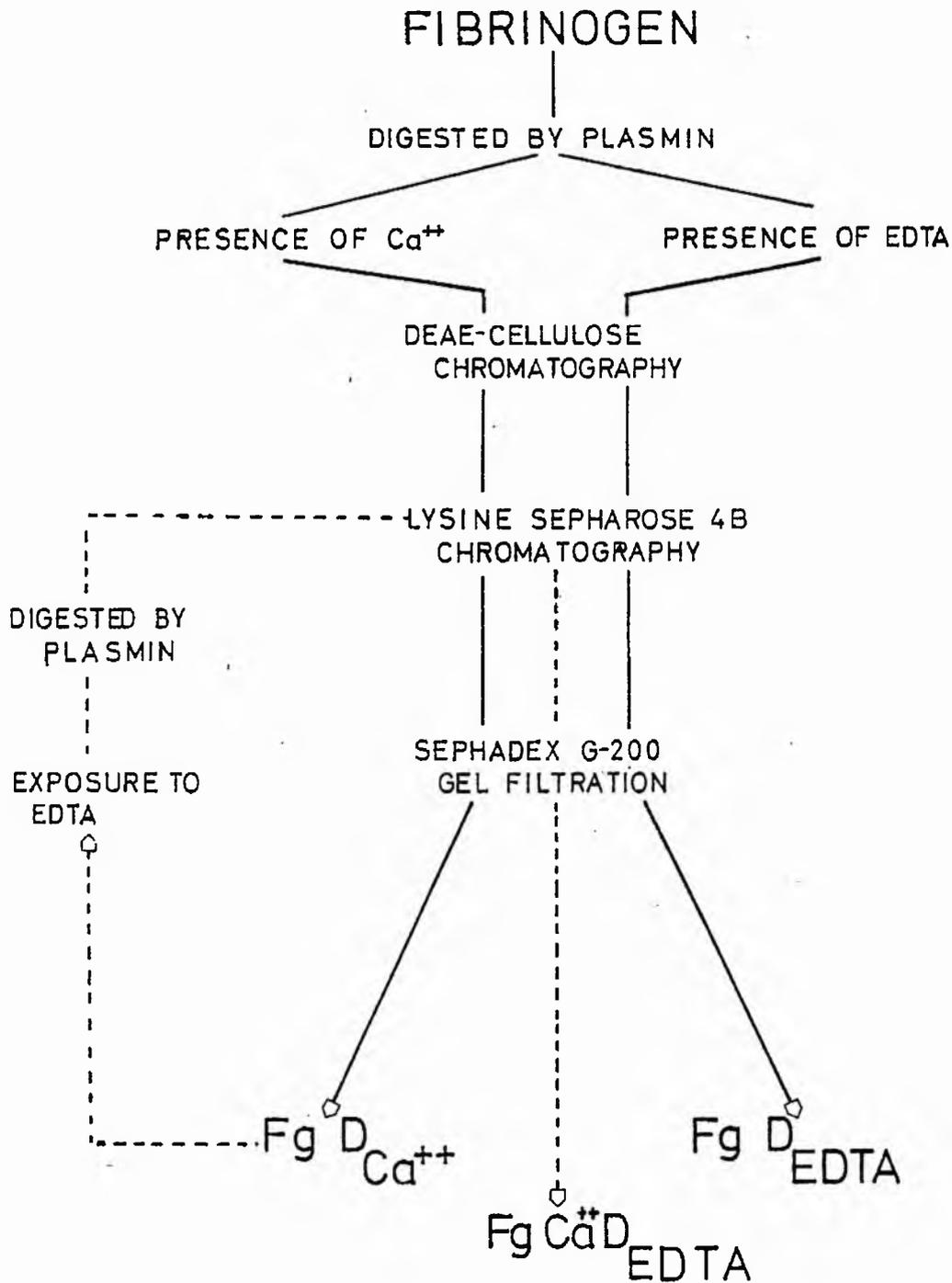
DISCUSSION

The initial Sections of this Division were concerned with the development of a procedure for the isolation of fragment $D_{Ca^{2+}}$ under conditions compatible with the presence of free Ca^{2+} . A method employing successively DEAE-cellulose ion-exchange chromatography (to remove fragment E), lysine-Sepharose 4B affinity chromatography (to remove contaminating plasmin(ogen)) and finally Sephadex G-200 gel filtration (to remove both higher and lower molecular weight species) has been described. This operation accomplished the isolation of a fragment $D_{Ca^{2+}}$ which displayed identical properties to those of the fragment $D_{Ca^{2+}}$ characterised from the fibrinogen digestion studies performed in the foregoing work, Division 1.

The simple omission of Ca^{2+} from the buffer solution employed in the isolation of fragment $D_{Ca^{2+}}$ however, did not permit the direct application of this procedure to the preparation and isolation of a fragment D_B , and for reasons which have been discussed, the method was modified by the incorporation of the Ca^{2+} - chelating agent, EDTA, in all buffer solutions. Examination of the fragment D thereby isolated i.e. fragment D_{EDTA} revealed that despite being produced under identical

Fig. 6.1 The isolation of fragment D

The procedures employed to prepare and isolate fragments $D_{Ca^{++}}$, D_{EDTA} and $Ca^{++}D_{EDTA}$ are summarised.



conditions of plasmic digestion, it differed from fragment $D_{Ca^{2+}}$ in the extent of degradation of its constituent γ chain. Evidence that this fragment D may be particularly unstable was also presented. Both these observations are consistent with the proposed role of the Ca^{2+} in stabilising and protecting the fragment $D_{Ca^{2+}}$ molecule from further degradation by plasmin.

The proven vulnerability of fragment $D_{Ca^{2+}}$ to further attack by plasmin following the sequestration of the bound Ca^{2+} was exploited in the preparation of a further type of fragment D - namely fragment $Ca^{2+}D_{EDTA}$.

The three fragment D isolation procedures described above are summarised in Fig. 6.1. In each case the isolation of one of the three stages in the digestion of fragment D by plasmin described in Fig 7.1, Division 1 has been accomplished.

Fragments $D_{Ca^{2+}}$ and D_{EDTA} were selected for further comparative studies to be described in the ensuing Division. Perhaps the choice of the fragment D_{EDTA} preparation could be criticised on the grounds that an unproven assumption has been made that the action of EDTA during the course of fibrinogen digestion is restricted specifically to Ca^{2+} chelation. The possibility that EDTA had some additional influence on the course of fibrinogen digestion cannot be ignored.

Thus fragment D_{EDTA} may not be identical to fragment D_B . However the study of fragment D_{EDTA} can be justified on the grounds of evidence presented in Division 1.

Fibrinogen digestion studies indicated that in the absence of added Ca^{2+} the pathway of fragment D digestion was identical both in the presence and absence of EDTA. Furthermore the subunit composition of each of these fragments D was identical to that of the fragment D prepared by the addition of both plasmin and EDTA to a sample of fragment $D_{Ca^{2+}}$. Therefore the evidence from SDS-gel electrophoresis studies is consistent with the view that the influence of EDTA on the course of fibrinogen digestion is restricted to its ability to chelate Ca^{2+} .

PART B
DIVISION **3**

SECTION 1

INTRODUCTION

In Part B (Division 1) of this work the report made by Haverkate & Timan (1977) that a single high molecular weight form of fragment D was produced by the plasmic digestion of fibrinogen in the presence of 2 mM-CaCl₂ was confirmed. A desire to characterise further this fragment D_{Ca²⁺} by techniques in addition to that of SDS-polyacrylamide gel electrophoresis and also in the absence of other fibrinogen digestion intermediates instigated the development of a method for the isolation of a fragment D prepared both in the absence and in the presence of Ca²⁺ (Part B, Division 2). This final Division of Part B recounts the results of studies designed to compare the properties of the fragment D_{Ca²⁺} and the fragment D_{EDTA} so prepared.

SECTION 2

CHARACTERISATION OF FRAGMENTS $D_{Ca^{2+}}$ AND D_{EDTA}

2.1 Introduction

The studies described in Part B, Division 1 of this work led to the development of a model for the fragment $D_{Ca^{2+}}$ molecule in which Ca^{2+} , bound to the constituent γ chain, conferred on the molecule a resistance to further attack by plasmin. A population of fragment D molecules, heterogeneous with respect to molecular weight, (a feature attributed by various investigators to the result of the progressive degradation of the constituent γ chain from its COOH-terminus) was not produced in the presence of 2 mM- $CaCl_2$. Thus it may be proposed that the one distinctive feature of the fragment $D_{Ca^{2+}}$ molecule compared to that of the fragment D_{EDTA} molecule must be an intact constituent γ chain. This characteristic would account for the lower electrophoretic mobility i.e. higher molecular weight displayed by the fragment $D_{Ca^{2+}}$ molecule.

The initial experiments reported below were designed to investigate the validity of this reasoning by determining the NH_2 -terminal amino acids and by estimating the molecular weights by ultracentrifugation, of both fragment $D_{Ca^{2+}}$ and fragment D_{EDTA} .

Finally the results from isoelectric focussing studies are presented. It seems feasible to propose that bound Ca^{2+} , by virtue of its positive charge, might alter the

isoelectric point of the fragment $D_{Ca^{2+}}$ molecule. This has been confirmed for the fibrinogen molecule by Godal (1960a) but the effect of Ca^{2+} on the isoelectric point of fragment D has not been investigated.

2.2. Methods

2.2.1 SDS-polyacrylamide gel electrophoresis

This technique was performed as previously described (Division 1, Section 3.2.1).

2.2.2 NH_2 -terminal amino acid determination

NH_2 -terminal amino acids were determined employing dansyl chloride using a previously described technique (Part A, Section 2.2.5).

2.2.3 Ultracentrifugation

The molecular weights of fragments $D_{Ca^{2+}}$ and D_{EDTA} were determined by meniscus depletion sedimentation equilibrium analysis (Yphantis, 1964). The instrument used was a Spinco Model E analytical ultracentrifuge (Beckman). Suitable aliquots of fragment D (0.2-0.4mg/ml) were dissolved in 0.05 M-Tris/HCl buffer, pH 7.5, (containing 2mM- $CaCl_2$ where appropriate) and then dialysed exhaustively against several changes of this buffer at 4°C.

Conditions for ultracentrifugation were;

Temperature: 20°C
Speed: 17,980 rev./min
Cell: 12mm with double-sector centre
piece (two 2.5° sectors)

Optical System: Rayleigh interference optics.

Fringe displacements were measured using a travelling microscope (Projectoscope PG Ltd.). Readings commencing at the meniscus were taken at 200 micron intervals along the X-scale until a deflection of more than 10 microns between consecutive readings occurred on the Y-scale. Readings were then made at 100 micron intervals along the X-scale. The programme of Yphantis & Roark (1972) was employed in the computation of molecular weights from experimental data using a value of 0.725ml/g (Nussenzweig *et al.*, 1961) for the partial specific volume of fragment D and 1.00g/ml for the density of the buffer solution.

2.2.4 Isoelectric focussing

Isoelectric focussing of fragments D was carried out with modifications to the methods of Arneson (1974) and Wrigley (1969).

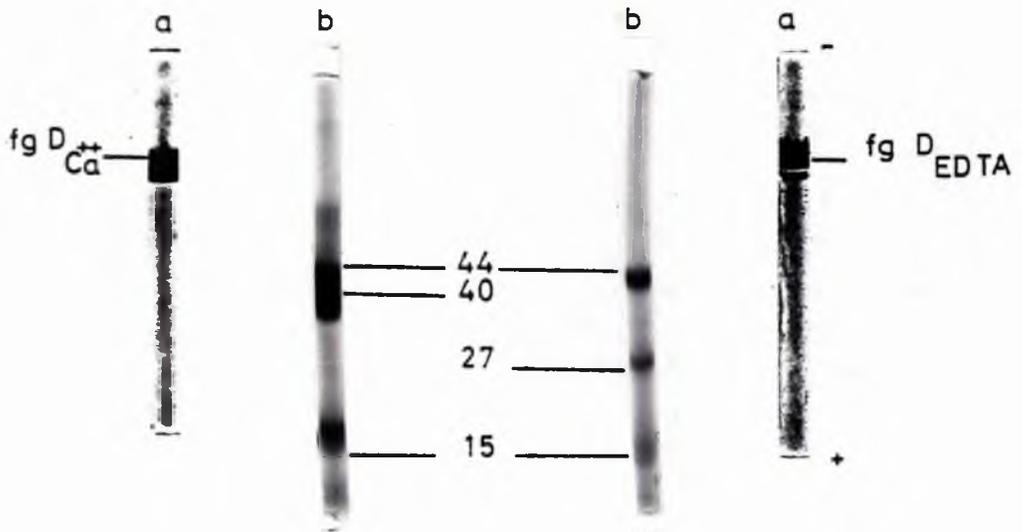
The protein sample was embedded in the gel matrix. Gels comprised 4.75% (w/v) acrylamide (12.5% (w/w) methylenebisacrylamide) 4.5 M-urea, 2% (v/v) ampholine (pH range 3.5-10) and 500 µl protein sample (30-300 µg). Polymerisation was initiated by the addition of 200 µl

ammonium persulphate (final concentration 0.083% (w/v)) and the gels were then cast in acid-cleaned glass tubes (0.5 x 10cm). (A gel, containing 500 μ l of 0.05 M-Tris/HCl buffer, pH 7.5, in place of protein was also prepared to estimate the established pH gradient).

After a period of one hour, electrofocussing was performed in a water-cooled Shandon electrophoresis chamber containing 0.02 M- H_2SO_4 in the upper (anode) compartment and 0.08 M-NaOH in the lower (cathode) compartment. Current was supplied by a Shandon Southern power pack. The voltage was gradually increased during the first hour to maintain a current of 2 mA/gel (to a maximum of 250v). The focussing was terminated after 8h and the gels placed in 5% (w/v) trichloroacetic acid for a period of 18h. This solution served both to fix protein bands and to remove carrier ampholytes. The gels were then washed for 1h in acetic acid/ethanol/water (10:50:40, by vol.) prior to being stained with 0.1% (w/v) Coomassie blue R250 in acetic acid/ethanol/water (10:45:45, by vol.) for 1h. The gels were destained over a period of 48h with acetic acid/ethanol/water (10:25:65, by vol.) and then scanned at 570nm using a Vitatron TLD-100 densitometer and photographed. The isoelectric point of the various protein bands was estimated from comparison with the pH gradient established in the non-protein-containing gel which was evaluated as follows. The gel was cut into slices of 9mm

Fig. 2.1

SDS-polyacrylamide gel patterns
of fragment D



SDS-gel analysis of fragments D_{Ca⁺⁺} and D_{EDTA}
(a) unreduced and (b) reduced. Apparent
molecular weights ($\times 10^{-3}$) are shown. (5% gels).

which were each incubated in water (0.5ml) for 18h at 20°C. The pH of each solution was then estimated using a Radiometer (Russell) pH meter (Copenhagen).

2.3 Results

2.3.1 SDS-polyacrylamide gel electrophoresis and 2.3.2 NH₂-terminal amino acid determination

The results of SDS-polyacrylamide gel electrophoresis of the samples fragment D_{Ca²⁺} and fragment D_{EDTA} are shown in Fig. 2.1 and suggest the following subunit compositions: fragment D_{Ca²⁺} (β chain: 44,000, γ chain: 40,000 and α chain 15,000) which implies a molecular weight of 99,000 for the intact fragment; fragment D_{EDTA} (β chain: 44,000, γ chain: 27,000 and α chain 15,000) suggesting a molecular weight of 86,000 for the intact fragment D_{EDTA} molecule.

The following NH₂-terminal amino acids were detected for both fragments D_{EDTA} and D_{Ca²⁺}; valine, aspartate, serine/threonine (these two amino acids could not be resolved) and a very faint spot corresponding to methionine. In addition the fragment D_{EDTA} sample exhibited a faint spot attributable to glycine.

2.3.3 Ultracentrifugation

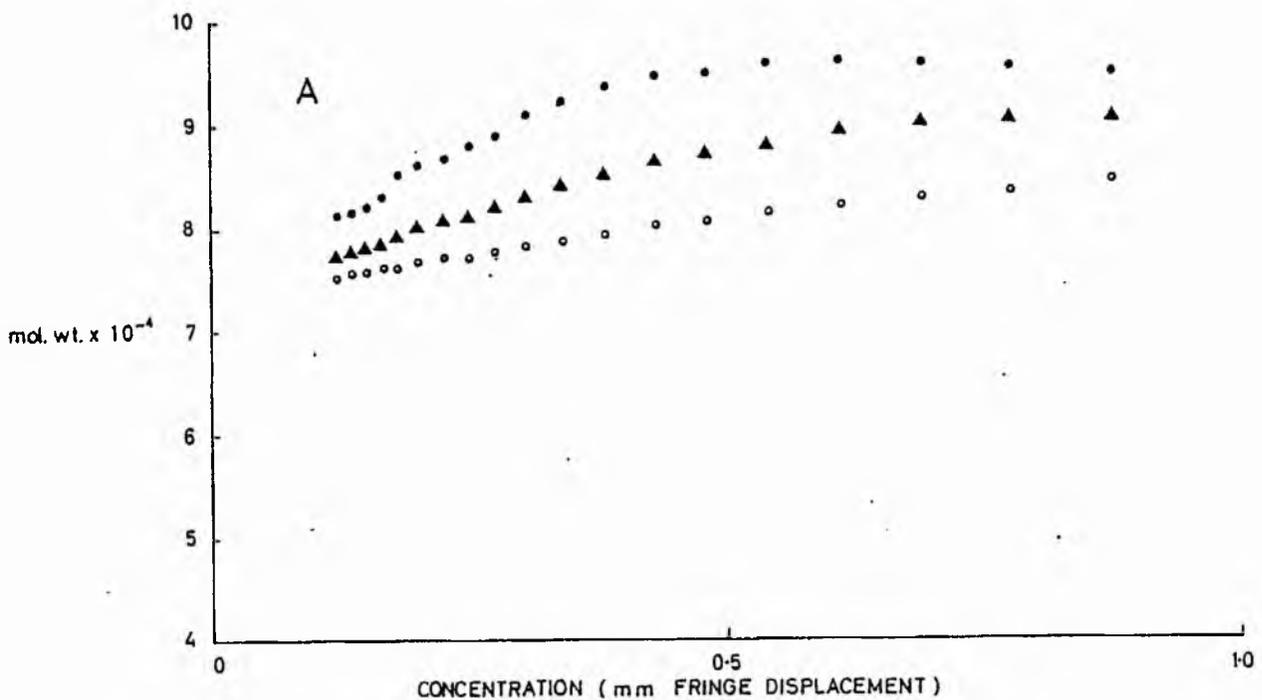
The reliability of protein molecular weight values obtained from SDS-gel electrophoresis is questionable -

Fig 2.2. Sedimentation equilibrium patterns of
fragments $D_{Ca^{++}}$ and D_{EDTA}

The point-average molecular weight moments
for fragments $D_{Ca^{++}}$ and D_{EDTA} as a function of
concentration at the same point.

- A fragment $D_{Ca^{++}}$ sample 1
- B fragment $D_{Ca^{++}}$ sample 2
- C fragment D_{EDTA} sample

M_N (o) number average, M_W (▲) weight average and
 M_Z (●) Z average mol. wts.



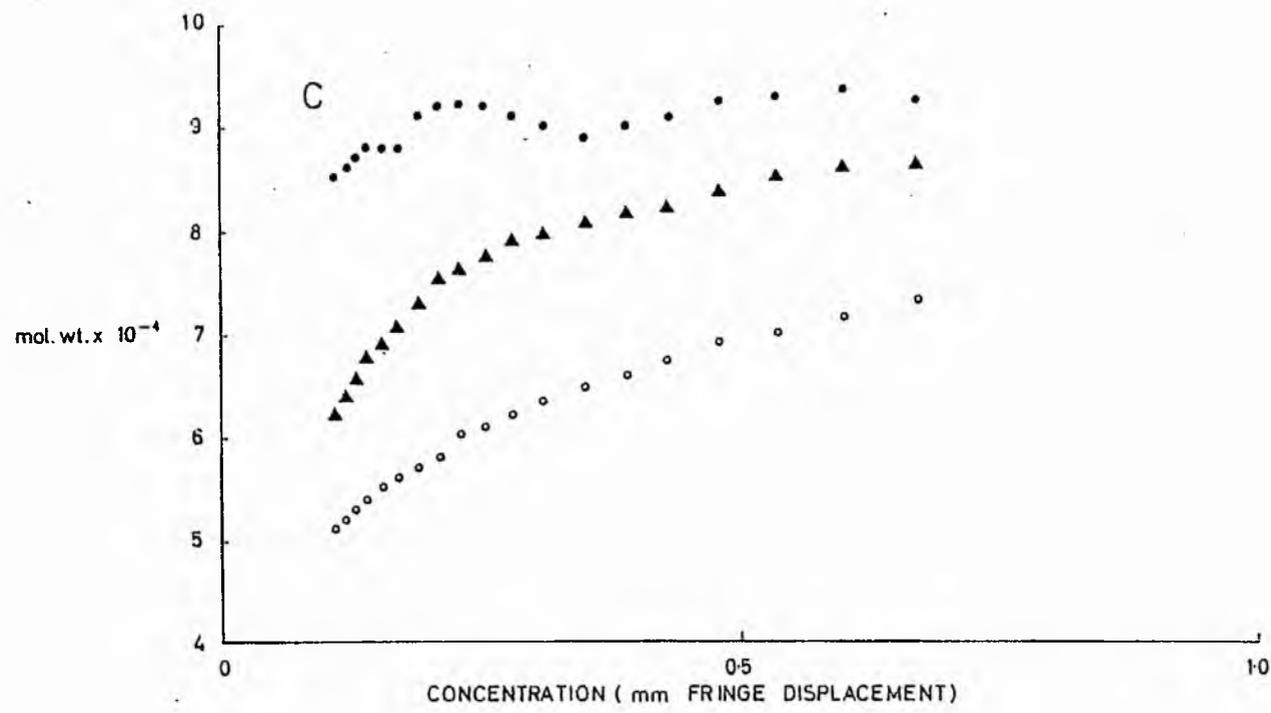
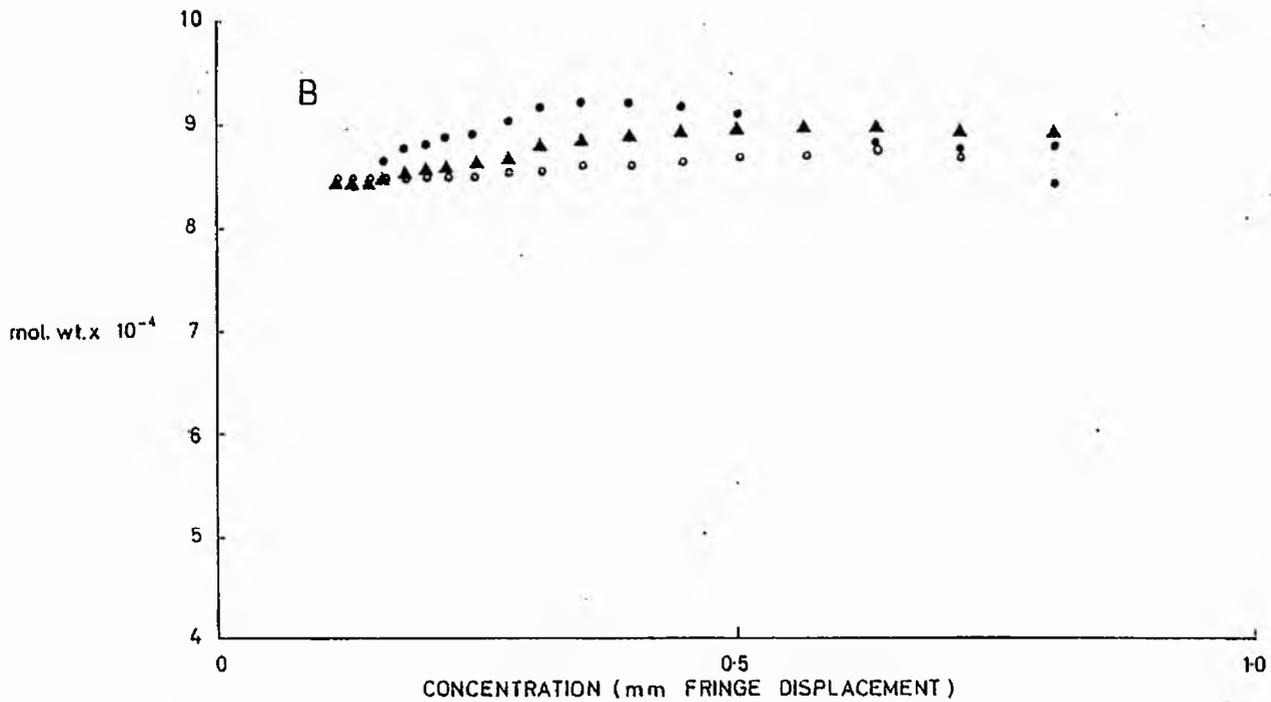


Table 2.1 Molecular weight analyses of fragments D

FRAGMENT D SPECIES	MOLECULAR WEIGHTS											
	SDS-GEL ANALYSIS		ULTRACENTRIFUGAL ANALYSIS OF NON-REDUCED FRAGMENT D									
	SUBUNIT CHAINS	SUMMATION OF CHAINS	mid-point									
			M_w	mol.wt. at INF DILUTION								
Fg D _{Ca⁺⁺}	44,000 40,000 15,000	99,000	84,712	<table style="display: inline-table; vertical-align: middle;"> <tr> <td>M_N</td> <td rowspan="3"> </td> <td>73,000</td> </tr> <tr> <td>M_w</td> <td rowspan="3"> </td> <td>76,000</td> </tr> <tr> <td>M_Z</td> <td></td> </tr> </table>	M_N		73,000	M_w		76,000	M_Z	
M_N		73,000										
M_w			76,000									
M_Z												
Fg D _{Ca⁺⁺}	44,000 40,000 15,000		99,000	87,497	<table style="display: inline-table; vertical-align: middle;"> <tr> <td>M_N</td> <td>85,000</td> </tr> <tr> <td>M_w</td> <td>83,500</td> </tr> <tr> <td>M_Z</td> <td>78,000</td> </tr> </table>	M_N	85,000	M_w	83,500	M_Z	78,000	
M_N	85,000											
M_w	83,500											
M_Z	78,000											
Fg D _{EDTA}	44,000 (36,000) 27,000 15,000	(95,000) 86,000	79,839	<table style="display: inline-table; vertical-align: middle;"> <tr> <td>M_N</td> <td>40,000</td> </tr> <tr> <td>M_w</td> <td>47,000</td> </tr> <tr> <td>M_Z</td> <td>74,000</td> </tr> </table>	M_N	40,000	M_w	47,000	M_Z	74,000		
M_N	40,000											
M_w	47,000											
M_Z	74,000											

Comparison of the molecular weights of fibrinogen fragments D_{Ca⁺⁺} and D_{EDTA} by SDS gel electrophoresis (10% gels) and meniscus depletion sedimentation equilibrium.

a problem which has been discussed (Part A, Section 2.2.3). An alternative method of obtaining molecular weight data is that of sedimentation equilibrium ultracentrifugation and the results presented here describe the analysis of two fragment $D_{Ca^{2+}}$ and one fragment D_{EDTA} sample by the meniscus depletion method of Yphantis (1964). Using interference optics the displacement of fringes within the ultracentrifuge cell can be used to calculate molecular weight averages; M_N (number average); M_W (weight average) and M_Z (Z average). These averages are plotted as a function of solute concentration (mm fringe displacement) in Fig. 2.2, A (fragment $D_{Ca^{2+}}$, sample 1), B (fragment $D_{Ca^{2+}}$, sample 2) and C (fragment D_{EDTA}). Each curve was extrapolated to zero concentration and the value corresponding to each of the molecular weight types at infinite dilution thereby determined. These values and the computed "mid-point weight average" molecular weight values are presented in Table 2.1. Data obtained from the parallel SDS-gel electrophoresis study are also shown.

The values of the mid-point M_W (i.e. the weight average molecular weight at the mid-point of the ultracentrifuge cell) suggest that fragment $D_{Ca^{2+}}$ is indeed of a higher molecular weight than fragment D_{EDTA} . Both values being approximately 14,000 less than the value obtained by electrophoresis. However, the more accurate molecular weight values obtained at infinite dilution indicate that the real situation is more complex.

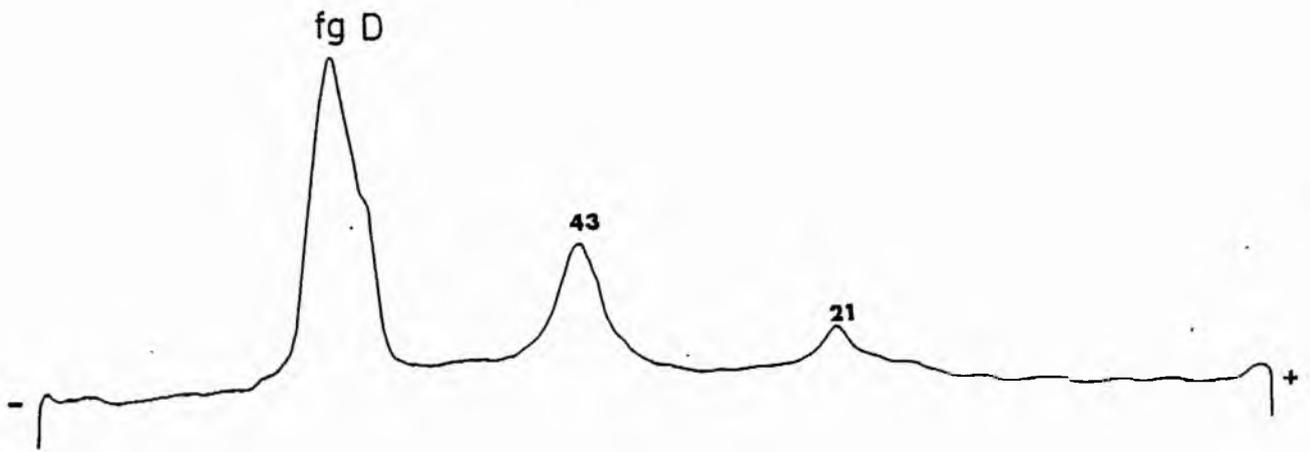
The values of M_W , M_N and M_Z for a pure protein should in theory be identical at infinite dilution. If not, then the inference is that the sample is heterogeneous due to either the presence of contaminating molecules or to the association of the protein. The value of M_N should then correspond most closely to the molecular weight of the most predominant species while that of M_Z should reflect the molecular weight of the heaviest component.

The results obtained from analysis of the fragment D_{EDTA} sample will be considered first. The values produced by extrapolation of each of the curves shown in Fig. 2.2C reveal that the average molecular weight of all species present i.e. the number average molecular weight M_N is 40,000. The curve of M_Z values suggests that the sample also contains a range of species of molecular weights 40,000 to approximately 90,000. SDS-gel analysis proposed that a major fragment D_{EDTA} species of molecular weight approximately 86,000 was contaminated by a slight amount of a fragment D of molecular weight 95,000 i.e. it was indeed heterogeneous but a wide discrepancy is obvious between the molecular weight values predicted by each method.

There are two possible interpretations of these results. The results obtained by electrophoresis may be very inaccurate. The true molecular weight of fragment D_{EDTA} may be approximately 44-47,000 and the species of molecular weight 90,000 may represent a dimeric structure.

Fig. 2-3 SDS-polyacrylamide gel electrophoresis of fragment D_{EDTA}

Fragment D_{EDTA} was incubated at 20°C for 48 h and then examined (unreduced) on a 5% gel. Apparent molecular weights of the various densitometer scan peaks are shown ($\times 10^{-3}$).



Alternatively degradation of fragment D_{EDTA} may have occurred during analysis. (Marder et al. (1976) in a similar study of fragment D added the plasmin inhibitor 6-amino-n-hexanoic acid to the fragment D solution. However to avoid a possible detrimental action on the binding of Ca^{2+} to the fragment $D_{Ca^{2+}}$ molecule, no such addition was made in the present study to the fragment $D_{Ca^{2+}}$ sample and, to maintain identical conditions, neither was it made to the fragment D_{EDTA} sample.)

To test if degradation of fragment D_{EDTA} during ultracentrifugation was a possible explanation for the low value predicted for its molecular weight, a sample was maintained at 20°C for 48h and then examined, unreduced, by SDS-gel electrophoresis. The densitometer scan of this 5% polyacrylamide gel is shown in Fig. 2.3. Two peaks are obvious in addition to the major (split) peak of fragment D and display apparent molecular weights of 43,000 and 21,000 suggesting that further degradation of fragment D_{EDTA} had indeed occurred under conditions similar to those employed during ultracentrifugation. An alternative explanation for the molecular weight values predicted by sedimentation equilibrium experiments being less than their true or ideal values is that the solution behaved in a non-ideal manner (Yphantis & Roark, 1972). This point will be reconsidered later.

The SDS-gel analysis of the fragment $D_{Ca^{2+}}$ samples

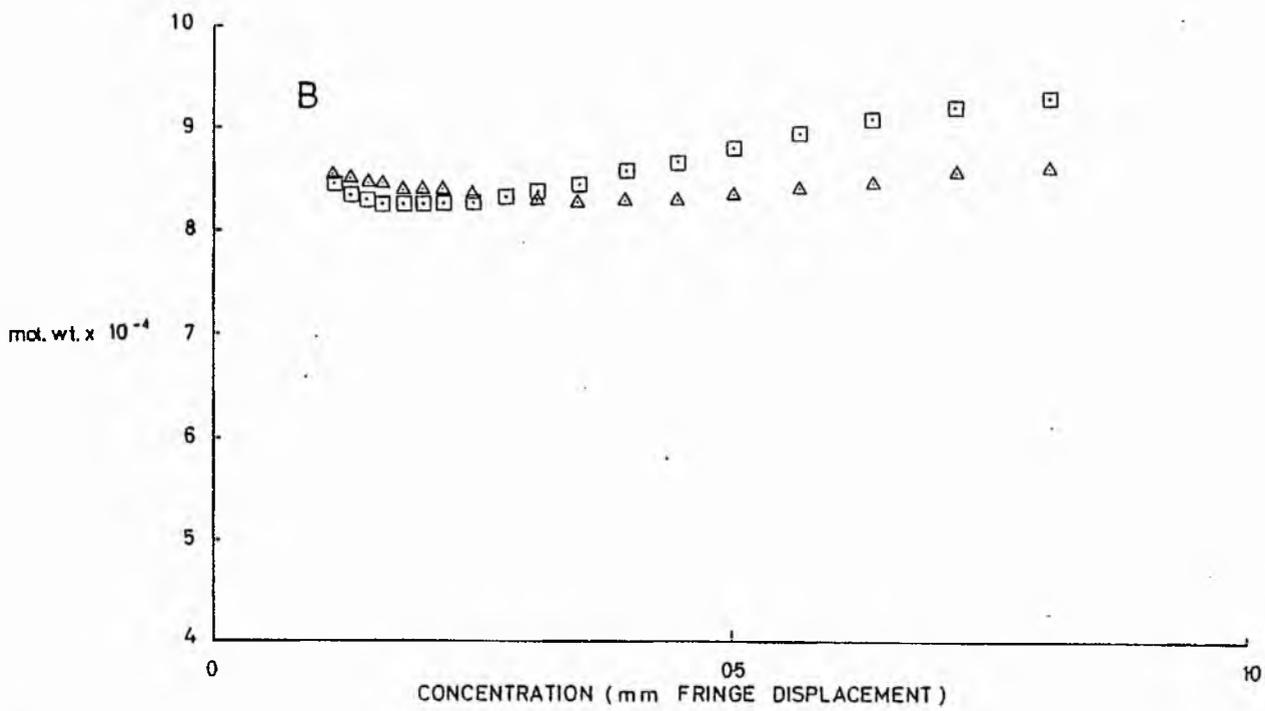
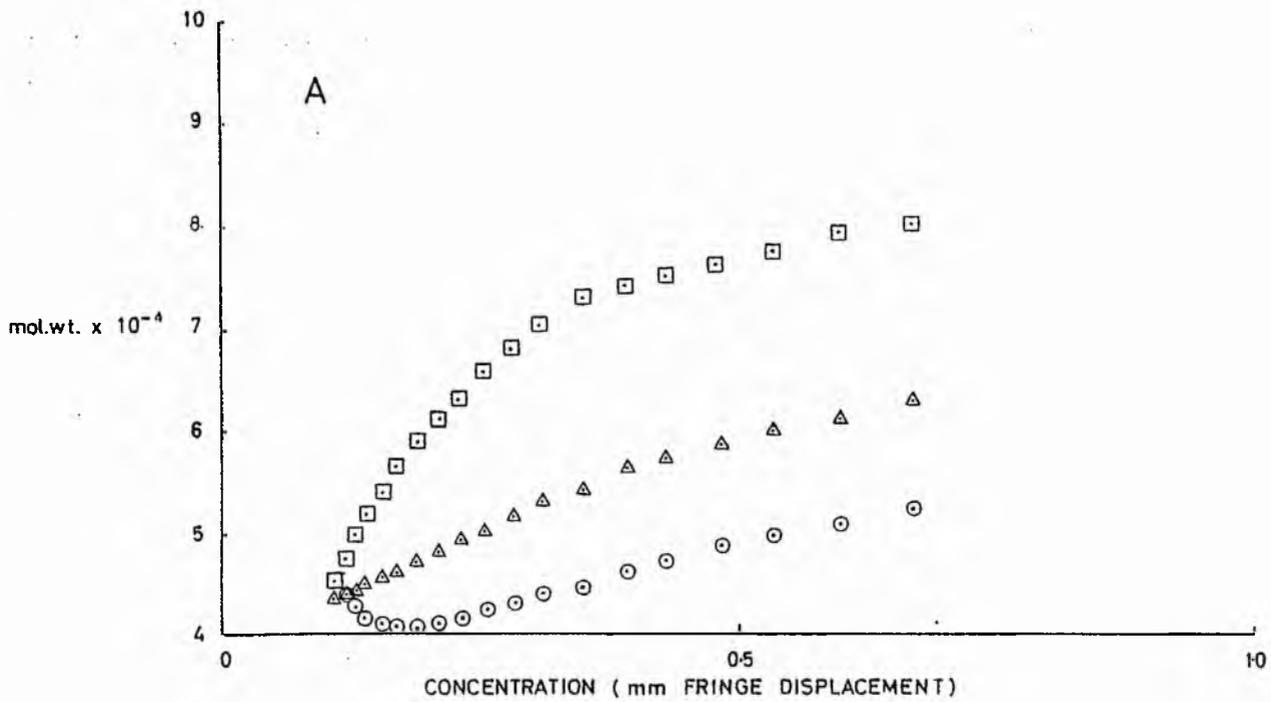
implied that each was apparently homogeneous i.e. at the concentration applied to the gel no protein bands other than those attributed to fragment $D_{Ca^{2+}}$ (constituent chains β , molecular weight 44,000; γ , molecular weight 40,000; α molecular weight 15,000) were observed. The results obtained from sedimentation equilibrium studies of fragment $D_{Ca^{2+}}$ sample 1 (Fig. 2.2 A) reveal that the values of M_W and M_N correspond at infinite dilution (as would be expected for a pure sample) and predict a molecular weight for fragment $D_{Ca^{2+}}$ of 73,000. The graph corresponding to the M_Z values would however suggest that some higher molecular weight (76,000) species are also present. These values are less than those predicted for fragment $D_{Ca^{2+}}$ from the results of SDS-gel electrophoresis and from published amino acid sequence data (88,000; to be detailed in Section 4). Analysis of the second fragment $D_{Ca^{2+}}$ sample (Fig. 2.2 B) revealed higher values for each of the molecular weight types M_N , M_W and M_Z at infinite dilution (Table 2.1). However a greater disparity between the three values is obvious when compared to the results obtained with the first fragment $D_{Ca^{2+}}$ sample. The molecular weight values calculated for the second fragment $D_{Ca^{2+}}$ sample are again less than that predicted by gel electrophoresis.

It has been stated that the examination of a non-ideal solution may result in the derived molecular weights being

Fig. 2.4 Sedimentation equilibrium patterns
of fragments $D_{Ca^{++}}$ and D_{EDTA}

The ideal point average molecular weight moments
for fragments $D_{Ca^{++}}$ and D_{EDTA} as a function of
concentration at the same point.

A fragment D_{EDTA} sample
B fragment $D_{Ca^{++}}$ sample 2
 M_{Y8} (\circ) M_{Y1} (Δ) and M_{Y2} (\square)



lower than their true or ideal values. This non-ideality factor becomes more critical as the concentration of protein in the ultracentrifuge cell increases and promotes a recognizable effect on the shape of the graph of molecular weight against concentration. The shape is that of a curve, the slope of which is reduced and eventually inverted at high concentrations of protein. However this problem can be overcome with the aid of further data supplied by computer analysis. These data M_{Y8} , M_{Y1} and M_{Y2} , three additional molecular weight moments, can be considered as being analogous to M_N , M_W and M_Z respectively, but are not influenced by non-ideality (Yphantis & Roark, 1972). Fig. 2.4A illustrates a plot of M_{Y8} , M_{Y1} and M_{Y2} as a function of solute concentration (mm fringe displacement) for fragment D_{EDTA} while data for M_{Y1} and M_{Y2} values for fragment $D_{Ca^{2+}}$, sample 2 are similarly presented in Fig. 2.3B. The curves of Fig. 2.3A (fragment D_{EDTA}) converge at a point corresponding to a molecular weight of 43,000. However the graph would also predict the presence of molecules ranging in molecular weight from 43,000 to 80,000. This interpretation of results is similar to that made previously for fragment D_{EDTA} . Thus non-ideality does not appear to account for the observed low molecular weight value of fragment D_{EDTA} .

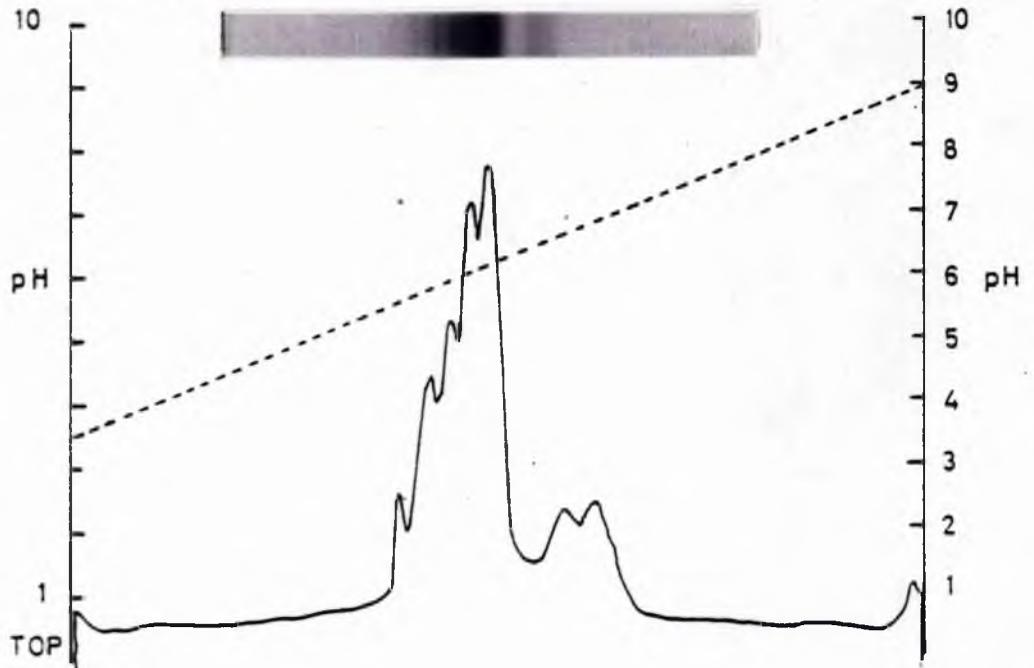
The analogous treatment of the data corresponding to the fragment $D_{Ca^{2+}}$, sample 2 (Fig. 2.4B) reveals a molecular weight value, at the point of convergence of the

Fig. 2.5

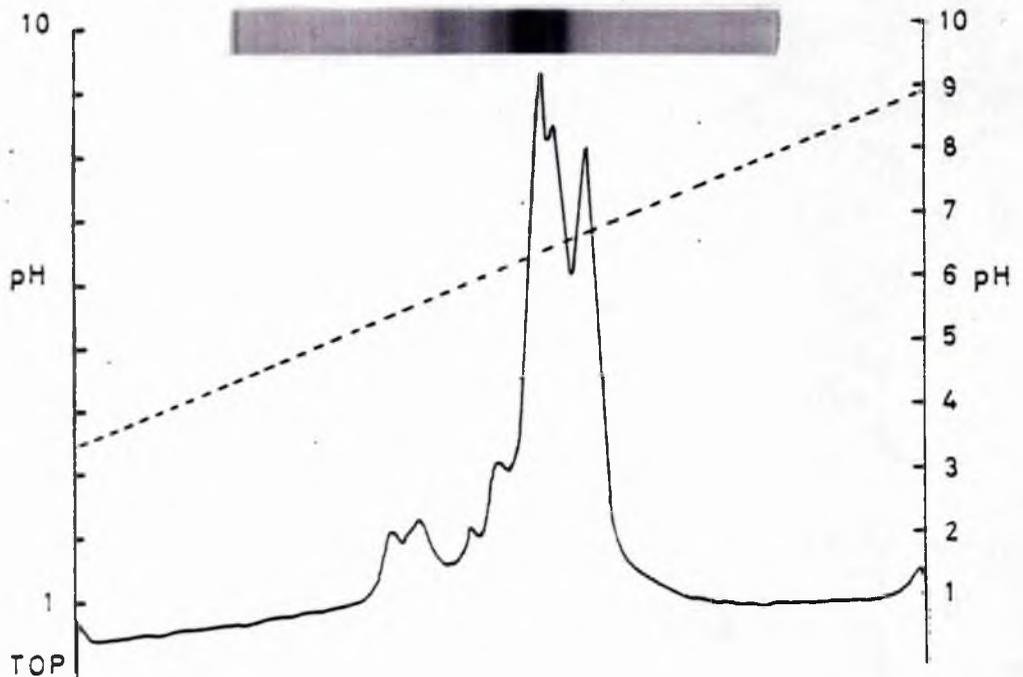
Isoelectric focussing of fragments D

Densitometric tracings and photographs of fragments D separated by isoelectric focusing in polyacrylamide gel. Gels were stained with Coomassie blue. Top: anode

a) fragment D_{EDTA}



b) fragment D_{Ca⁺⁺}



two curves, of 86,000. (Data for the values of M_{Y8} were unavailable for this sample.) The graphs presented in Fig. 2.4B do not reveal that the fragment $D_{Ca^{2+}}$ was significantly heterogeneous. Similar treatment of the data corresponding to fragment $D_{Ca^{2+}}$ sample 1 produced a result resembling that illustrated by Fig. 2.2A i.e. the non-ideality of the solution was not a critical factor in the analysis of this sample. A molecular weight value of 73,000 was predicted at infinite dilution.

2.3.4 Isoelectric focussing

The results from the isoelectric focussing of both fragments $D_{Ca^{2+}}$ and D_{EDTA} are presented in Fig. 2.5. Each sample focussed as 7 bands although the relative intensity of the bands differed. For the fragment $D_{Ca^{2+}}$ sample there were 3 intense bands located in the pH region 6.2 to 6.5 while the fragment D_{EDTA} sample produced four major bands in the pH region 5.4 to 6.0.

2.4 Discussion

The results of polyacrylamide gel electrophoresis of the reduced fragments $D_{Ca^{2+}}$ and D_{EDTA} is in accordance with the proposal of Furlan et al. (1975) that heterogeneous molecular weight forms of fragment D contain identical β and α subunit chains but differ in their respective constituent δ chains. The apparent molecular weight

attributed to the δ chain of fragment D_{EDTA} was 27,000 and this value implies that the intact fragment D_{EDTA} corresponds to a "late" fragment D digestion stage. The sample of fragment D_{EDTA} subjected to ultracentrifugation contained in addition a small amount of a δ chain of apparent molecular weight 36,000.

The failure to detect significant differences between the NH_2 -terminal amino acid composition of the two fragments D suggests that the molecular weight disparity between these two molecules is attributable to differences in their respective constituent polypeptide chains at their COOH-terminal regions. In addition the results from the SDS-electrophoretic studies infer that the region of heterogeneity is the COOH-terminus of the δ chain. The NH_2 -terminal amino acids identified for fragments D_{EDTA} and $D_{Ca^{2+}}$ agree with those reported recently by Van Ruijven-Vermeer et al. (1979) for human fibrinogen fragment $D_{Ca^{2+}}$ and D_{EDTA} but are different to those reported by the same authors for rat fragments D.

However further discussion of the present NH_2 -terminal analysis results is warranted. Furlan et al. (1975) and Doolittle et al. (1977a) have proposed that the NH_2 -terminal amino acid of the D- α chain is valine. The initial NH_2 -terminal amino acid of this chain is in

fact aspartate but six residues are split from the NH₂-terminus resulting in the NH₂-terminal amino acid valine (Furlan et al., 1975; Doolittle et al., 1977a). In the case of the D- γ chain it is proposed that a similar situation occurs. An NH₂-terminal alanine residue is removed by further plasmic digestion to be replaced by a serine residue (Collen et al., 1975). The NH₂-terminal residue of the D- β chain is aspartate (Henschen & Lottspeich, 1976). The β chain remnant of fragment D appears to be most resistant to further attack from plasmin. These findings would imply that in the present study both fragments D_{Ca²⁺} and D_{EDTA} contain the "late" NH₂-terminal amino acids of the α and γ chains (assuming that the unresolved spot of serine/threonine was in fact serine); i.e. both fragments D must have undergone degradation, specifically at the NH₂ termini of their constituent α and γ chains. Both samples also displayed a faint spot corresponding to methionine and this finding may indicate that, in addition, the α chain had undergone degradation at its COOH-terminus, since Doolittle et al. (1977a) identified a plasmic cleavage point within the D- α chain (α lys 205-206 Met). The faint spot of methionine may therefore be attributable to this released peptide produced during storage of fragment D

samples.

The detection of a faint spot identified as glycine in the fragment D_{EDTA} sample is of particular interest in relation to the interpretation of polyacrylamide gel electrophoresis results. Henschen et al. (1978) proposed that the plasminic cleavage of a lysine/glycine bond occurred within a disulphide-linked region of the δ chain. Thus an additional NH₂-terminal amino acid would be exposed without degradation of the unreduced sample being detected. A molecular weight of approximately 37,500 was calculated for the intact D - δ chain from available sequence data (to be detailed in Section 4). The lysine/glycine bond identified by Henschen et al. is situated in the δ chain at a position corresponding to approximately 6,000 "molecular weight units" from the NH₂-terminus. Therefore the detection of a δ chain remnant of molecular weight 31,000 by SDS-gel electrophoretic analysis of a reduced fragment D sample may not be indicative, as has been hitherto assumed, of a D - δ chain minus a COOH-terminal portion (approximately 6,000 "molecular weight units") removed by plasmin. Alternatively the 31,000 δ chain remnant may have arisen from cleavage of this lysine/glycine bond and may contain an intact COOH-terminal region. Thus if two unreduced fragment D molecules are poorly resolved by SDS-gel electrophoresis, the examination of their

reduced chain composition may not necessarily identify their respective subunit structures.

To summarise, the NH_2 -terminal amino acid analysis results failed to detect major differences between fragments $\text{D}_{\text{Ca}^{2+}}$ and D_{EDTA} . If the comparison with published results was made correctly, the conclusion is that the α and γ chains of both fragments D had undergone limited degradation at their NH_2 -termini. Thus if Ca^{2+} does indeed protect the γ chain of fragment $\text{D}_{\text{Ca}^{2+}}$ from plasmic attack, such an influence must be restricted to the COOH -terminal region.

The data provided by ultracentrifugation of the fragment D_{EDTA} were complicated by factors reflecting the heterogeneity of the sample. Two proposals were made to account for this phenomenon. In view of the reports by numerous investigators of a molecular weight for fragment D of 70-100,000 the suggestion that the true molecular weight of fragment D_{EDTA} is approximately 40,000 seems untenable. The second proposal i.e. that degradation of fragment D_{EDTA} had occurred appears more acceptable especially since a species of molecular weight 43,000 was detected in this sample by SDS-gel analysis following its subjection to conditions similar to those employed during ultracentrifugation. However SDS-gel analysis did not imply that the 43,000 molecular weight species was the major component of the fragment D_{EDTA} sample. A considerable amount of fragment D was in

fact detected. In order to reconcile this observation with the molecular weight value predicted by ultracentrifugation (40,000 for M_N) the possibility that the SDS-gel electrophoresis result may have "concealed" an extensively degraded form of fragment D_{EDTA} which displayed an apparent molecular weight of 72,000 but had a true molecular weight of 43,000 must be considered. (A similar anomaly attributed to SDS-gel analysis of an unreduced form of an extensively degraded fragment D molecule was noted in Part A, Section 6.4).

Evidence for the proposal that the molecular weight of fragment $D_{Ca^{2+}}$ is greater than that of fragment D_{EDTA} is inconclusive. The results are complicated by the apparent ongoing degradation of the fragment D_{EDTA} sample and further characterisation of the origin of this heterogeneity is required. The results obtained with the first fragment $D_{Ca^{2+}}$ sample suggested a molecular weight value of 73,000 (error $\pm 3,700$). Correction for non-ideality of the sample solution did not alter this value. The value obtained with the second fragment $D_{Ca^{2+}}$ sample was higher and suffered from the effects of the non-ideality of the sample solution. After correction for this factor a molecular weight value of 82,500 (error $\pm 4,500$) was predicted for fragment $D_{Ca^{2+}}$. Additional data are therefore required to establish a reliable estimate of the molecular weight of fragment $D_{Ca^{2+}}$.

Nevertheless, the present studies do substantiate the proposal that Ca^{2+} confers stability on the fragment $\text{D}_{\text{Ca}^{2+}}$ molecule. Under identical experimental conditions fragment D_{EDTA} appeared unstable.

Mihalyi et al. (1976) employing an identical procedure proposed a molecular weight for fragment D of 84,000. They reported that their fragment D preparation was not heterogeneous however it was prepared by tryptic fragmentation of fibrinogen in a phosphate buffer system. Thus their fragment D cannot be considered to be identical to either of the fragments D analysed in the present experiments. Marder et al. (1976) employing low speed column sedimentation equilibrium (according to Yphantis & Roark (1972), a system less conducive to the detection of molecular heterogeneity) reported a molecular weight value for fragment D of $103,500 \pm 11,000$. The corresponding result from SDS-gel analysis was 95,000. In agreement with the present findings for the fragment D_{EDTA} molecule both Niléhn (1967) and Collen et al. (1975) reported from ultracentrifugation studies considerable molecular weight heterogeneity of their fragment D samples. In each case the sample was examined in a buffer system incompatible with the presence of free Ca^{2+} .

In summary, ultracentrifugation studies have revealed considerable molecular weight heterogeneity of fragment D samples prepared in the absence of Ca^{2+} . This finding is in accordance with the proposed protective role of Ca^{2+}

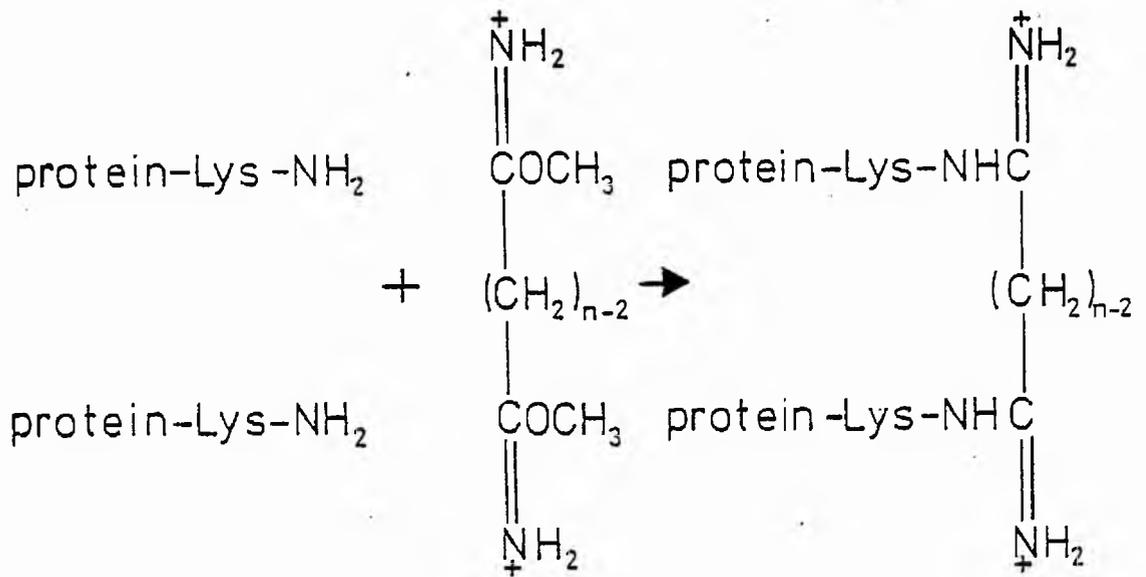
in preventing degradation of fragment D. Further studies are required to establish a molecular weight for the fragment $D_{Ca^{2+}}$ molecule.

Finally the results of an investigation of the isoelectric points of fragments $D_{Ca^{2+}}$ and D_{EDTA} were presented. The values obtained (6.2 to 6.5 for fragment $D_{Ca^{2+}}$ and 5.4 to 6.0 for fragment D_{EDTA}) are in agreement with published results. Jamieson & Pepper (1970) described the resolution of approximately 6 bands within the pH range 5 to 6.5, while Catanzaro *et al.* (1972) reported 8 bands. Arneson (1974) obtained 5-8 bands within the pH range 6.05-6.45. The finding in the present study of a higher pI value for fragment $D_{Ca^{2+}}$ compared with fragment D_{EDTA} is consistent with the report by Godal (1960a) that Ca^{2+} affected the isoelectric point of fibrinogen causing a shift to a more alkaline value. This effect of Ca^{2+} may be mediated via its contribution of positive charge to the overall charge of the molecule. However this explanation seems unlikely since the ampholytes within the gel chelate free metal ions and it is improbable that the Ca^{2+} remains bound to the fragment $D_{Ca^{2+}}$ molecule. The different values of pI for the fragment $D_{Ca^{2+}}$ molecule compared with those of fragment D_{EDTA} may instead reflect the contribution to the overall charge of the molecule made by those amino acids at the COOH-terminus of the constituent γ chain whose removal by plasmin, it is proposed, is prevented by bound Ca^{2+} .

The heterogeneity in isoelectric points of fragment D preparations hitherto described by various investigators may be due, in part, to the preparation of fragment D from fibrinogen in the presence of varying amounts of Ca^{2+} and may therefore reflect differing extents of degradation of the constituent γ chain.

The present results suggest that the separation of fragment $\text{D}_{\text{Ca}^{2+}}$ from other forms of fragment D may be achieved by column electrofocussing. However, more recently, Van Ruijven-Vermeer et al. (1979) performed a similar study but did not report the different isoelectric focussing patterns described for fragments $\text{D}_{\text{Ca}^{2+}}$ and D_{EDTA} in the present work.

Fig. 3.1 Scheme for the reaction of a diimidoester with protein amino groups



DIIMIDO ESTER

DMA n = 6
DMS n = 8

SECTION 3

CHEMICAL CROSSLINKING OF FRAGMENTS D

3.1 Introduction

Chemical crosslinking reagents have been employed to investigate the structures and functional relationships of a variety of proteins (Peters & Richards, 1977). One class of such bifunctional reagents is the alkyl imidates (imidoesters). These compounds react specifically with the primary amino group (probably the lysyl ϵ -amino group) in proteins (Hunter & Ludwig, 1962) to produce stable products. No change of charge occurs at the modified residue and the reaction is therefore unlikely to disrupt the tertiary structure of the protein (Perham, 1973).

The reaction of an alkyl imidate with protein lysine groups is depicted in Fig. 3.1. The reaction of the first imidoester group of the reagent with a protein primary amino group may be followed by an identical reaction between the second group and another protein amino group thereby forming a crosslinked protein. However, if no protein amino group is available or if it is located outwith the domain of bonding of the reagent no crosslink will be formed and the second ester bond of the reagent will be hydrolysed.

The imidate reagents employed in the present study were dimethyl suberimidate, DMS (n=8) and dimethyl adipimidate, DMA, (n=6) (n refers to the formula shown in Fig. 3.1). The approximate dimensions between the reactive imidoester

groups in the fully extended reagent molecules are; DMS 9.7 Å; DMA 7.3 Å (Hajdu et al., 1976). The fibrinogen fragment D molecule contains approximately 63 lysine residues (see following section, Section 4) and should therefore react with diimidoester compounds. It is proposed (Part B, Section 1) that Ca^{2+} binds to fragment D and induces an altered conformation of the molecule as a consequence. Thus the distribution and position of lysine residues accessible to the crosslinking reagent may also be affected. The following section of work describes the results of an investigation of the crosslinking pattern produced by each of the reagents with a) fragment $\text{D}_{\text{Ca}^{2+}}$ and b) fragment D_{EDTA} .

3.2 Methods

3.2.1 Crosslinking of fragment D

Fragment $\text{D}_{\text{Ca}^{2+}}$ and D_{EDTA} protein samples were prepared for crosslinking by dialysis at 4°C for 18h against 0.2 M-triethanolamine/HCl buffer, pH 8.15, containing 2 mM- CaCl_2 . A stock solution of crosslinking reagent (either DMS or DMA) in 0.5 M-triethanolamine/HCl buffer, pH 8.5, was prepared immediately prior to use. After the mixing of suitable volumes of the reagent stock solution and protein solution the crosslinking reaction was allowed to proceed at 20°C for 2h. The final concentration of reagent was 0.7-13 mM-DMS, 1-20 mM-DMA and that of each protein sample

(estimated spectrophotometrically at 280nm after dialysis)

1.0-3.6mg/ml.

Samples of crosslinked proteins were then dialysed against 0.1 M-Tris/HCl buffer, pH 7.5, containing 0.2% SDS for 4h at 20°C or examined immediately, both reduced and unreduced, by polyacrylamide gel electrophoresis (5% gels).

3.3 Results

3.3.1 Crosslinking of fragments D with dimethyl adipimidate (DMA)

The SDS-polyacrylamide gel patterns corresponding to both reduced and unreduced samples of DMA-treated fragments D_{EDTA} and $D_{Ca^{2+}}$ are shown in Fig. 3.2. The results relating to the analysis of non-crosslinked fragments D are included for comparison - these gels are labelled "A".

Inspection of the gels corresponding to the unreduced DMA-treated fragment D samples reveals an obvious difference in the pattern displayed by each type of fragment D. The fragment D_{EDTA} -containing sample displays dimeric, trimeric and tetrameric species in significant amounts at all three DMA concentrations employed (5, 16 and 20 mM). By contrast the DMA-treated fragment $D_{Ca^{2+}}$ samples exhibit evidence of dimer formation but only a very faint band corresponding to a trimeric component is obvious. No increased oligomer formation was induced by increasing the concentration of DMA.

Fig. 3.2 Crosslinking of fragment D with
dimethyl adipimidate

SDS-polyacrylamide gel electrophoresis of fragments $D_{Ca^{2+}}$ and D_{EDTA} before and after treatment with dimethyl adipimidate (DMA). Samples were examined both reduced and unreduced on 5% polyacrylamide gels.

Gels A : untreated fragment D

Gels B : 5 mM-DMA

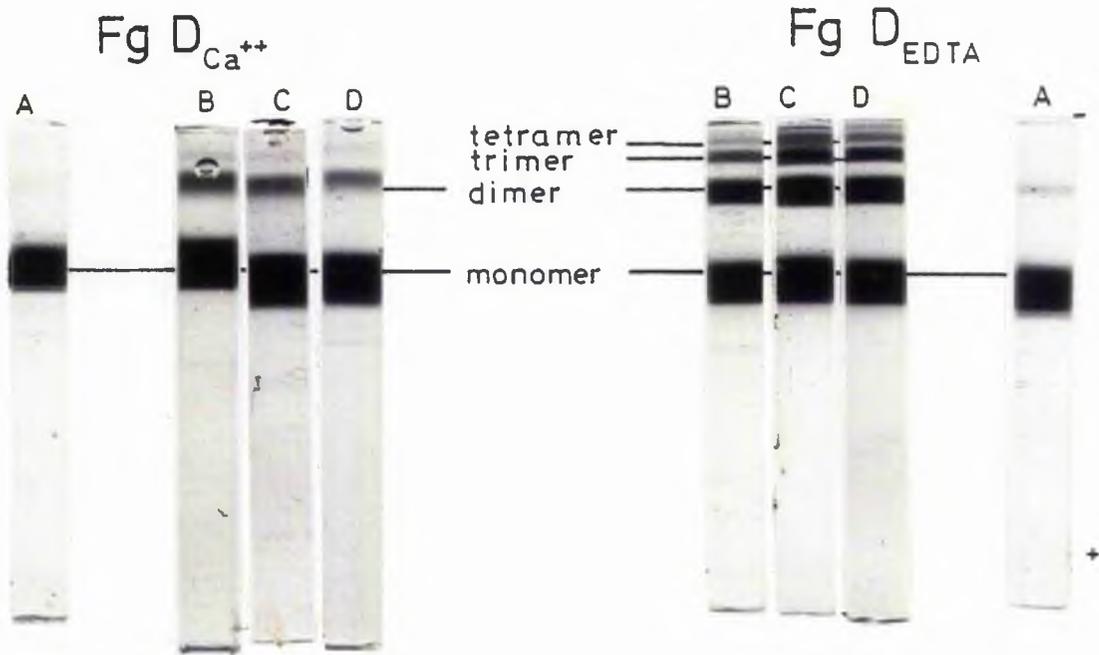
Gels C : 16 mM-DMA

Gels D : 20 mM-DMA

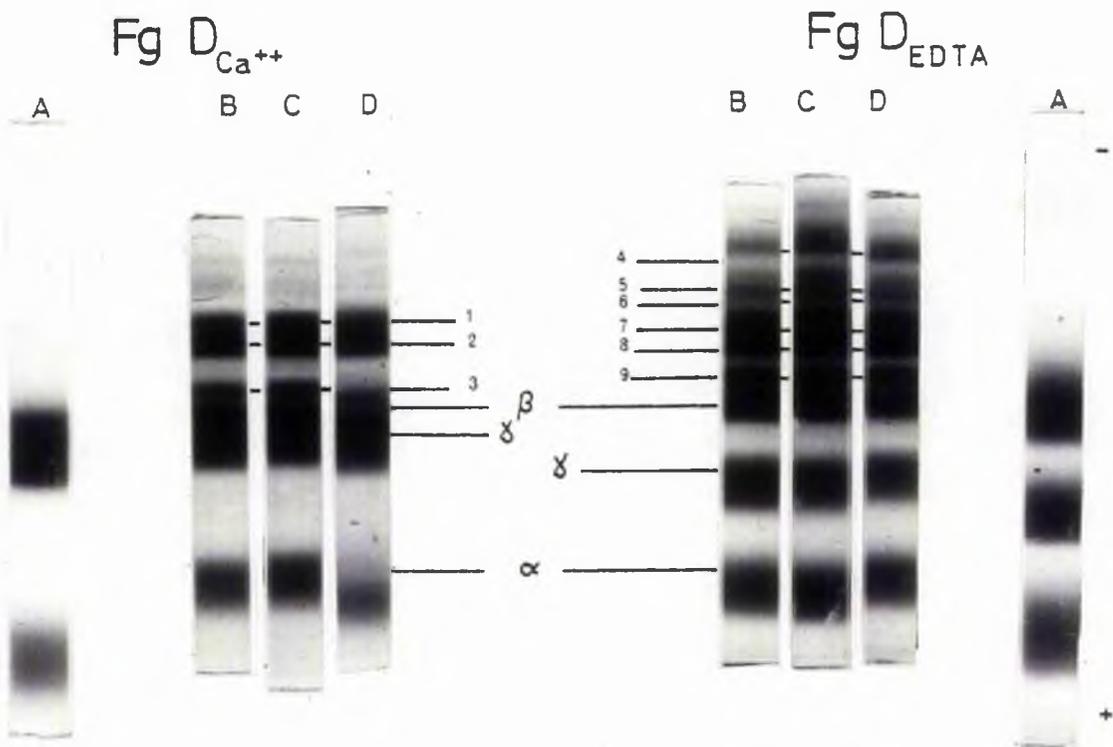
For other details see text.

Fig. 3.2

UNREDUCED



REDUCED



These results imply that the two types of fragment D differ in their tendency to be inter-molecularly crosslinked.

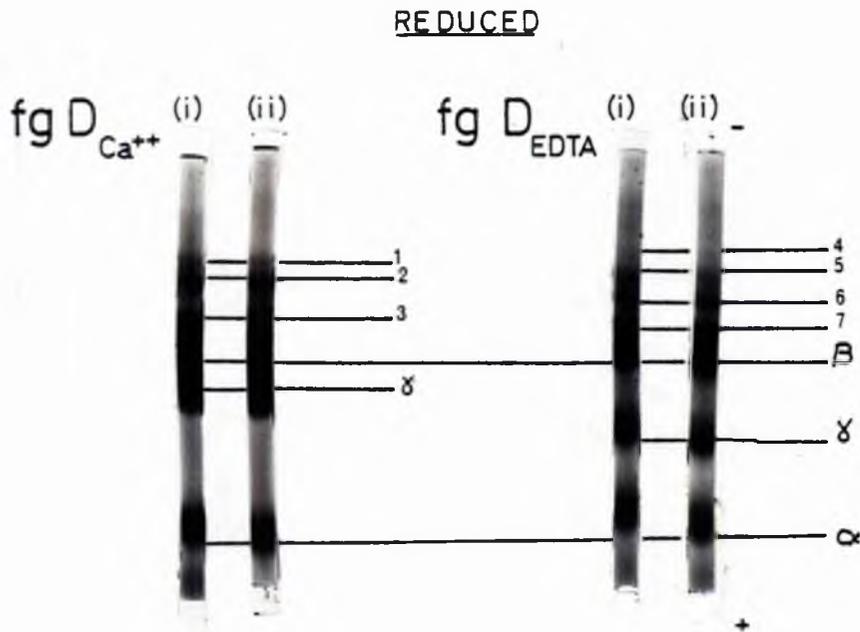
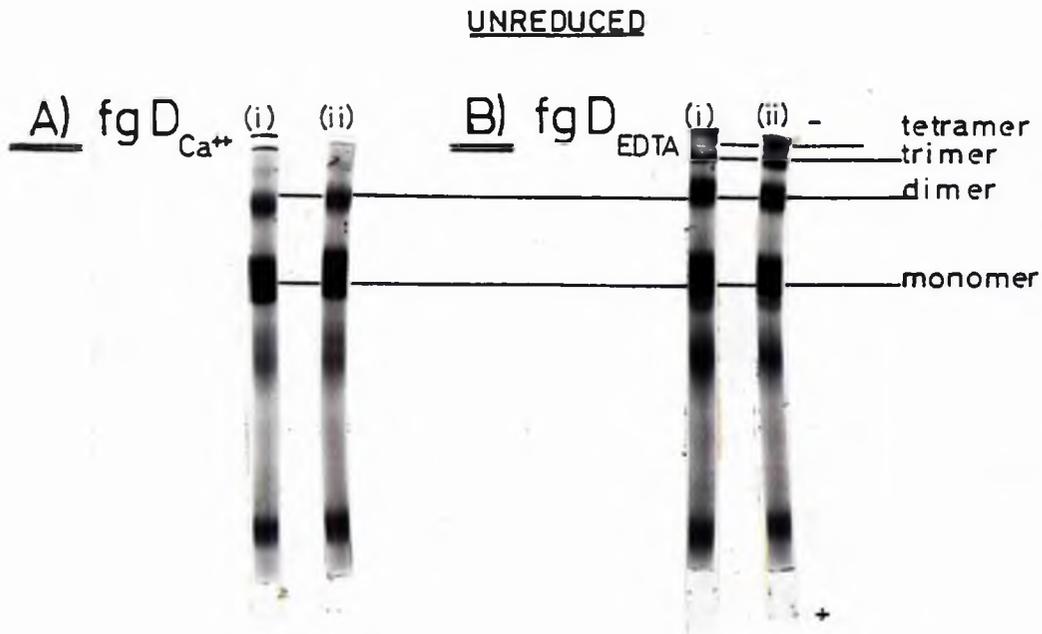
The reduced samples corresponding to each of the DMA-treated fragment D types however, both display evidence of crosslinked protein chains. The interpretation of these gel patterns is facilitated by comparison with the appropriate untreated fragment D sample (labelled A). The subunit chains of untreated fragment $D_{Ca^{2+}}$ have apparent molecular weights of 44,000, 40,000 and 14,000 and, by analogy with previous results, these represent the β , δ and α chains respectively. Examination of the corresponding DMA-treated samples reveals three additional protein bands and the prevalence of the fragment $D_{Ca^{2+}}$ monomer in the unreduced DMA-treated sample implies that the predominant type of crosslink formed is intra-molecular. The estimated molecular weights of these three additional protein bands (labelled 1-3) are 88,000, 81,000 and 56,000 and a possible, but equivocal, interpretation would be that these represent $\beta - \beta$, $\delta - \delta$ or $\beta - \delta$ and $\beta - \alpha$ or $\delta - \alpha$ crosslinked protein chains respectively. The inference is that the 88,000 molecular weight band has arisen from a $\beta - \beta$ inter-molecularly crosslinked fragment $D_{Ca^{2+}}$ species and the faint intensity of this 88,000 molecular weight band is consistent with the failure to detect significant amounts of crosslinked dimeric molecules in the unreduced sample.

An alternative interpretation is that one of these three higher molecular weight bands represents a completely intra-molecularly crosslinked fragment $D_{Ca^{2+}}$ molecule with all three constituent chains intact.

The reduced DMA-treated fragment D_{EDTA} samples display 6 bands in addition to those assigned to the β chain (molecular weight 44,000), γ chain (molecular weight 27,000) and α chain (molecular weight 14,000). This increased number of combinations of crosslinked fragment D_{EDTA} constituent chains is an observation consistent with the greater level of inter-molecular crosslinked species described above for the unreduced fragment D_{EDTA} sample compared with that of the fragment $D_{Ca^{2+}}$ sample. The assignation of the fragment D_{EDTA} subunit chain components of each of these six extra bands cannot be made accurately. The molecular weights were estimated as follows; band 4, 120,000; 5, 97,000; 6, 92,000; 7, 76,000; 8, 70,000; 9, 55,000. (The mobilities corresponding to bands 4 and 5 are outwith the range of the molecular weight standards employed during electrophoresis.) If the fragment D_{EDTA} molecule had only been intra-molecularly crosslinked then the greatest number of additional protein bands would have been 4 ($\alpha - \beta - \gamma$, $\beta - \gamma$, $\gamma - \alpha$ and $\beta - \alpha$ crosslinked species). Therefore there is considerable evidence that inter-molecular crosslinking of fragment D_{EDTA} molecules was induced by DMA-treatment.

To summarise, only the fragment D_{EDTA} DMA-treated sample

Fig. 3.3 Crosslinking of fragment D with dimethyl suberimidate



SDS-Polyacrylamide gel electrophoresis patterns of A) $fgD_{Ca^{++}}$ and B) fgD_{EDTA} after crosslinking with dimethyl suberimidate, (5% gels). (i) 1.4 mM, (ii) 1.1 mM dimethyl suberimidate.

β, γ, α refer to the reduced protein bands characteristic of non-cross-linked fragment D. Additional reduced protein bands are labelled 1-7.

displays evidence of significant polymer formation. Nevertheless crosslinked subunit chains are obvious in reduced samples of both DMA-treated fragments $D_{Ca^{2+}}$ and D_{EDTA} . This suggests that the type of crosslinking predominating in the fragment $D_{Ca^{2+}}$ samples must therefore be intra-molecular while in fragment D_{EDTA} -containing samples considerable inter-molecular crosslinking has also occurred.

3.3.2 Crosslinking of fragments D with dimethyl suberimidate (DMS)

The gel patterns resulting from SDS-electrophoretic analysis of DMS-treated samples of both fragments $D_{Ca^{2+}}$ and D_{EDTA} are shown in Fig. 3.3. Two DMS concentrations namely 1.4 mM and 1.1 mM were employed and both reduced and unreduced DMS-treated samples were examined.

The gel band patterns displayed by the DMS-treated fragment D samples are very similar to those described previously for samples treated with DMA. The DMS-treated fragment D_{EDTA} samples display evidence of the formation of inter-molecular crosslinks since bands of mobilities appropriate to dimeric, trimeric and tetrameric species are obvious. However the fragment $D_{Ca^{2+}}$ samples exhibit only faint bands attributable to trimeric species and inter-molecular crosslinking has been restricted mainly to the formation of dimeric structures. Thus there is a greater tendency for the fragment D_{EDTA} molecules to form inter-molecular crosslinks than those of fragment $D_{Ca^{2+}}$.

Reduction of the DMS-treated fragment $D_{Ca^{2+}}$ samples revealed 3 protein bands in addition to those assumed to represent the non-crosslinked constituent subunit chains of the molecule. Their respective apparent molecular weights are; 1, 84,000; 2, 76,000 and 3, 52,500. These values are similar to those detected with DMA. The presence of three additional protein bands implies that crosslinking of the fragment $D_{Ca^{2+}}$ molecule was induced by DMS treatment. However as with DMA, the absence of considerable polymeric species in the corresponding unreduced samples infers that the predominant mode of crosslinking of the fragment $D_{Ca^{2+}}$ molecule is intra-molecular.

Like the analogous fragment $D_{Ca^{2+}}$ samples the reduced DMS-treated fragment D_{EDTA} sample displays evidence of crosslinked subunit chains. However the preponderance of the polymeric species in the unreduced sample suggests that these have arisen from the formation of inter-molecular crosslinks. A similar conclusion was reached from studies with DMA. In this instance the reduced DMS-treated fragment D_{EDTA} contains four bands (labelled 4-7) in addition to those assigned as the α , β and γ subunit chains of fragment D_{EDTA} . Their apparent molecular weights are 78,000, 68,000, 60,000 and 49,000. Both the number and the molecular weights of these additional bands differ from the analogous result obtained with DMA. This disparity may be accounted for by two factors which might

influence the type and number of crosslinks formed. These are firstly that different ratios of concentration of crosslinking reagent to fragment D were employed with DMS compared to DMA and, secondly, the differing types of crosslink formed may result from some function of the dimensions of the reagent molecules. The fully extended DMS molecule is 2.4 Å longer than that of DMA (Hajdu et al., 1976).

However, it should be noted that inspection of the gel photographs in Fig. 3.3 corresponding to both samples of unreduced fragment D reveals that each preparation contains two contaminants, one below the fragment D band and the other near the bottom of the gel. The possibility that these proteins may have influenced the crosslinking reaction must be considered; if this were so it would detract from the validity of the above conclusions. However both contaminants were present in each fragment D sample. Furthermore the present pattern of results is very similar to that obtained by DMA-treatment of pure fragment D samples. It therefore seems unlikely that the crosslinking of fragment D with DMS has been significantly influenced by these contaminants.

To conclude, the pattern of crosslinking obtained with DMS is similar to that obtained with DMA. The predominant type of crosslinking induced in fragment $D_{Ca^{2+}}$ by DMS was intra-molecular while significant inter-molecular crosslinking was induced in fragment D_{EDTA} samples by the same concentration of reagent.

3.3.3 The effect of varying both the concentration of dimethyl suberimidate (DMS) and of fragment $D_{Ca^{2+}}$ on the extent of crosslinking.

One possible explanation of the differing susceptibilities of the two fragments D to crosslinking is that different concentrations of each protein had been compared. The contrasting electrophoretic patterns obtained would then reflect, not some property of the two fragment D molecules, but instead an effect related simply to the employment of differing concentration ratios of crosslinking reagent to protein.

Thus the extent of formation of polymeric species by the treatment of fragment $D_{Ca^{2+}}$ with DMS was investigated:- (A) at a constant concentration of fragment $D_{Ca^{2+}}$ but with varying concentration of DMS and (B) vice versa. Fig. 3.4 illustrates the results obtained following the analysis of the samples (unreduced) by SDS-gel electrophoresis. In no case was a significant amount of trimeric fragment $D_{Ca^{2+}}$ produced. Increasing the concentration of DMS failed to increase the extent of inter-molecularly crosslinked fragment $D_{Ca^{2+}}$ molecules. The greatest intensity of the dimeric species was produced at a DMS concentration of 3 mM. A further increase in the concentration of the crosslinking reagent promoted, instead, a decrease in the amount of dimeric species formed. This situation may correspond to the saturation of the protein primary amino groups by the reagent. Consequently less free amino groups are available to react with the second reactive group of the DMS molecule

to form dimeric, crosslinked molecules.

The effect of varying the concentration of fragment $D_{Ca^{2+}}$ at a constant concentration of DMS on the amount of dimeric species formed is illustrated by part B of Fig. 3.4. No further increment in oligomer formation could be induced by raising the fragment $D_{Ca^{2+}}$ concentration from 2.4 to 3.6mg/ml. Instead, this concentration change promoted a decrease in the amount of dimeric fragment $D_{Ca^{2+}}$ species produced.

Thus it is unlikely that either an insufficient concentration of fragment $D_{Ca^{2+}}$ or of crosslinking reagent can account for the decreased ability of this protein to form polymeric structures.

3.4 Discussion

The bifunctional crosslinking reagents DMS and DMA react specifically with protein primary amino groups (Hunter & Ludwig, 1962). Hajdu et al. (1976) employed these reagents to investigate the distances between lysine residues in subunits of oligomeric proteins, while Furlan et al. (1977) utilised their properties to investigate the conformational changes involved in the expression of factor VIII activity. Thus since fibrinogen fragment $D_{Ca^{2+}}$ may contain approximately 63 lysine residues per molecule (as deduced from published sequence data to be described in Section 4) it should react with these reagents

and thereby reveal information relative to its molecular conformation.

The observed failure to induce significant inter-molecular crosslinking of fragment $D_{Ca^{2+}}$ was not due to the inability of the crosslinking reagent to react with this protein. Significant amounts of crosslinked fragment $D_{Ca^{2+}}$ constituent chains were identified by analysis of the reduced DMA or DMS-treated fragment $D_{Ca^{2+}}$ samples and these had, presumably, been formed by intra-molecular linkages. However considerable inter-molecular crosslinks were formed by similar treatment of a fragment D_{EDTA} sample. Thus it can be proposed that the fragment $D_{Ca^{2+}}$ molecule may exhibit a more compact conformation than the fragment D_{EDTA} molecule. This theory is based on the following evidence. Following the initial reaction of the crosslinking reagent with a primary amino group on the fragment $D_{Ca^{2+}}$ molecule it appears that the second reaction of the reagent is more likely to occur with another amino group within the fragment $D_{Ca^{2+}}$ molecule, i.e. this second amino group is more readily available to the reagent than that from a second fragment $D_{Ca^{2+}}$ molecule. In the instance of the fragment D_{EDTA} molecule the second reactive group of the bound crosslinking reagent is readily accessible to primary amino groups on secondary fragment D_{EDTA} molecules thereby forming inter-molecular crosslinks. This reasoning suggests a more open conformation for the fragment D_{EDTA} molecule compared to that of fragment $D_{Ca^{2+}}$.

The site of attachment of the crosslinking reagent molecule is also a point of attack of the enzyme plasmin on fragment D. Thus if the present model is correct and the lysine residues of fragment $D_{Ca^{2+}}$ are held within a folded conformation of the molecule i.e. they are not located on the exposed extremities, it may also illustrate the proposed insusceptibility of fragment $D_{Ca^{2+}}$ to the action of plasmin. Whether this folded conformation of the fragment $D_{Ca^{2+}}$ molecule is maintained directly by Ca^{2+} or by an interaction of the amino acids comprising the COOH-terminal region of the constituent γ chain (whose removal by plasmin, it is proposed, is prevented by bound Ca^{2+}) is not revealed by the present results.

The less folded structure envisaged for the fragment D_{EDTA} molecule should cause the exposure of a greater number of potential plasmin attack points. This theory would explain the observed increased susceptibility of this fragment to degradation by plasmin.

The possibility however, that these findings were an artefactual result of employing different relative concentrations of reagent and protein for each type of fragment D is acknowledged. However, additions of DMS or DMA to the protein solutions were made from a single stock solution. Identical protein concentrations of each fragment D type were assured by measuring the absorbance at 280nm of each sample before the addition of the crosslinking reagent. Further evidence that the different gel patterns obtained were not the result of studying

different relative amounts of fragments D was provided by studies which suggested that neither an increase in the concentration of fragment $D_{Ca^{2+}}$ or of crosslinking reagent would induce significant increases in the amount of inter-molecular contacts.

However several other points relating to the experimental procedure invite discussion. Fragment D_{EDTA} may not be the most suitable fragment D for comparison with fragment $D_{Ca^{2+}}$. It is produced from fibrinogen in the presence of an "unnatural" compound i.e. EDTA. Thus the present results may in fact represent peculiarities of the fragment D_{EDTA} molecule. Digestion studies described in Part B, Division 1, implied that the γ chain remnant of the present fragment D_{EDTA} molecule is normally produced following two plasmic digestion 'attacks' on the fragment $D_{Ca^{2+}}$ γ chain. A more valid comparison would therefore have been made if crosslinking of a fragment D containing the intermediate γ chain component had been available for study.

A possible action of EDTA, present in the fragment D_{EDTA} sample, on the crosslinking process must also be considered. Both fragment D_{EDTA} and $D_{Ca^{2+}}$ samples were dialysed against a buffer containing $CaCl_2$ to ensure identical sample composition and to remove EDTA present in the fragment D_{EDTA} sample. However if this treatment

was not successful residual EDTA might decrease the effectiveness of the crosslinking reagent by either a) electrostatic interactions with the positively charged imido ester group or b) promote the hydrolysis of the reagent; a process favoured at low pH (Peters & Richards, 1977). In both cases decreased crosslinking of the molecule would be predicted. The opposite situation was in fact observed. Similarly a nonspecific inhibitory action of Ca^{2+} on the crosslinking process may have occurred and might thereby account for the decreased level of crosslinking induced in the fragment $\text{D}_{\text{Ca}^{2+}}$ - containing sample. However this possibility seems unlikely since a preliminary dialysis step was performed to ensure that both types of fragment D sample contained equal concentrations of the ion.

Finally one further precaution which must be exercised when interpreting the results of crosslinking reactions is to acknowledge that the formation of a crosslink may in itself alter the conformation of the molecule. Thus in the present context the detection of increased amounts of polymeric species prepared by crosslinking of fragment D_{EDTA} as opposed to fragment $\text{D}_{\text{Ca}^{2+}}$ may more simply represent the ability of bound reagent to "manipulate" the molecular structure of fragment D_{EDTA} . This reasoning by the same token, must also imply that the fragment $\text{D}_{\text{Ca}^{2+}}$ molecule is less amenable to manipulation

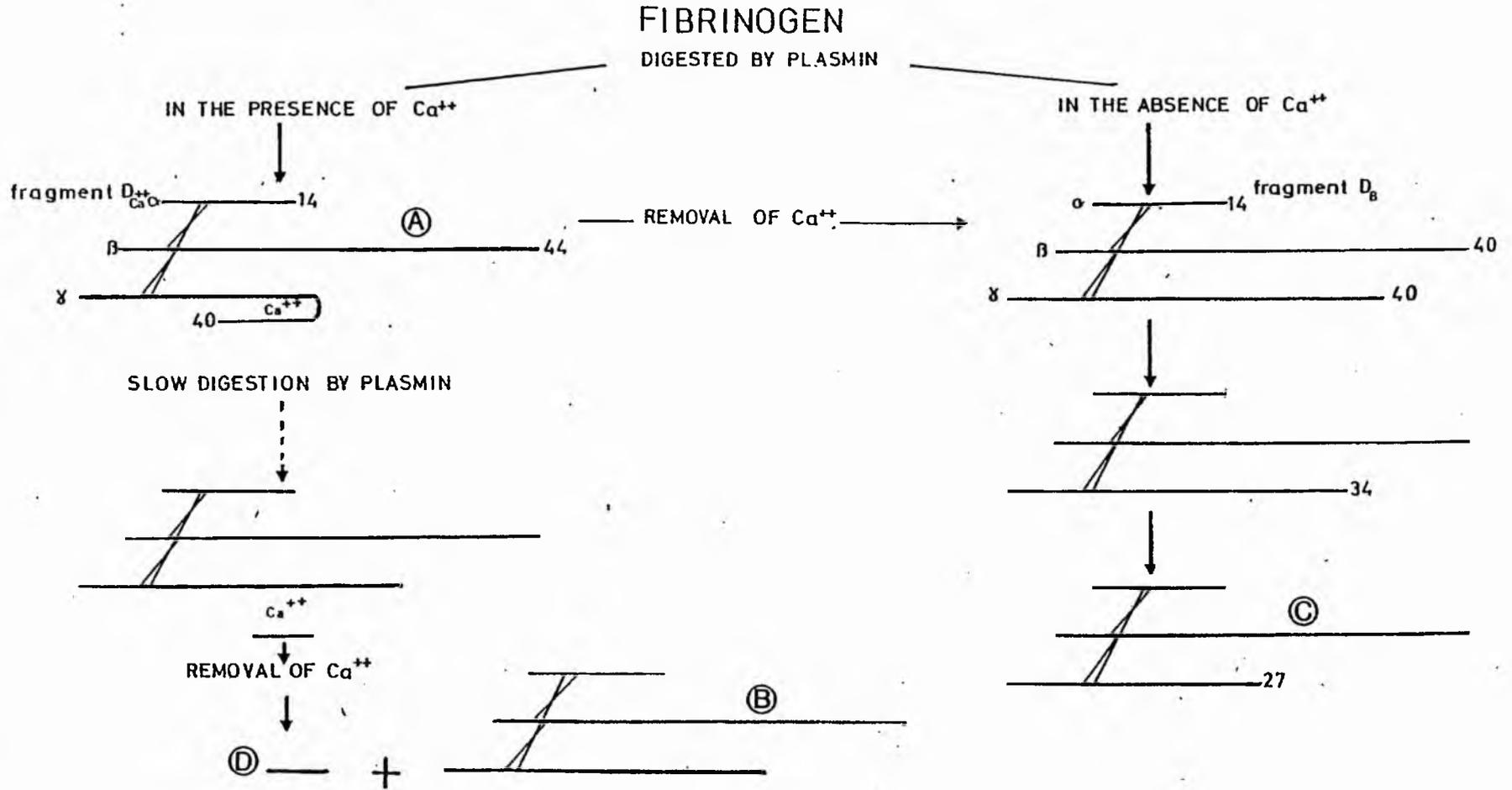
i.e. it has a more rigid conformation.

In conclusion the present results predict a more compact structure for the fragment D molecule prepared by digestion of fibrinogen in the presence of Ca^{2+} than in its absence.

Fig. 4.1

Schematic diagram of the effect of Ca^{++} on the digestion of fragment D

This diagram is based on Fig. 7.1 of Division 1. The structures proposed for the four samples (A,B,C and D) subjected to amino acid analysis are shown.



SECTION 4
AMINO ACID ANALYSIS

4.1 Introduction

The results of the amino acid analysis of four samples relating to fragment D are presented in this Section.

Fig. 4.1 summarises the scheme proposed in Division 1 for the digestion of fragment D both in the presence and the absence of Ca^{2+} and serves to illustrate the hypothetical composition of each of the four samples (A-D) analysed. Plasmin digestion of fibrinogen in the presence of Ca^{2+} produces a single, high molecular weight form of fragment D, fragment $\text{D}_{\text{Ca}^{2+}}$ (sample A) while digestion in the absence of Ca^{2+} proceeds to a fragment D which has undergone further degradation of the constituent δ chain. The lowest molecular weight form of this type of fragment D which was described in Division 1 contained a constituent δ chain of molecular weight 27,000. This is the structure predicted by SDS-gel electrophoresis for sample C.

It has been suggested (Division 1) that fragment $\text{D}_{\text{Ca}^{2+}}$ may not be completely resistant to further degradation by plasmin and that limited digestion of the constituent δ chain occurs. However the peptide lysed from the δ chain remains attached to the rest of the molecule by virtue of the Ca^{2+} -bridge. This degraded, minor component of the fragment $\text{D}_{\text{Ca}^{2+}}$ preparation could be distinguished from the intact fragment $\text{D}_{\text{Ca}^{2+}}$ molecules either by reduction at 100°C

(which revealed a γ chain of molecular weight approximately 6,000 less than that of the constituent γ chain of the undigested fragment $D_{Ca^{2+}}$ molecule) or by the addition of EDTA (the minor, degraded component of the fragment $D_{Ca^{2+}}$ samples being revealed as a faint band which did not exhibit the characteristic electro-:phoretic mobility decrease, but instead migrated immediately below the original fragment $D_{Ca^{2+}}$ band). EDTA and heat treatment, it is assumed, disrupt the Ca^{2+} -bridge and thereby release the lysed peptide. This proposition implies that the lysed peptide is intimately involved in the binding of Ca^{2+} to fragment D. Accordingly it was of interest to reinvestigate the amino acid composition of fragment $D_{Ca^{2+}}$ after dialysis against buffer containing EDTA to effect the release and removal of the lysed peptide (sample B).

During the course of studies employing fragment $D_{Ca^{2+}}$ it was noted that the changes in the electrophoretic mobility pattern of fragment $D_{Ca^{2+}}$ attributed to the release of the lysed peptide could also be induced by subjecting fragment $D_{Ca^{2+}}$ to repeated freezing and thawing. In addition SDS-gel analysis revealed a faint, high mobility component of approximate molecular weight 9,000. The possibility that this low molecular weight component of a pure sample of fragment $D_{Ca^{2+}}$ represented the peptide released from the γ chain by the disruption

of the Ca^{2+} -bridge, prompted the isolation and subsequent amino acid analysis of this species from a sample of fragment $\text{D}_{\text{Ca}^{2+}}$ (sample D).

4.2 Methods

4.2.1 The isolation of a peptide released from fragment $\text{D}_{\text{Ca}^{2+}}$

Purified fibrinogen fragment $\text{D}_{\text{Ca}^{2+}}$ -containing samples were thawed and pooled (6ml, 1.3mg/ml). Gel filtration was performed at room temperature on a column (2.5 x 40cm) of Sephadex G-100 equilibrated and eluted with 0.05 M-Tris/HCl buffer, pH 7.5 at a flow rate of 55ml/h. The absorbance of the eluant was monitored at 280nm and fractions (5min) were collected. Fractions 22-41 (i.e. those fractions eluted between the fragment D-containing peak and the marker dye bromophenol blue) were pooled and dialysed against water before being freeze dried. The reconstituted sample (1ml, $A_{280} = 0.6$) was examined by amino acid analysis, SDS-polyacrylamide gel electrophoresis and immunoelectrophoresis.

4.2.2 Preparation of fragment D samples for amino acid analysis

The three fragment D samples (samples A-C) were dialysed prior to analysis against distilled water containing in the case of sample A (fragment $\text{D}_{\text{Ca}^{2+}}$)

2 mM-CaCl₂ and in the case of samples B (fragment D_{Ca²⁺}) and C (fragment D_{EDTA}) 5 mM-EDTA.

4.2.3 Amino acid analysis

Samples for amino acid analysis (fragment D 0.7-0.8mg; peptide 0.3mg) were hydrolysed in 6 M-HCl containing 0.01 M-thioglycollic acid for 24h at 110°C. After hydrolysis the tubes were cooled, opened and the contents evaporated at 37°C using a Büchler Evapo-mix. The tubes were stored for 18h over NaOH pellets in an evacuated dessicator before being analysed. Analyses were performed on a Locarte amino acid analyser (single column operation). Standard colour values for amino acids were obtained from the chromatography of suitable volumes of a standard solution of amino acids (Sigma Chem. Co. Ltd., London). Amino acids were quantitated manually from chromatograms.

4.3 Results

4.3.1 Amino acid analysis of fragments D (samples A, B and C)

The amino acid composition of each of the three fragment D samples (A-D_{Ca²⁺}, B-D_{Ca²⁺} dialysed against buffer containing EDTA and C-D_{EDTA}) is presented in Table 4.1 (expressed as residues per 1000 residues) and Table 4.2 (expressed as residues per mol of fragment D). The amino acid composition of a high molecular weight

Table 4.1

AMINO ACID ANALYSIS OF FRAGMENT D-1

RESULTS ARE EXPRESSED AS RESIDUES PER 1000 RESIDUES.

AMINO ACID	A Fg D _{Ca⁺⁺}	B Fg D _{Ca⁺⁺} [■]	C Fg D _{EDTA}	THEORETICAL VALUES
Asp	119.2	145.7	126.7	136.0
Thr ^h	53.0	53.7	49.5	54.4
Ser ^h	68.0	56.2	53.1	65.3
Glu	113.7	113.2	110.3	122.4
Pro	50.2	26.7	40.3	39.4
Gly	101.8	92.5	93.2	89.8
Ala	44.3	49.2	49.2	46.2
Val	58.4	48.8	51.7	53.0
Met	39.0	30.6	31.1	31.3
Ile	50.8	50.2	53.8	55.8
Leu	63.9	72.5	76.8	66.6
Tyr	47.3	54.6	49.4	47.6
Phe	29.4	32.8	30.4	32.6
Lys	84.0	86.9	96.1	85.7
His	23.4	25.4	23.7	21.7
Arg	53.1	61.0	64.7	49.0

Eg D_{Ca⁺⁺}[■] represents fragment D_{Ca⁺⁺} dialysed against 5 mM-EDTA prior to analysis.

THEORETICAL VALUES were prepared from published sequence data.

^h: corrected for hydrolytic loss.

Table 4.2

AMINO ACID ANALYSIS OF FRAGMENT D-2

RESULTS ARE EXPRESSED AS RESIDUES PER MOL OF FRAGMENT D.

AMINO ACID	A Fg D _{Ca⁺⁺}		B Fg D _{Ca⁺⁺} [■]		C Fg D _{EDTA}	THEORETICAL VALUES
	v	w	v	w	x	z
Asp	106	94	127	113	95	100
Thr ^h	47	41	47	41	37	40
Ser ^h	60	53	49	43	40	48
Glu	101	90	99	88	83	90
Pro	44	40	23	20	30	29
Gly	90	80	81	71	70	66
Ala	39	35	43	38	37	34
Val	52	46	43	38	39	39
Met	34	31	27	24	23	23
Ile	45	40	44	39	40	41
Leu	57	50	63	56	58	49
Tyr	42	38	48	42	37	35
Phe	26	23	29	25	23	24
Lys	74	66	76	67	72	63
His	21	18	22	19	18	16
Arg	48	42	53	47	49	36

Fragment D_{Ca⁺⁺}[■] represents fragment D_{Ca⁺⁺} dialysed against 5mM-EDTA prior to analysis.

Molecular Weights used in calculations:

THEORETICAL VALUES (z) = 88000 (from published sequence data).

Fg D_{Ca⁺⁺} (v) = 99000 (from SDS-gel analysis of subunit chains).

Fg D_{Ca⁺⁺}[■] (w) = 88000 (from (z) above).

Fg D_{EDTA} (x) = 85000 (from SDS-gel analysis of subunit chains).

^h: corrected for hydrolytic loss.

form of fragment D predicted from available sequence data (to be detailed in the Discussion of this Section) is also shown in Tables 4.1 and 4.2.

The highest molecular weight form of fragment D analysed was fragment $D_{Ca^{2+}}$. The amino acid composition of this sample, sample A, calculated by assuming the theoretical molecular weight of 88,000, agrees favourably with the composition of the theoretical fragment D molecule. However notable exceptions are the values for proline, methionine, valine and glycine. The calculation of the values for proline was complicated by the skew shape of the chromatogram peak.

The transition of fragment $D_{Ca^{2+}}$ to D_{EDTA} is attributed to degradation of the constituent χ chain from a molecular weight of 40,000 to 27,000. The data presented in Table 4.2 suggest that this change is accompanied by a decrease in the levels of aspartate, threonine, serine, glutamate, proline, glycine, valine and methionine. However the data relating to the theoretical amino acid composition of fragment D suggest that the degradation to fragment D_{EDTA} is accompanied by a reduction in the levels of aspartate threonine serine and glutamate - residues with polar or acidic side chains.

The data presented in Table 4.2 relating to a fragment $D_{Ca^{2+}}$ which had been exposed to EDTA (sample B) have been prepared assuming identical molecular weight

values to those of the untreated fragment $D_{Ca^{2+}}$ (sample A). Thus if a polypeptide is released from fragment $D_{Ca^{2+}}$ by exposure to EDTA the molecular weight of fragment $D_{Ca^{2+}}$ should decrease and accordingly the values presented in Table 4.2 for sample B may be overestimated. Sample B contains less of the following amino acids than sample A; serine, proline, glycine, valine and methionine. A slight decrease in the amounts of threonine, glutamate, isoleucine, lysine and histidine is also indicated. However for the reasons mentioned above, the changes in the levels of the amino acids proline, glycine, valine and methionine may not be significant. A comparison of the composition of sample B with the theoretical values suggests that exposure of fragment $D_{Ca^{2+}}$ to EDTA may promote the loss of serine and also to a lesser extent threonine, glutamate, isoleucine, valine and phenylalanine residues. These results predict that the peptide which, it is assumed, is released from fragment $D_{Ca^{2+}}$ by EDTA-treatment, must have a predominantly hydrophilic composition. Further consideration of these amino acid analysis results will be delayed until the Discussion of this Section.

4.3.2 The isolation of the peptide released from fragment $D_{Ca^{2+}}$

The elution profile relating to the Sephadex G-100

Fig. 4.2 Elution profile for Sephadex G-100 gel filtration of fragment D_{Ca}⁺⁺

Fragment D_{Ca}⁺⁺ (7.8mg) was applied to a column (2.5 x 40cm) of Sephadex G-100 equilibrated and then eluted with 0.05 M-Tris/HCl buffer, pH 7.5. SDS gel electrophoresis of the fragment D_{Ca}⁺⁺ sample (a) and of the pooled eluted fractions (indicated by the horizontal bar) (b) are shown (5% gels). Flow rate: 55ml/h. Fractions 5min. \square : elution of marker dye.

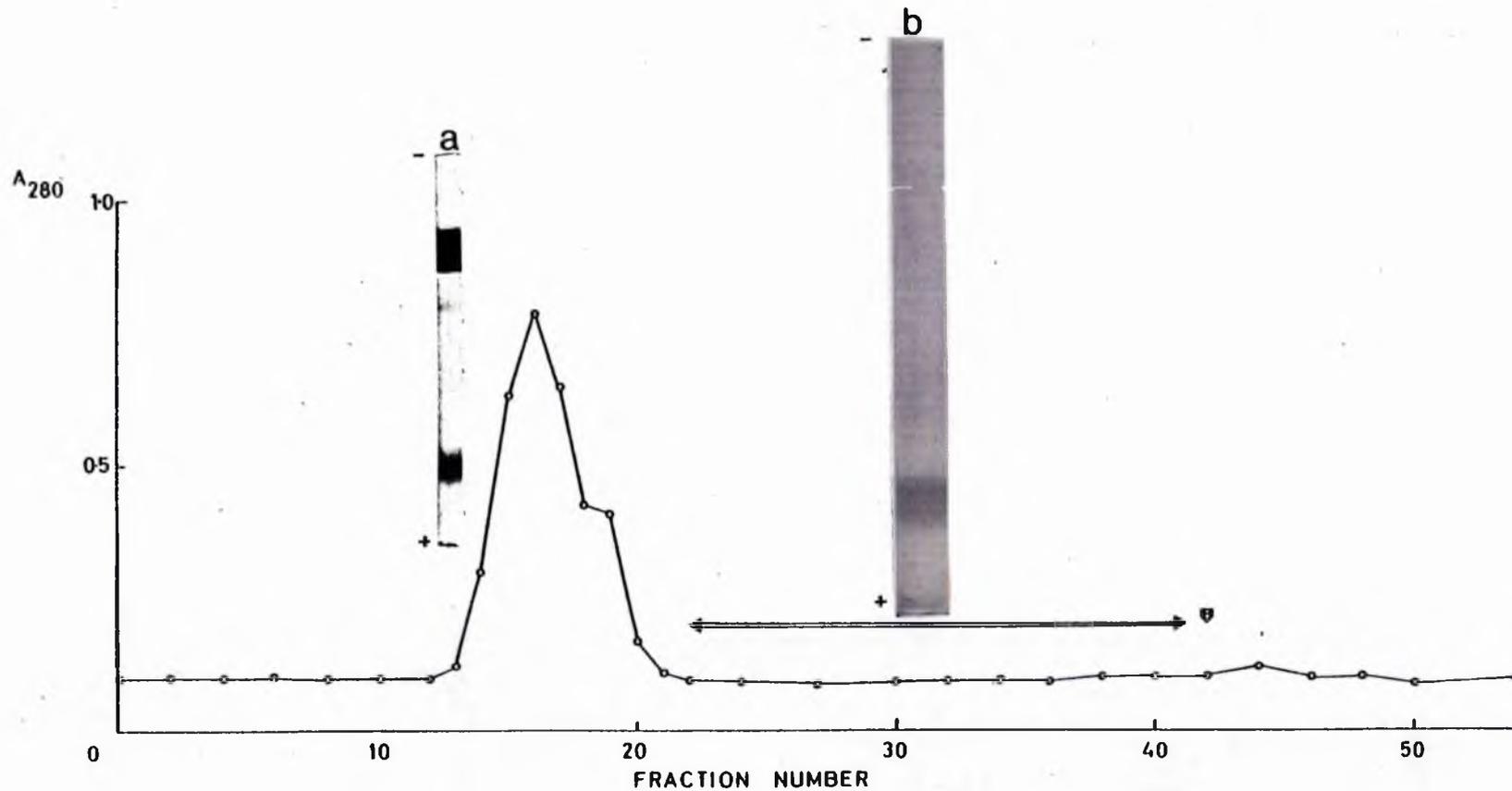


Table. 4.3 Amino acid analysis of isolated peptide

AMINO ACID	RESIDUES PER 1000 RESIDUES			RESIDUES PER MOL OF FRAGMENT ^m		
	A	C	N	A	C	N
Asp	93.2	85.0	138.5	6	4	9
Thr ^h	54.5	127.7	46.2	3	6	3
Ser ^h	182.8	42.6	107.7	11	2	7
Glu	166.7	63.8	138.5	10	3	9
Pro	T	42.6	30.8	T	2	2
Gly	184.0	127.7	-	11	6	-
Ala	90.9	63.8	61.5	6	3	4
Val	51.3	-	15.4	3	-	1
Met	T	42.6	46.2	T	2	3
Ile	37.3	106.4	107.7	2	5	7
Leu	46.2	42.6	92.3	3	2	6
Tyr	T	42.6	61.5	T	2	4
Phe	T	21.3	-	T	1	-
Lys	93.1	106.4	107.7	6	5	7
His	T	42.6	15.4	T	2	1
Arg	T	42.6	30.8	T	2	2

Results presented in column A were obtained from analysis of a fragment isolated from a preparation of fragment D_{Ca⁺⁺}.

Columns C and N represent the amino acid composition of sections located at the C- and N- termini of the δ -chain of fragment D (obtained from published sequence data).

^mThe following molecular weights were used in the calculations
A 6000 C 5167 N 7422.

^hCorrected for hydrolytic loss.

T: Trace

gel filtration of a sample of fragment D_{Ca}^{2+} is presented in Fig. 4.2. The somewhat overloaded SDS-gel pattern corresponding to a sample of fragment D_{Ca}^{2+} which had been subjected to repeated freezing and thawing is shown (labelled a). The major fragment D_{Ca}^{2+} band is split and in addition a faint band is obvious near the bottom of the gel corresponding to an apparent molecular weight of 9,000. (This value cannot be quoted with confidence due to the severe limitations of molecular weight estimations relating to this high mobility region of the gel.) The pooling and subsequent concentration of those fractions eluted between the main fragment D_{Ca}^{2+} peak and the marker dye permitted the isolation of the material analysed by SDS-gel electrophoresis in gel (b). A high mobility, low molecular weight band is obvious. The isolated material did not react with anti-human fibrinogen antiserum. The amino acid analysis results are presented in Table 4.3 and imply a predominantly hydrophilic amino acid composition for the peptide. The most prevalent amino acids being serine, glycine and glutamate.

4.4 Discussion

To aid the interpretation of the amino acid analysis results the data from several literature reports were combined to prepare the amino acid sequence of an early human fibrinogen fragment D (Fig. 4.3). The data

Fig. 4.3 The amino acid sequence of fibrinogen fragment D

Proposed amino acid sequence of an 'early' fibrinogen fragment D from data published by several authors (see text).

The single letter code used is:

A, Ala; B, Asx; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; Z, Glx.

$\overset{\text{CHO}}{|}$: Carbohydrate attachment site.
▼ Δ : Crosslinking acceptor and donor sites.
Ⓟ : Plasmin cleavage points.
* : Lysine residue.
— : Disulphide bond.

HOVK*^{*}IRK*^{*}LYEIRSR*^{*}LDES*^{*}V*^{*}R*^{*}NY*^{*}T*^{*}ND*^{*} D_α
 I QLL*^{*}OK*^{*}N*^{*}V*^{*}RA*^{*}OL*^{*}VD*^{*}MD*^{*}K*^{*}RL*^{*}EV*^{*}DI*^{*}DI*^{*}K*^{*}IR*^{*}S*^{*}RG*^{*}S*^{*}SR*^{*}AL*^{*}ARE*^{*}V*^{*}DL*^{*}K*^{*}D*^{*}Y*^{*}E*^{*}OO*^{*}K*^{*}OL*^{*}OO*^{*}VI*^{*}AK*^{*}DL*^{*}LP*^{*}RS*^{*}DR*^{*}OH*^{*}LP*^{*}LI*^{*}AK*^{*}PK*^{*}P*^{*}VP*^{*}DL*^{*}VP*^{*}GN*^{*}FK*^{*}SO*^{*}LO*^{*}K*^{*}VP*^{*}PE*^{*}WK*^{*} P

INSN*^{*}V*^{*}T*^{*}E*^{*}DI*^{*}LQ*^{*}H*^{*}KE*^{*}LES*^{*}SY*^{*}EN*^{*}V*^{*}V*^{*}NN*^{*}END*^{*} D_β
 P TNLR*^{*}VL*^{*}RS*^{*}ILE*^{*}N*^{*}LR*^{*}SK*^{*}IO*^{*}K*^{*}LE*^{*}SD*^{*}V*^{*}SA*^{*}Q*^{*}ME*^{*}Y*^{*}RT*^{*}P*^{*}VS*^{*}Q*^{*}IP*^{*}V*^{*}VS*^{*}G*^{*}KE*^{*}EE*^{*}II*^{*}R*^{*}KG*^{*}GET*^{*}SE*^{*}MY*^{*}LI*^{*}OP*^{*}D*^{*}SS*^{*}V*^{*}K*^{*}PY*^{*}R*^{*}V*^{*}Y*^{*}Q*^{*}DM*^{*}NT*^{*}EN*^{*}GG*^{*}WT*^{*} V
 G YBK*^{*}G*^{*}DT*^{*}NT*^{*}AV*^{*}NG*^{*}FG*^{*}G*^{*}K*^{*}YP*^{*}D*^{*}WK*^{*}RG*^{*}FD*^{*}VS*^{*}GD*^{*}OR*^{*}NO*^{*} I
 LPGE*^{*}Y*^{*}WL*^{*}GB*^{*}B*^{*}IK*^{*}IS*^{*}Z*^{*}LR*^{*}M*^{*}GP*^{*}TE*^{*}LL*^{*}IE*^{*}ME*^{*}D*^{*}WK*^{*}G*^{*}DK*^{*}V*^{*}KA*^{*}HY*^{*}GG*^{*}FT*^{*}V*^{*}Q*^{*}NE*^{*}AN*^{*}K*^{*}Y*^{*}Q*^{*}IS*^{*}V*^{*}N*^{*}K*^{*}Y*^{*}RG*^{*}T*^{*} CHO
 A HOR*^{*}BY*^{*}WW*^{*}GG*^{*}DE*^{*}K*^{*}S*^{*}Q*^{*}QR*^{*}RP*^{*}D*^{*}ST*^{*}L*^{*}WG*^{*}D*^{*}ND*^{*}RD*^{*}Y*^{*}TS*^{*}F*^{*}M*^{*}GN*^{*}HI*^{*}TM*^{*}TR*^{*}NE*^{*}GM*^{*}L*^{*}Q*^{*}S*^{*}AG*^{*}D*^{*}ML*^{*}ANG*^{*} A
 ANPN*^{*}GR*^{*}Y*^{*}YW*^{*}GG*^{*}Q*^{*}Y*^{*}T*^{*}WD*^{*}MA*^{*}K*^{*}H*^{*}G*^{*}T*^{*}BD*^{*}GV*^{*}V*^{*}WM*^{*}NN*^{*}WK*^{*}GS*^{*}W*^{*}YS*^{*}MR*^{*}K*^{*}MS*^{*}M*^{*}K*^{*}IR*^{*}PF*^{*}FP*^{*}Q*^{*}Q

I SAEY*^{*}K*^{*}M*^{*}IE*^{*}EL*^{*}M*^{*}K*^{*}R*^{*}S*^{*}KL*^{*}TA*^{*}AD*^{*}IM*^{*}NP*^{*}K*^{*}S*^{*}SE*^{*} D_γ
 P LTH*^{*}SS*^{*}IR*^{*}YL*^{*}Q*^{*}E*^{*}I*^{*}NS*^{*}NN*^{*}O*^{*}K*^{*}I*^{*}V*^{*}N*^{*}L*^{*}K*^{*}E*^{*}V*^{*}A*^{*}Q*^{*}EA*^{*}Q*^{*}Q*^{*}EP*^{*}Q*^{*}DT*^{*}V*^{*}Q*^{*}I*^{*}H*^{*}DI*^{*}T*^{*}G*^{*}K*^{*}D*^{*}Q*^{*}IA*^{*}N*^{*}K*^{*}GA*^{*}K*^{*}OS*^{*}GL*^{*}Y*^{*}FI*^{*}K*^{*}PL*^{*}KA*^{*}NO*^{*}Q*^{*}FL*^{*}Y*^{*}Q*^{*}E*^{*}ID*^{*}GS*^{*}GN*^{*}GW*^{*}TV*^{*}F*^{*}Q*^{*}K*^{*}RL*^{*}D*^{*}GS*^{*}V*^{*}DF*^{*}K*^{*}R*^{*}N*^{*}WI*^{*}Q*^{*} Y
 D AEP*^{*}GV*^{*}K*^{*}F*^{*}M*^{*}AY*^{*}D*^{*}AT*^{*}STR*^{*}GN*^{*}W*^{*}DE*^{*}LE*^{*}V*^{*}RL*^{*}AY*^{*}PI*^{*}A*^{*}S*^{*}OT*^{*}S*^{*}IL*^{*}HI*^{*}K*^{*}EN*^{*}GL*^{*}WF*^{*}ET*^{*}T*^{*}G*^{*}T*^{*}PS*^{*}L*^{*}H*^{*}G*^{*}F*^{*}GE*^{*}K*^{*} G
 P KY*^{*}RL*^{*}TY*^{*}AY*^{*}F*^{*}AG*^{*}GD*^{*}AG*^{*}DA*^{*}FG*^{*}DF*^{*}GD*^{*}DD*^{*}PS*^{*}DK*^{*}FF*^{*}T*^{*}SH*^{*}NG*^{*}M*^{*}Q*^{*}F*^{*}ST*^{*}W*^{*}D*^{*}ND*^{*}ND*^{*}DK*^{*}FE*^{*}GN*^{*}Q*^{*}AE*^{*}Q*^{*}D*^{*}GS*^{*} G
 P IIR*^{*}M*^{*}TT*^{*}K*^{*}MS*^{*}Y*^{*}WR*^{*}T*^{*}K*^{*}(IA,W,I)IG*^{*}ND*^{*}Y*^{*}GN*^{*}PT*^{*}SA*^{*}K*^{*}(S,Y,T)GG*^{*}O*^{*}Y*^{*}Y*^{*}GN*^{*}L*^{*}H*^{*}G*^{*}A*^{*}H*^{*}OK*^{*}N*^{*}M*^{*}(W)W*^{*} G
 P FNRL*^{*}T*^{*}IG*^{*}EG*^{*}O*^{*}QH*^{*}HL*^{*}GG*^{*}A*^{*}K*^{*}Q*^{*}AG*^{*}D*^{*}V*^{*} P

relating to the δ chain were provided by the work of Lottspeich & Henschen (1977). The β chain sequence data were published by Henschen & Lottspeich (1977) and by Watt et al. (1978) while the work of Doolittle et al. (1977a) and Lottspeich & Henschen (1978) was consulted to prepare the α chain sequence. The identity of the COOH and NH₂-terminal amino acids and of the plasmic cleavage points was provided by several published reports. The COOH-terminal glutamine residue of the β chain of fibrinogen appears to be preserved even in the "late" forms of fragment D (Furlan et al., 1975) while the NH₂-terminal amino acid, aspartate, was reported by Collen et al., (1975). In the δ chain a pentapeptide is split from the COOH-terminus during the formation of fragment D (Takagi & Doolittle, 1975) (this pentapeptide is shown in brackets in Fig. 4.3). The initial peptide bond split between fragments D and E is δ 62Lys-63Ala (Takagi & Doolittle, 1975) but this δ chain NH₂-terminal alanine residue of fragment D is replaced by a serine residue following further plasmin digestion (Collen et al., 1975). This NH₂-terminal serine survives further plasmic treatment while the D- δ chain is progressively "nibbled" from the COOH-terminus (Furlan et al., 1975). A further plasmic cleavage point has been reported in the δ chain between lysine and glycine residues located approximately 74 residues from

the NH_2 -terminus (Henschen et al., 1978). In the D- α chain the original NH_2 -terminal residue is aspartate but this is replaced after further digestion by a valine residue (Doolittle et al., 1977a). Plasmic cleavage may also occur at the COOH-terminus at arginine-serine and arginine-glutamine bonds (Lottspeich & Henschen, 1978) and lysine-methionine and lysine-serine bonds (Doolittle et al., 1977).

The arrangement of the disulphide bonds within fragment D is based upon the reports by Henschen (1978), Bouma et al. (1978) and Gårdlund et al. (1977).

The sequence data presented in Fig. 4.3 display the "earliest" fragment D molecule. The molecular weight of this fragment D (minus the γ chain terminal pentapeptide) is 91,000. However the NH_2 -terminal estimation results described in Section 2.3.2 suggest that both fragment $\text{D}_{\text{Ca}^{2+}}$ and D_{EDTA} contain α and γ chains which have undergone plasmic attack at the NH_2 -termini and here a molecular weight of 88,000 is predicted. This latter figure and its associated sequence data were employed in the preparation of Tables 4.1 and 4.2. No allowance was made when calculating the molecular weight values for carbohydrate known to be associated with the β chain (Töpfer-Peterson et al., 1976) of fragment D.

The present conception of the fragment $\text{D}_{\text{Ca}^{2+}}$ molecule infers that this species should most closely represent the theoretical "early fragment D" molecule

depicted in Fig. 4.3 (minus an NH_2 -terminal peptide in the α and γ chains). Two molecular weight values were employed in the presentation of the amino acid analysis data relating to fragment $\text{D}_{\text{Ca}^{2+}}$. The first value 99,000 was implied by SDS-gel electrophoresis while the second, 88,000 was calculated from the primary sequence data. This second lower value produced a greater degree of similarity between the amino acid composition of fragment $\text{D}_{\text{Ca}^{2+}}$ (sample A) and the fragment D represented by Fig. 4.3. However notable exceptions were the values attributable to proline, valine, glycine and methionine. Further data are required to clarify the origin of this dissimilarity.

The amino acid analysis of fragment D_{EDTA} (sample C) suggested that the more degraded forms of fragment D i.e. those molecules which have undergone plasminic attack at the COOH-terminal regions of the constituent γ chain contain fewer polar and negatively charged amino acids. This conclusion agrees with the findings of Ferguson et al. (1975) who proposed that the COOH-terminal region of the γ chain which is digested by plasmin lies on the surface of the fragment D molecule. However they also reported the loss of proline and phenylalanine residues.

The results pertaining to the second fragment $\text{D}_{\text{Ca}^{2+}}$ sample (sample B) and to the peptide isolated from fragment $\text{D}_{\text{Ca}^{2+}}$ (sample D) must be interpreted with caution. Several assumptions have been made in designing this experiment not least being the validity of the structure envisaged for the

Table 4.4 The amino acid composition predicted for a peptide isolated from fragment D_{Ca}^{++}

SAMPLES	DIFFERENCES IN AMINO ACID COMPOSITION	
	MAJOR	MINOR
Fg D_{Ca}^{++} (sample B) and Fg D_{Ca}^{++} (sample A)	Ser	Thr Glu His Ile Lys
Fg D_{Ca}^{++} (sample B) and theoretical values	Ser	Thr Glu Val Ile Phe
Isolated peptide	Ser Gly Glu	Thr Val Ile Asp Leu Ala Lys

second type of fragment $D_{Ca^{2+}}$ molecule which has undergone limited plasmic degradation (Fig. 4.1). Given these limitations the structure proposed for the fragment $D_{Ca^{2+}}$ molecule infers that the difference in amino acid composition between samples A and B should reflect the amino acid composition determined for the isolated peptide. These results are summarised in Table 4.4. The composition of the peptide is largely predicted from the results obtained with the fragment $D_{Ca^{2+}}$ samples.

The results presented in Division I led to the proposal that the region of binding of Ca^{2+} to fragment $D_{Ca^{2+}}$ may be the COOH-terminus of the δ chain. Accordingly it was of interest to compare the amino acid composition of the isolated peptide with that predicted from the sequence data of Fig. 4.3 for two peptides derived from the NH_2 and COOH-termini of the fragment $D - \delta$ chain. The location of the peptides is presented in Fig. 4.4 and the respective amino acid compositions are included in Table 4.3. The isolated peptide cannot be assigned with certainty to either of these regions of the fragment $D_{Ca^{2+}}$ δ chain. The high glycine content of the peptide concurs with that of the δ chain COOH-terminal region while conversely the content of valine glutamate and serine residues is more akin to that of the NH_2 -terminal region. Therefore the possibility that the isolated peptide originated from

some other region of the fragment $D_{Ca^{2+}}$ molecule or indeed from some contaminant of the fragment $D_{Ca^{2+}}$ sample must be considered. However the sequence data presented in Fig. 4.3 are not consistent with the peptide having arisen from either the COOH or NH_2 -termini of the β or α chains of fragment D. Furthermore the possibility that the peptide may be ascribed to contamination of the fragment $D_{Ca^{2+}}$ sample by Trasylol appears unlikely. The predominantly acidic nature of the isolated peptide is not in keeping with the basic properties of Trasylol. However, there remains the possibility that the peptide arose from contamination of fragment $D_{Ca^{2+}}$ by some peptide released from the plasminogen and streptokinase molecules during the digestion stage or alternatively from the degraded fibrinogen molecule.

The amino acids which have been implicated in the binding of Ca^{2+} to fibrinogen are histidine, aspartate or glutamate and tyrosine (Earland et al., 1972). Endres & Scheraga (1972) proposed a role for carboxyl groups and possibly the hydroxyl groups of serine or threonine. Marguerie et al. (1977) also suggested that carboxyl groups were involved along with histidine residues in the binding of Ca^{2+} . The amino acid composition of the peptide presented in Table 4.4 is not incompatible with these proposals.

In conclusion the amino acid analysis results have suggested a predominantly hydrophilic composition for a

peptide isolated from fragment D_{Ca²⁺}. However the examination of the primary amino acid sequence of fragment D has failed to confirm the proposal that the origin of the peptide was the COOH-terminus of the γ constituent chain and the exact source of the peptide has not been established. No reaction of the isolated peptide with anti-human fibrinogen was detected. Therefore the relevance of this result to the binding of Ca²⁺ to the fragment D molecule must await the investigation of the validity of the models for the fragment D_{Ca²⁺} molecules and in addition further characterisation of the isolated peptide. However it is interesting to note in passing that Cottrell & Doolittle (1976) reported the isolation of a peptide from the COOH-terminus of the A α chain of fibrinogen (the region of binding of the third Ca²⁺ according to Marguerie (1977)). One third of that peptide was composed of serine, glycine and glutamate residues - also the most prevalent amino acids recorded for the peptide isolated from fragment D_{Ca²⁺}.

SECTION 5

DISCUSSION

Two aspects of the fibrinogen fragment D molecule - the size and conformation are reflected in the results from the foregoing studies.

The heterogeneity of fragment D preparations both in charge and size has been well documented. The variation in molecular weight of fragment D molecules has been ascribed to the effect of the sequential attack of plasmin on the COOH-terminal end of the constituent γ chain remnant. The inference from the studies reported in Division I and the results of Haverkate & Timan (1977) is that this phenomenon may be a consequence of the degradation of fibrinogen in a medium containing minimal levels of Ca^{2+} , since a single high molecular weight form of fragment D ($\text{D}_{\text{Ca}^{2+}}$) is produced in the presence of near physiological Ca^{2+} concentrations. A model of the fragment $\text{D}_{\text{Ca}^{2+}}$ molecule was developed in Division I in which, it was proposed that further degradation of the γ subunit chain was prevented by the binding of Ca^{2+} at the plasmin-vulnerable, COOH-terminus. The validity of this model is strengthened by the foregoing NH_2 -terminal amino acid analysis results. Furthermore the results from the isoelectric focussing studies suggest that the reported charge heterogeneity of the D fragments of fibrinogen may also be attributed, in part, to the preparation of fragment D in the presence of varying amounts of Ca^{2+} .

It was suggested that the plasmin-resistance of fragment $D_{Ca^{2+}}$ may be derived from the particular conformation of the molecule induced by bound Ca^{2+} . This same conformational effect of bound Ca^{2+} seemed to explain the anomalous electrophoretic mobilities displayed by fragments $D_{Ca^{2+}}$ and Y and the constituent γ chains of fibrinogen and fragment $D_{Ca^{2+}}$. The results from the chemical crosslinking studies do indeed strongly imply an important role for Ca^{2+} in maintaining a more compact, thereby plasmin-resistant and stable fragment $D_{Ca^{2+}}$ molecule. This stability displayed by fragment $D_{Ca^{2+}}$ was also indicated from ultracentrifugation studies. Under identical experimental conditions the fragment D_{EDTA} molecule is unstable and degradation occurs.

This model for the fragment $D_{Ca^{2+}}$ molecule has wider implications for the structure of the parent molecule, fibrinogen and this aspect of the conformation of fragment D will be considered in the General Discussion (Part C).

PART C

GENERAL DISCUSSION

This Discussion reviews the pertinent issues arising from the experimental work which have bearing on the structure of fragment D and concludes with a consideration of the relevance of this information to the structure of fibrinogen.

The study of the plasmin-derived fibrinogen degradation product fragment D is complicated by the heterogeneity of the preparation. This factor may be attributed both to the instability of fragment D and to the effect of differing extents of plasminic degradation of the constituent δ chain. In the experimental work described in Part A this problem was manifested by the identification of three major molecular weight forms of fragment D. Three fragment D species of differing molecular weights were also described by Furlan et al. (1975) and by Ferguson et al. (1975). However it is possible to reconcile these results with the fibrinogen digestion studies performed in the presence of Ca^{2+} and reported in Part B. There a single high molecular weight form of fragment D ($\text{D}_{\text{Ca}^{2+}}$) was produced from fibrinogen but, upon removal of Ca^{2+} , two lower molecular weight species of fragment D were identified. These three fragment D molecules differed apparently only in the extent of degradation of the constituent δ chain. Therefore the three forms of fragment D referred to in Part A must have arisen as a consequence of degrading

fibrinogen in a medium containing insufficient Ca^{2+} to saturate completely the Ca^{2+} binding sites within the fibrinogen molecule. The normal total concentration of Ca^{2+} in human blood plasma is approximately 2.5 mM of which about one half is in the ionized state, the remainder being in combination with protein. A value of approximately 9×10^{-6} M has been reported for the dissociation constant of the Ca^{2+} binding site associated with fragment D (Lindsey et al., 1978; Nieuwenhuizen et al., 1979). Thus it can be predicted that the structure described for fragment $\text{D}_{\text{Ca}^{2+}}$ in Part B must represent the more physiologically important form of the molecule. Fibrinogen preparations are normally contaminated by factor XIII and prothrombin. Hitherto it has been normal practice to avoid any possible detrimental effect on the fibrinogen molecule by inhibiting the activation of these agents through the addition of a Ca^{2+} -chelating agent. Consequently the importance of Ca^{2+} both to the degradation and to the structure of fibrinogen has yet to be fully recognised. In this context the present studies strongly advocate that a reevaluation of many of the reports describing the biological effects of fragment D is warranted.

Several lines of evidence drawn from the foregoing work suggest that Ca^{2+} may play a crucial role in the conformation and therefore by implication, in the properties of the fragment $\text{D}_{\text{Ca}^{2+}}$ molecule viz. the

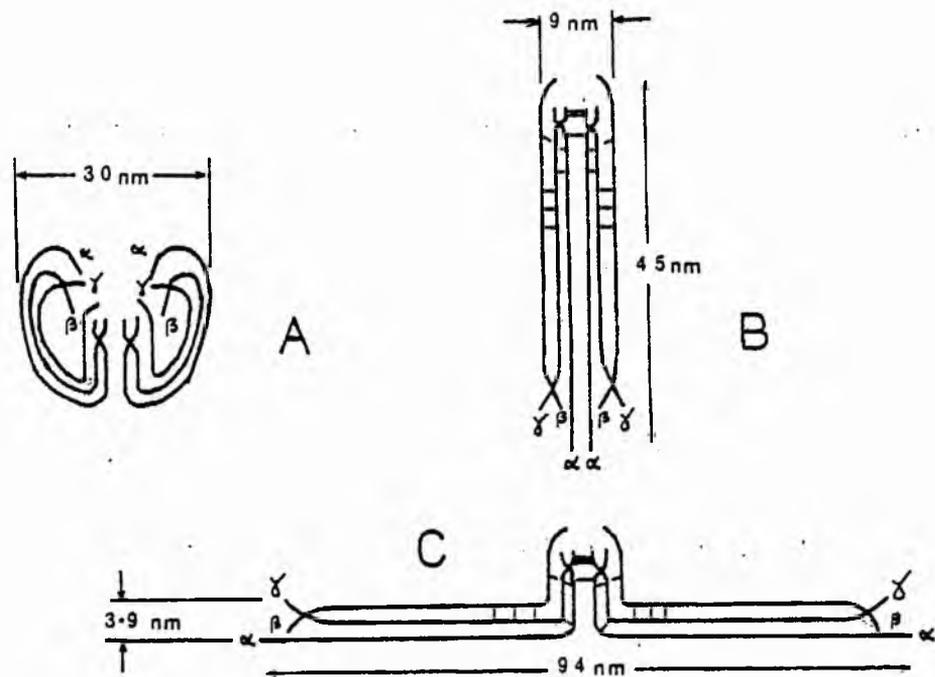
resistance to degradation by plasmin, the anomalous electrophoretic mobility and the disinclination to form significant intermolecular contacts. Each of these observations is compatible with a model of the fragment $D_{Ca^{2+}}$ molecule in which Ca^{2+} favours a more compact thereby stable structure. The evidence from fibrinogen digestion studies and amino-terminal amino acid analysis results strongly suggests that the site of Ca^{2+} binding to fragment $D_{Ca^{2+}}$ is located towards the COOH-terminus of the constituent δ chain. This proposal is supported by the work of Haverkate et al. (1979) who demonstrated that fragment $D_{Ca^{2+}}$ exhibits anticlotting activity. The crosslinking site on the δ chain is located at the COOH-terminus, and must be preserved in the fragment D molecule prepared in the presence of Ca^{2+} . The fragment D produced in the absence of Ca^{2+} was ineffective as an anticlotting agent.

These proposals for the influence of Ca^{2+} on the conformation of the fragment D molecule may have implications for the structure of the parent molecule fibrinogen.

The present state of knowledge concerning the shape of the fibrinogen molecule was reviewed in the General Introduction. A variety of shapes has been proposed for the fibrinogen molecule, ranging from the trinodular model of Hall & Slayter (1959) and the cylindrical model

suggested by Bachmann et al. (1975) to the spherical shape described by Marguerie et al. (1975), Marquerie & Stuhmann (1976) and by Hudry-Clergeon et al. (1975), but an unequivocal description of the shape of the molecule has yet to be achieved. This problem may be due in part to the failure of many investigators to appreciate the importance of Ca^{2+} to the properties of the fibrinogen molecule. Marguerie & Stuhmann (1976) reported their results not from the electron microscopy of dehydrated fibrinogen specimens but from neutron small angle scattering studies with fibrinogen solutions containing certain defined levels of Ca^{2+} . The spherical model proposed for the fibrinogen molecule by this group of workers involves the folding of the constituent $\text{A}\alpha$, $\text{B}\beta$ and γ chains to form a spherical shape which is maintained, in part, by interactions between the COOH -terminal and NH_2 -terminal regions of the constituent chains. Marguerie (1977) suggested from a study of the early stages of fibrinogen digestion by plasmin that one of the three Ca^{2+} binding centres was located within the COOH -terminal region of the $\text{A}\alpha$ chain. It seems reasonable, although speculative, to propose that this Ca^{2+} and the two Ca^{2+} bound at the COOH -terminus of the γ chain may be located at a similar point on the spherical fibrinogen structure and thereby contribute to the proposed interactions between the NH_2 and COOH -terminal regions of the folded molecule. This line of reasoning also offers a means of reconciling

Fig.(i) Models proposed for the fibrinogen molecule



Three different isomeric structures of fibrinogen.

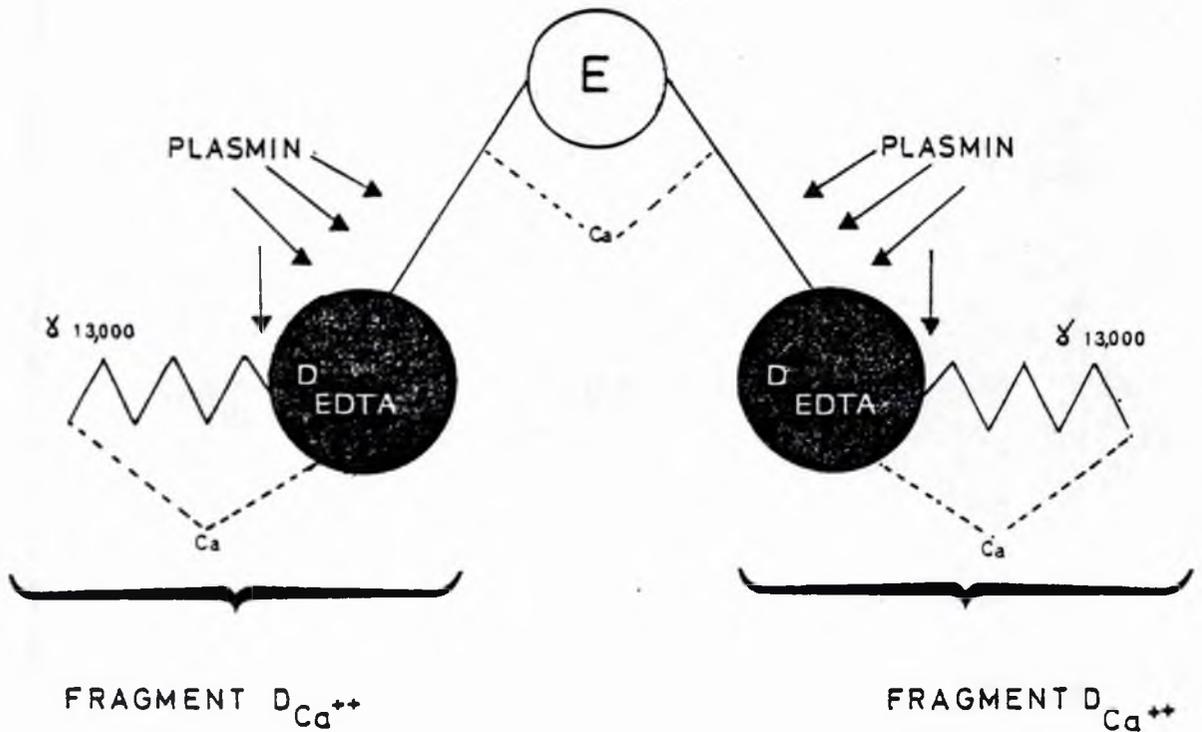
A, the banana-like model of Marguerie and associates;
B, the folded rod of Bachmann; C, the stretched rod
described by Mueller and Burchard.

From Mueller and Burchard (1978).

several of the apparently incompatible shapes reported for the fibrinogen molecule. Just as the molecular weight heterogeneities of fragment D preparations may be attributed to the digestion of fibrinogen in the presence of differing levels of Ca^{2+} , so the intact fibrinogen molecule may display one of several isomeric structures depending on the level of available Ca^{2+} . Thus the spherical shape of the fibrinogen molecule suggested by Marguerie and associates may be converted to the elongated trinodular shape described by Hall & Slayter (1959) upon the removal of Ca^{2+} and the subsequent unfolding of the molecule. A similar proposal was made by Mueller & Burchard (1978) (Fig. (i)) who also suggested that the cylindrical shape proposed by Bachmann et al. (1975) for fibrinogen may be caused by the intramolecular association, possibly via hydrophobic and hydrogen bond interactions, between the rod-like sections of the molecule.

However it must be conceded that not all the investigators who have recognised the importance of Ca^{2+} to the structure and properties of the fibrinogen molecule have supported the model proposed by Marguerie and associates. Van Ruijven-Vermeer et al. (1978) studied rat fibrinogen and although they reported the location of a Ca^{2+} binding site within the fragment D moiety of fibrinogen, confirming the present results with human fibrinogen, they do not accept the proposal by

Fig.(ii) The binding of Ca^{++} to rat fibrinogen



Schematic representation of the three high-affinity binding sites in rat fibrinogen. The solid lines between the fragment D and E regions of the molecule represent parts of all three chains; $\text{A}\alpha$, $\text{B}\beta$ and δ . The dotted lines indicate a binding of Ca^{++}
 (Modified from Van Ruijven-Vermeer *et al.* (1978))

Marguerie (1977) that the COOH-terminal regions of the A α chains are involved in the binding of Ca²⁺. Instead they envisage that the third Ca²⁺ bound to fibrinogen forms an interdimeric bridge with binding sites in the polypeptide chains between the fragment D and E regions. Accordingly their model for the fibrinogen molecule (Fig. (ii)) may be compatible with either that of Hall & Slayter (1959) or that of Marguerie and associates. Furthermore the proposals of Marguerie for the location of the third Ca²⁺ binding site are inconsistent with the report by Stemberger & Jilek (1976) that rat fibrinogen contains a shorter A α chain than human fibrinogen and yet Van Ruijven-Vermeer et al. (1978) have reported the binding of three Ca²⁺ to this molecule. Furthermore Lindsay et al. (1978) reported that human fibrinogen displaying heterogeneity of the constituent A α chains still bound three Ca²⁺ per molecule. Marguerie's proposals for the A α chain Ca²⁺ binding site were based on evidence from fibrinogen digestion studies. The velocity of the splitting of the earliest plasmin-susceptible bonds was reduced in the presence of Ca²⁺. However the initial attack of plasmin on fibrinogen may remove a polypeptide of about 40,000 to 50,000 molecular weight (Furlan & Beck, 1972; Mills & Karpatkin, 1972) which contains the COOH-terminus but constitutes approximately two thirds of the A α chain. Therefore the observations of Marguerie (1977) do not preclude the

possibility that the Ca^{2+} binding site is in fact nearer the NH_2 -terminus than the COOH -terminus of the $\text{A}\alpha$ chain.

Conjecture regarding the identity of the third site of Ca^{2+} binding to the fibrinogen molecule may be made by considering the proposals for the binding of Ca^{2+} to fragment D. The present work has strongly implied a Ca^{2+} binding site towards the COOH -terminus of the δ chain. Purves et al. (1978) have demonstrated that fragment $\text{D}_{\text{Ca}^{2+}}$ can be crosslinked by factor XIII. Crosslinking of δ chains occurs between glutamine and lysine residues located respectively 14 and 6 residues from the COOH -terminus (Doolittle, 1973). Therefore although the bound Ca^{2+} maintains a conformation of the fragment D molecule in which the plasmin-vulnerable sites in the δ chain are masked, the crosslink site must be accessible to factor XIII. It can be proposed that there may be three not unrelated functions of bound Ca^{2+} at this COOH -terminal region of the δ chain; (i) to maintain a conformation of the chain amenable to the crosslinking by factor XIII (ii) to inhibit the action of plasmin and thereby prevent the digestion of the peptide containing the factor XIII-susceptible amino acids and (iii) the presentation of Ca^{2+} for the activation of factor XIII. The Ca^{2+} -requirement for the activation of factor XIII (Lorand, 1972) was shown by Credo et al. (1978) to be lowered by fibrinogen.

Thus the binding of Ca^{2+} proximal to the crosslinking site within the δ chain may not be coincidental.

Following this line of reasoning it can be considered that the third Ca^{2+} may be bound near the crosslinking site of the fibrinogen $\text{A}\alpha$ chain. The amino acid sequence of this chain has recently been reported and two glutamine crosslink acceptor sites at positions 328 and 366 i.e. within the terminal two thirds of the chain initially removed by plasmin, have been identified (Doolittle et al., 1979). Marguerie et al. (1977) suggested that histidine residues may be involved in the binding of Ca^{2+} to fibrinogen. Two of the fifteen histidine residues present in the $\text{A}\alpha$ chain are located at positions 353 and 368. Furthermore Marguerie (1977) suggested that the Ca^{2+} binding centres of fibrinogen are located in regions which do not involve α -helical structural parts of the molecule. The region of the $\text{A}\alpha$ chain suggested above as the site of Ca^{2+} binding does not contain computer predicted α -helical regions (Doolittle et al., 1979). The implied relationship between the binding of Ca^{2+} to fibrinogen and the generation of factor XIII activity has interesting reverberations of physiological significance to the final stages of blood coagulation.

To conclude, the importance of Ca^{2+} to the structure and properties of the fibrinogen molecule has yet to be

fully realised. The results of the investigation of the binding of Ca^{2+} to fragment D suggest that the identification of the site of binding of the third Ca^{2+} will provide valuable information of significance to the in vivo properties of the fibrinogen molecule.

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HCl in 10ml of methanol and diluting to 100ml with butan-1-ol; just before use, 0.1 g of sodium dithionite was added. The dried electrophoresis sheets were sprayed with the reagent and developed at 120°C for 15 min.

With 4- and 6-sulphated disaccharides a strong colour-positive reaction occurred with *p*-anisidine at the highest amount examined (20 µg) and a detectable coloured product was observed at the lowest amount (156 ng). The unsulphated disaccharide produced a weaker reaction, but was detected at a concentration of 1 µg. The *p*-anisidine reagent provided more than 50 times the sensitivity of alkaline AgNO₃. The best of several alkaline AgNO₃ reagents examined was that described by Trevelyan *et al.* (1950). Cartilage extracts of glycosaminoglycans, enzymes and buffers did not cause interference with the disaccharide-anisidine reaction. In an examination of the monosaccharide components of glycosaminoglycans, the amino sugars, glucosamine and galactosamine did not react with *p*-anisidine. Glucose, galactose and uronic acids gave a positive (brown) reaction confirming previous reports that this reagent reacts with reducing monosaccharides (Merck, 1966). Under the assay conditions described monosaccharides were not produced from the chondroitin sulphate.

The electrophoresis conditions described produced migration distances of 9.5 cm for unsulphated disaccharide with the 4-sulphated disaccharide located between 12.5 and 14.5 cm and the 6-sulphated disaccharide between 13.0 and 15.0 cm. The desulphation of the 4-sulphated disaccharide by chondro-4-sulphatase readily permitted separation and identification of mixtures containing the two disaccharides.

The depolymerization of chondroitin sulphate from articular cartilage extracts with chondroitin ABC lyase produced *p*-anisidine-detectable sulphated disaccharides after 45 min at 37°C. The nature of the reaction between *p*-anisidine and the chondroitin sulphate disaccharides was not resolved. For the quantitative determination of microgram quantities of 4- and 6-sulphated disaccharides the periodate/thiobarbituric acid and borate/*p*-dimethylaminobenzaldehyde assays were used (Elliott & Gardner, 1977).

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Evidence for the Presence of a Calcium-Ion-Binding Site within Fibrinogen Fragment D

JAN S. LAWRIE and GRAHAM KEMP

Department of Biochemistry, University of St. Andrews, St. Andrews KY16 9AL, Fife, Scotland, U.K.

Recent reports have emphasized the importance of Ca²⁺ to native fibrinogen structure. The results of Marguerie (1977) and Marguerie *et al.* (1977) suggest the existence of three strongly bound Ca²⁺ ions per fibrinogen molecule. The position of these within the molecule is not established, but Marguerie (1977) has suggested that a binding site exists in the C-terminal portion of the (A)α-chain. Haverkate & Timan (1977) showed that in the presence of Ca²⁺ the digestion of fibrinogen by plasmin proceeded more slowly than in their absence, and, significantly, that only a single molecular-weight species of fragment D was produced. This is in contrast with the digestion in the absence of Ca²⁺, where three D fragments of mol.wts. 94000 (D₁), 88000 (D₂) and 83000

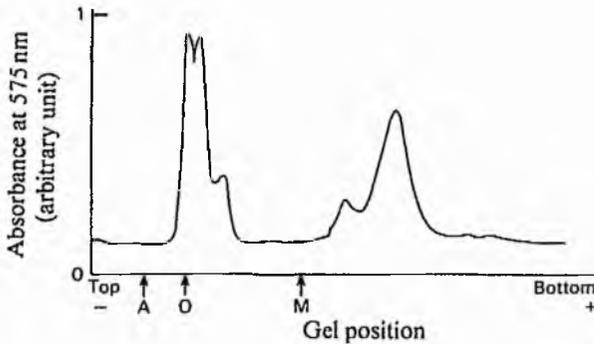


Fig. 1. Densitometric traces of 10% acrylamide gels subjected to SDS/polyacrylamide-gel electrophoresis

The sample was reduced. The arrows indicate the positions of proteins of known molecular weight run on accompanying gels. A, bovine serum albumin, mol.wt. 68000; O, ovalbumin, mol.wt. 43000; M, myoglobin, mol.wt. 17000.

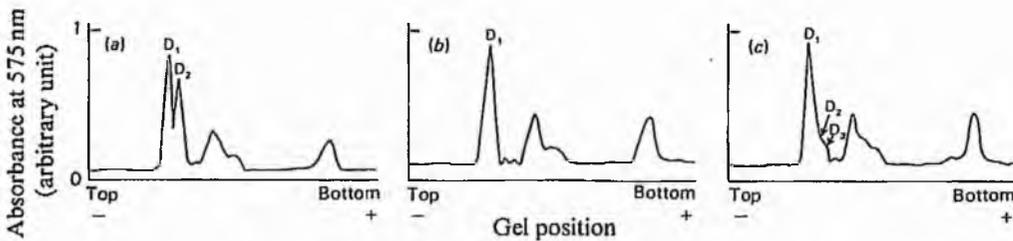


Fig. 2. Densitometric traces of 5% gels subjected to SDS/polyacrylamide gel electrophoresis

Samples were not reduced, and were treated as follows. (a) Adjusted to 1.5% SDS/4M-urea immediately before application; (b) incubated for 30 min at 20°C in the presence of 1.5% SDS/4M-urea and 20mM-EDTA; (c) incubated for 18 h at 4°C in the presence of 1.5% SDS/4M-urea.

(D₃) are produced (Furlan *et al.*, 1975). Our studies of the plasmic degradation of fibrinogen in the presence of Ca²⁺ ions enable us to make some suggestions as to the locations of the calcium-binding sites.

The conditions used for the activation of plasminogen, digestion of fibrinogen and termination of the reaction with aprotinin were as described previously (Lawrie *et al.*, 1977), but with two modifications. CaCl₂ was added to concentration of 2mM (this concentration being maintained throughout), and 0.25 C.T.A. (Committee on Thrombolytic Agents) unit of plasmin was added per mg of fibrinogen. For polyacrylamide-gel electrophoresis, acrylamide and methylenebisacrylamide were dissolved directly in 6M-urea/0.1M-Tris/HCl/0.2% SDS,* pH7.4. Final concentrations of acrylamide were either 5% (w/w) (containing 5% bisacrylamide) or 10% (containing 3% bisacrylamide). The electrophoresis chamber contained 0.2% SDS in 0.1M-Tris/HCl buffer, pH7.4. Samples were reduced by mixing with an equal volume of a 3% (v/v) 2-mercaptoethanol/3% (w/v) SDS/8M-urea solution and heating at 100°C for 5 min. Samples run without reduction were normally mixed with an equal volume of a 3% SDS/8M urea solution immediately before application to the gel.

The results from a 3h digest are shown in Figs. 1 and 2(a). After reduction (Fig. 1)

* Abbreviation: SDS, sodium dodecyl sulphate.

the band pattern was in agreement with the results of Haverkate & Timan (1977). The presence of two peaks of approximately equal size, with mobilities corresponding to mol.wts. of 45000 and 40000, and only a small peak between those and the peak due to aprotinin towards the end of the gel, indicates that the major product is a fragment-D species of mol.wt. 94000, assuming a mol.wt. of 9000 for the α -chain remnant (Furlan *et al.*, 1975). A small amount of a lower-molecular-weight fragment-D species (83000) was also present in the digest. The results from the unreduced samples of the same digest (Fig. 2a) were in apparent contradiction to this. Two peaks of approximately equal size were present, corresponding to species of mol.wt. 94000 and 88000, as well as a shoulder with a mobility consistent with the 83000-mol.wt. species. Addition of disodium EDTA to a concentration of 20mM, after the addition of urea and SDS, and followed by incubation at 20°C for 30 min resulted in the disappearance of the faster-moving major peak with a concomitant increase in the size of the slower-moving peak (Fig. 2b). Prolonged incubation (18 h at 4°C) of the sample in 1.5% SDS/4M-urea resulted in a decrease in, but not the complete disappearance of, this faster-moving peak (Fig. 2c). Addition of EGTA had an effect similar to that of EDTA (results not shown).

These results strongly suggest that a binding site for Ca^{2+} is associated with fragment D. As one molecule of fibrinogen gives rise to two molecules of fragment D, then two of the three Ca^{2+} -binding sites proposed by Marguerie *et al.* (1977) are accounted for in this way. To accommodate the suggestion of Marguerie (1977) that the C-terminal portion of the (A) α -chain contains a Ca^{2+} -binding site, one must involve a Ca^{2+} bridge between the C-terminal portions of the two fibrinogen (A) α -chains. The largest-molecular-weight fragment D contains a γ -chain remnant with only a pentapeptide missing from the C-terminus (Tagaki & Doolittle, 1975), whereas in the absence of Ca^{2+} , digestion of fragment D proceeds by progressive proteolysis from the C-terminus (Furlan *et al.*, 1975). The fact that the presence of calcium inhibits this digestion of the γ -chain, even in the presence of 2M-urea (Haverkate & Timan, 1977) suggests that the C-terminal portion of the γ -chain is involved in the binding of Ca^{2+} . Whether this Ca^{2+} ion forms a bridge with another chain of fragment D, or whether it forms an internal bridge within the γ -chain is not yet clear.

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***N*-Methylindoxyl Acetate-Linked Stain for Acetylcholinesterase**

D. A. GALBRAITH and D. C. WATTS

Department of Biochemistry and Chemistry, Guy's Hospital Medical School, London SE1 9RT, U.K.

Acetylcholinesterase (EC 3.1.1.7) hydrolyses *N*-methylindoxyl acetate to give a fluorescent product. It can be used to locate acetylcholinesterase bands on gels with a standard u.v. lamp (approx. 350nm) (Seghatchian *et al.*, 1973). Quite heavy loading of an impure enzyme preparation such as solubilized stroma is required and the bands made visible are rather indistinct.

To improve this method we attempted to link various salts to the reaction to give coloured bands. The salts used were Fast Blue RR (from Edward Gurr Ltd.), Fast Blue BB, Fast Red TR and Fast Violet B (all from Sigma).

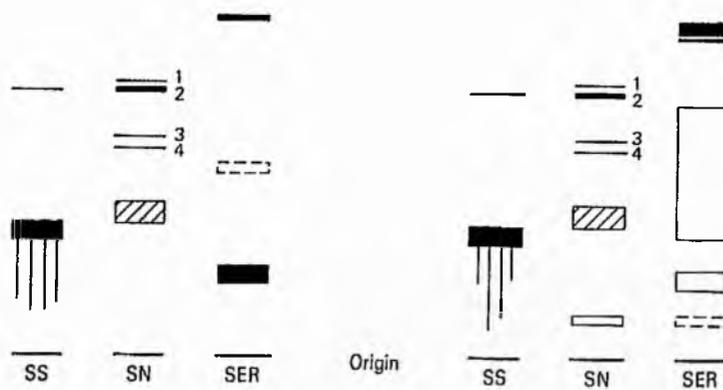


Fig. 1. (a) Solubilized stroma, supernatant and serum electrophoresed and stained with *N*-methylindoxyl acetate as substrate, and (b) solubilized stroma, supernatant and serum electrophoresed and stained with 2-naphthyl acetate as substrate

▨, Haemoglobin. Abbreviations used: SS, solubilized stroma; SN, supernatant; SER, serum.

Stroma and the supernatant from the first centrifugation after lysis of the erythrocytes were obtained by the method of Dodge *et al.* (1963) from fresh blood obtained by venipuncture collected into ACD plasma (aged citric acid/sodium citrate/dextrose) or heparin. The stroma was solubilized in a final concentration of 1% Lubrol WX.

The samples were analysed by electrophoresis on starch gels with Tris/borate/EDTA buffer, pH 8.7 (Seghatchian *et al.*, 1973) at 20 v/cm in a cold-room at 4°C for 4 h.

N-Methylindoxyl acetate, 6.4 mm in 0.2 M-phosphate buffer, pH 7.4, was prepared by first dissolving the *N*-methylindoxyl acetate in a small volume of methanol (not exceeding 4% of the final volume). The various coupling dyes were then dissolved in this to give concentrations of 50 mg/ml. After slicing, the gels were incubated in the staining solutions for periods up to 2 h.

A typical result obtained with Fast Blue RR salt is shown in Fig. 1(a).

Constituents stained with Fast Blue RR salt showed very distinct and narrow bands within 30 min. Fast Violet B and Fast Blue BB gave more diffuse bands and were slower-developing. Fast Red TR gave very poor results; it had poor linkage with the enzymic product.

Only the acetylcholinesterase in the solubilized stroma showed any smearing, apparently due to the incomplete solubilization procedure. Solubilized stroma showed only one large band of acetylcholinesterase and sometimes a faint fast band that appeared to have been attached to the membrane from the interior of the cell, since an identical band appeared very heavily stained in the supernatant electrophoretogram. Serum gave two heavily stained bands and one very faint one.

When gels were stained with 2-naphthyl acetate (see Simonarson & Watts, 1969) as substrate, additional bands were stained (Fig. 1b). The slow-moving one shown by supernatant is carbonic anhydrase. Out-of-date blood-bank blood gave an additional fast heavily stained band in the supernatant electrophoretogram.

No differences were found in the patterns given by serum, supernatant or solubilized stroma from blood collected into ACD plasma or heparin.

In an attempt to identify the bands produced by the supernatant, further starch gels were incubated in different inhibitor solutions for 30 min before staining with Fast Blue RR and *N*-methylindoxyl acetate.

Inhibitors used were acetylcholine chloride, tetramethylammonium chloride, eserine, *p*-chloromercuribenzoate, *p*-iodomercuribenzoate, phenyl acetate and di-isopropyl phosphorofluoridate. The results and tentative conclusions are summarized in Table 1.

Phenyl acetate was a competitive inhibitor to all the esterases, causing slowed appear-

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Purification of Fibrinogen and the Separation of its Degradation Products in the Presence of Calcium Ions

JAN S. LAWRIE, JOAN ROSS and GRAHAM D. KEMP

Department of Biochemistry and Microbiology, University of St. Andrews, North Street, St. Andrews, Fife KY16 9AL, Scotland, U.K.

A desire to investigate the influence of Ca^{2+} on the structure and properties of fibrinogen and its degradation products has necessitated the development of novel purification and separation procedures. The purification scheme of Mosesson & Finlayson (1963) yields a fibrinogen free from factor XIII and plasminogen, but involves the use of Tris/phosphate buffers and is therefore incompatible with the presence of Ca^{2+} . Separation of the plasmin degradation products, fragments D and E, can be achieved by using CM-cellulose (Kemp *et al.*, 1973) or DEAE-cellulose (Nussenzweig *et al.*, 1961). We wished to avoid the use of acidic buffers, since Marguerie *et al.* (1977) have reported that the binding of at least one Ca^{2+} ion is abolished at pH values below 6.5. Published DEAE-cellulose methods are again incompatible with Ca^{2+} , because they use phosphate-buffer systems.

The starting material was human fibrinogen (grade L) from KABI Pharmaceuticals (Stockholm, Sweden). Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out as previously described (Lawrie & Kemp, 1979). Fractions were tested for the presence of plasminogen as follows. Streptokinase was added to a final concentration of 200 i.u./ml and the test sample incubated at 37°C for 2h. Samples were examined for proteolytic degradation by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. In order to detect factor XIII, samples were incubated with a final concentration of 5 National Institute of Health units of thrombin/ml and 20mM- CaCl_2 for 2h at 37°C. The presence of cross-linked γ chains after electrophoresis of the reduced sample indicated the presence of factor XIII.

Fibrinogen purification

Fibrinogen at a concentration of 5 mg/ml was dialysed at 4°C against 0.05M-Tris/HCl buffer, pH 8.6, made 0.05M with respect to NaCl and 2mM with respect to CaCl_2 . At 4°C there was an extensive precipitate, which almost completely dissolved on warming to 37°C for 5 min. Any remaining precipitate was removed by centrifugation and discarded. The supernatant was applied to a column (1.5cm x 25cm) of DEAE-cellulose equilibrated with the Tris/NaCl/ CaCl_2 buffer. The column was eluted first with the equilibration buffer, followed by this buffer made 0.1M and then 1.0M in NaCl. The elution profile shown in Fig. 1 resulted.

As judged by gel electrophoresis, peak 1 was fibrinogen with very little degradation apparent. There was a faint band with a mobility between that of the (A) α and (B) β chains, but no other indication of (A) α -chain degradation. This peak contained no detectable plasminogen or factor XIII.

Peak 2 was a degraded fibrinogen showing little intact (A) α chain, but significant amounts of plasminogen and factor XIII.

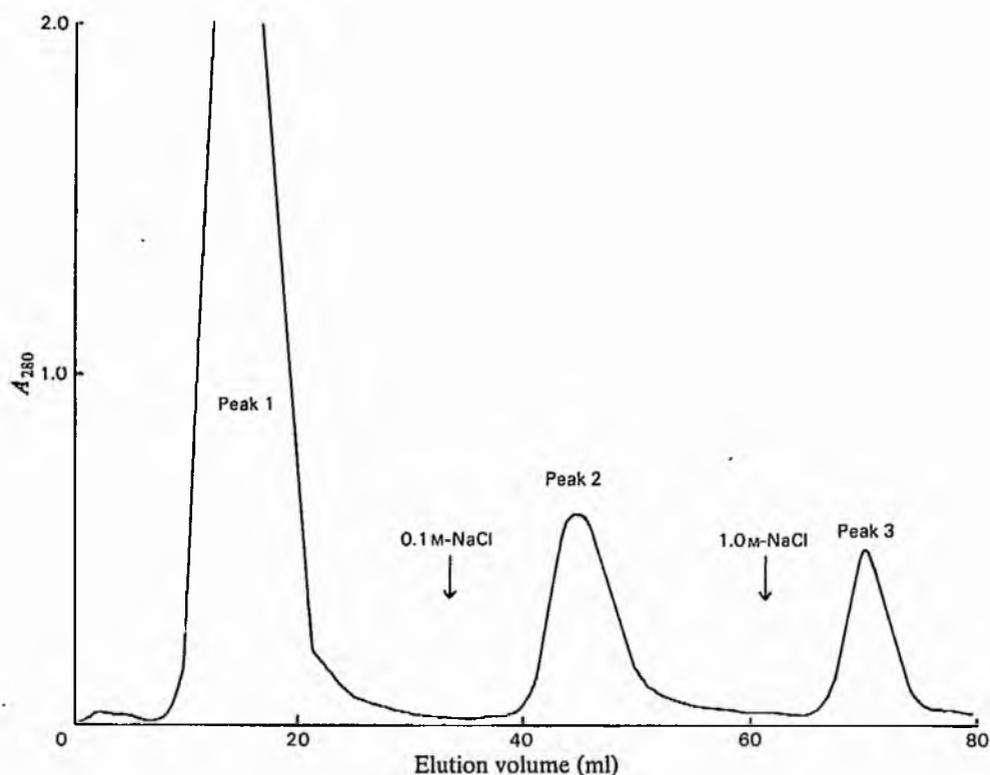


Fig. 1. DEAE-cellulose chromatography of fibrinogen

Stepwise elution was carried out with increases in NaCl concentration as indicated by the arrows.

Peak 3 was composed almost entirely of a high-molecular-weight component with a lower mobility than fibrinogen on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. After reduction, the mobility increased, but there was still one band. This material could be fibronectin, which is known to associate with fibrinogen (Yamada & Olden, 1978).

Separation of fragments D and E

Fibrinogen was digested in the presence of 2 mM-CaCl₂ as previously described (Lawrie & Kemp, 1979). The digest was dialysed against 0.05 M-Tris/HCl buffer, pH 7.5, 2 mM in CaCl₂, and applied to a column (20 cm × 2.5 cm) of DEAE-cellulose equilibrated with the same buffer. Fragment D passed straight through, but fragment E was eluted with the equilibration buffer, 0.3 M in NaCl. Fragment D was sometimes contaminated with fragment Y. In this case, gel filtration on Sephadex G-200 was necessary to separate them.

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weight to 29 000 was achieved with a significantly higher yield at 4°C than at 37°C, indicating that this reduction in mol. weight was not due to proteolysis.

Fig. 4 illustrates the role of Ca^{2+} in protecting fragment D from further digestion by plasmin. Gårdlund et al. [17] reported that the cyanogen bromide fragment Ho3-DSK, which they deduced was located entirely within the C-terminal region of the γ chain, contained an intrachain disulphide bond. The amino acid sequence of that region, reported by Henschen and Lottspeich [16], contained cysteine residues at positions 326 and 339 of the γ chain. It seems probable that these are the two components of the intrachain bond within Ho3-DSK. If this is the case then the disulphide bridge would be located in the region of the bend in the γ chain required to accommodate the Ca^{2+} bridge. It is tempting to suggest that the function of the disulphide bridge is to form the bend required for the Ca^{2+} stabilised hook at the end of the γ chain.

Marguerie et al. [1] reported the presence of three strongly bound Ca^{2+} per fibrinogen molecule. Since it is believed that one molecule of fibrinogen gives rise to two molecules of fragment D, the present study would thus suggest allocations for two of these three calcium ions.

Many reports have demonstrated that the initial attack of plasmin is directed at the C-terminal region of the (A) α chain of fibrinogen [11,18,19]. Marguerie [2] showed that the initial stages of digestion by plasmin proceed more slowly in the presence of Ca^{2+} , and on this basis he suggested that the (A) α chain is involved in the binding of Ca^{2+} . In addition Marguerie et al. [1] pointed out that since three Ca^{2+} are bound by a supposedly symmetrical molecule, one (or all three) must be linking the two halves of the molecule. Our results suggest one Ca^{2+} from an intrachain bridge at the C-terminus of each γ chain, where they limit the action of plasmin. On this basis it is reasonable to postulate that the remaining Ca^{2+} must link the C-termini of the (A) α chains.

Acknowledgement

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decrease in the intensity of the γ chain band, coupled with the appearance of an additional band of greater mobility when intact fibrinogen was reduced after dialysis against 2 mM CaCl_2 .

The Ca^{2+} bound by fragment D probably forms an intrachain bridge located towards the C-terminus of the γ chain. This statement can be justified as follows: Fig. 2a shows the pattern exhibited by fragment D produced by the action of plasmin on fibrinogen in the presence of Ca^{2+} . Addition of EDTA to this preparation immediately before electrophoresis caused a decrease in the mobility of the major fragment D band and the majority of the fragment D migrated as a single species. (Fig. 2b). However, following the addition of EDTA, a faint band appeared with a mobility greater than either of the other species. This must have resulted from a fragment D (molecular weight 93 000) which had been cleaved by plasmin, but in which the C-terminal γ chain peptide was still linked to the rest of the molecule by the Ca^{2+} bridge (Fig. 4). In Fig. 2c and 2d a faint band is visible and this has a mobility corresponding to a molecular weight of 26 000. The mobility of this band was not affected by EDTA. This band may be due to the γ chain remnant from the high mobility fragment D which appeared following the addition of EDTA (Fig. 2b). In other words one half of the Ca^{2+} binding site is in the γ -chain C-terminal fragment of molecular weight approximately 14 000, and the other half is in the remainder of this chain.

Evidence also comes from our attempts to replace the Ca^{2+} in various fragment D preparations which had been EDTA treated and reduced. The 40 000 γ chain remnant was the only chain to show evidence of rebinding Ca^{2+} (Fig. 3). This rebinding, which was manifest as an apparent reduction in molecular

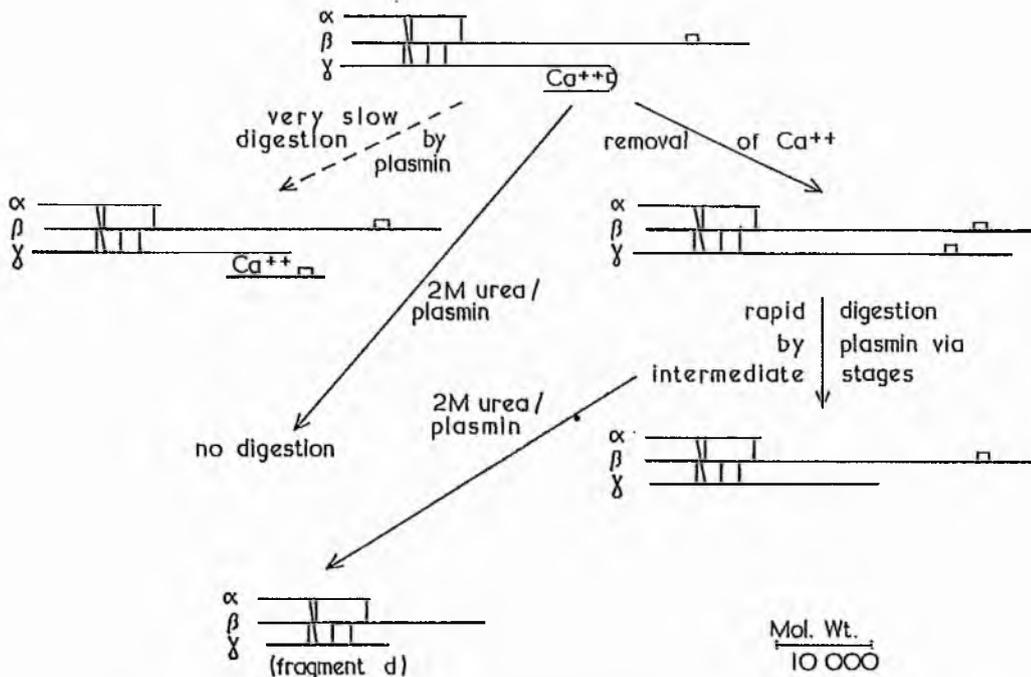


Fig. 4. Schematic diagram to illustrate the protective effect of Ca^{2+} within the γ chain of fragment D. Disulphide bonds are represented by the thinner lines. The positions are approximate, but are based on the data of Doolittle et al. [15] and Henschen and Lottspeich [16].

Ca^{2+} was heterogeneous. As reported by other authors [11–14] this heterogeneity is due to progressive degradation at the C-terminus of the γ chain. The mobilities of the γ chain remnants produced under our conditions (molecular weights, 24 000 and 26 000) were not affected by the addition of Ca^{2+} under conditions where the 40 000 γ chain remnant rebound Ca^{2+} .

The effect of 2 mM CaCl_2 on the proteolytic action of plasmin was examined using casein as a substrate. As can be seen from Table I, the caseinolytic activity was increased in the presence of Ca^{2+} and the addition of 2 mM CaCl_2 did not decrease the effectiveness of Trasylol as an inhibitor of plasmin.

Discussion

In the presence of Ca^{2+} , the fibrinogen degradation product, fragment D(Ca^{2+}) and the intact γ chain of reduced fibrinogen exhibited anomalously high mobilities on SDS polyacrylamide gel electrophoresis. We also found a similar dual mobility for fragment Y. These mobilities were decreased by the addition of chelating agents such as EDTA to values similar to those exhibited by the same species produced from fibrinogen in the absence of Ca^{2+} . It is our contention that these higher mobilities were due to differences in conformation of the fragment or chain caused by the presence of bound calcium ions. In the presence of Ca^{2+} , the molecule would be unable to unfold to the same extent under the influence of SDS, and as a consequence, the fragment or chain containing the bound Ca^{2+} would appear smaller and therefore migrate further.

In the presence of Ca^{2+} the action of plasmin on fibrinogen is restricted [2,3] and even in the presence of 2 M urea the end product is a fragment D of molecular weight 94 000, containing a γ chain remnant of molecular weight 38 000 [3]. Therefore the possibility that the effects described were due to Ca^{2+} influencing the action of plasmin must be considered. A direct inhibitory action of Ca^{2+} on plasmin would not be consistent with the results shown in Table I nor with the results of Haverkate and Timan using a chromogenic plasmin substrate [3]. There remains the possibility that an enhanced activity of plasmin, induced by Ca^{2+} , was responsible for the increased mobility observed in the presence of Ca^{2+} . In other words digestion might have occurred after the addition of urea/SDS and the increased mobility might genuinely reflect a decreased molecular weight. Addition of EDTA might prevent this additional digestion, a circumstance consistent with our unreported observations that, in the presence of EDTA, fibrinogen digestion was slower. This explanation however is not tenable for two reasons. Firstly, it does not explain the high mobility of the γ chain of intact fibrinogen following reduction at lower temperatures. Secondly, in a previous report [6], we showed that the mobility of fragment D decreased after 18 h at 4°C in the presence of urea and SDS. This observation is consistent with the eventual loss of bound Ca^{2+} under denaturing conditions, but is not consistent with additional digestion.

The smallest fragment which displayed an anomalous mobility in the presence of Ca^{2+} was the fragment D of molecular weight 94 000, and thus this fragment contains at least one bound Ca^{2+} . The results presented in Figs. 2 and 3 suggest that this Ca^{2+} is associated with the γ chain remnant and does not involve any of the other chains of fragment D. This view was confirmed by the

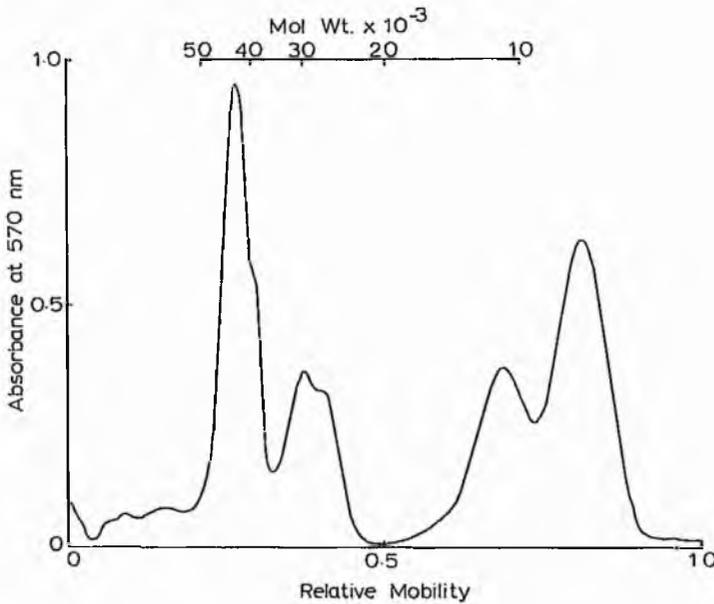


Fig. 3. Densitometric scan of fibrinogen, digested by plasmin in the presence of 2 mM CaCl_2 and separated by SDS polyacrylamide gel electrophoresis. The sample was reduced at 37°C for 30 min after being made 2 mM with respect to EDTA. Following reduction CaCl_2 was added to a final concentration of 12 mM (10% gel).

EDTA was added to this sample after reduction and immediately before electrophoresis the mobility of the γ chain remnant decreased to a value corresponding to a molecular weight of 40 000. (Fig. 2d). A band with an apparent molecular weight of 26 000 was also just discernible (Fig. 2c). This was probably the γ chain remnant from the high mobility fragment D which was visible in the unreduced fragment D(Ca^{2+}) following EDTA addition. The mobility of this γ chain remnant was not influenced by EDTA (Fig. 2d), suggesting that it did not bind Ca^{2+} .

When EDTA was added to fragment D(Ca^{2+}) before reduction, the γ chain remnant migrated with mobility equivalent to a molecular weight of 40 000. When an excess of CaCl_2 was added to this reduced sample before electrophoresis a proportion of the γ chain reverted to an apparent molecular weight of 29 000 (Fig. 3). Fragment D produced by plasmin digestion in the absence of

TABLE I

THE EFFECT OF CaCl_2 ON THE CASEINOLYTIC ACTIVITY OF PLASMIN AND ITS INHIBITION BY TRASYLOL

Each assay contained 1.02 units of plasmin, and the activity of this in the standard procedure was taken as 100%. Values given are an average of 2 determinations.

CaCl_2 concn. (mM)	TrasyloI concn. (units/ml)	Plasmin activity (%)
0	0	100
2	0	135
0	200	7
2	200	4

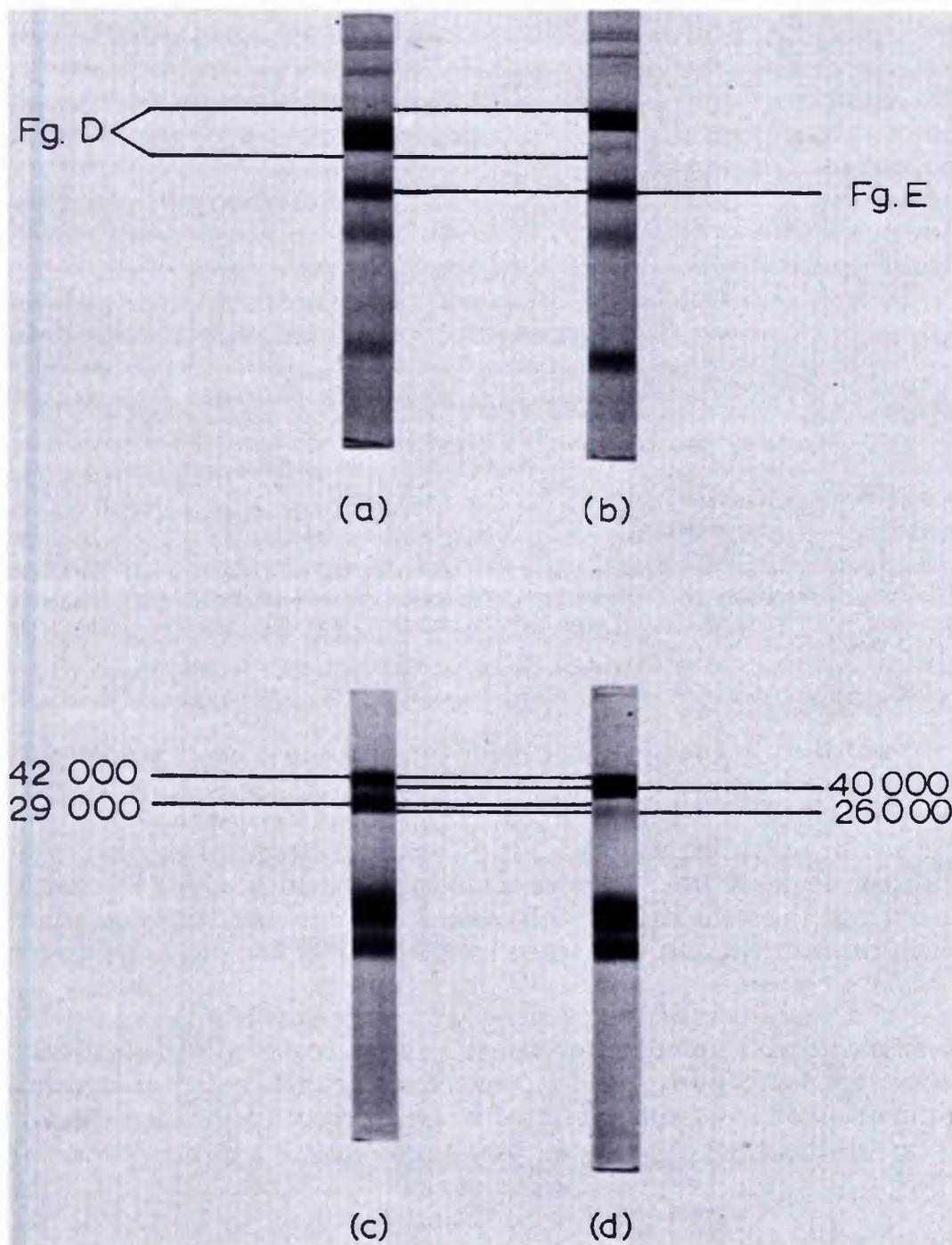
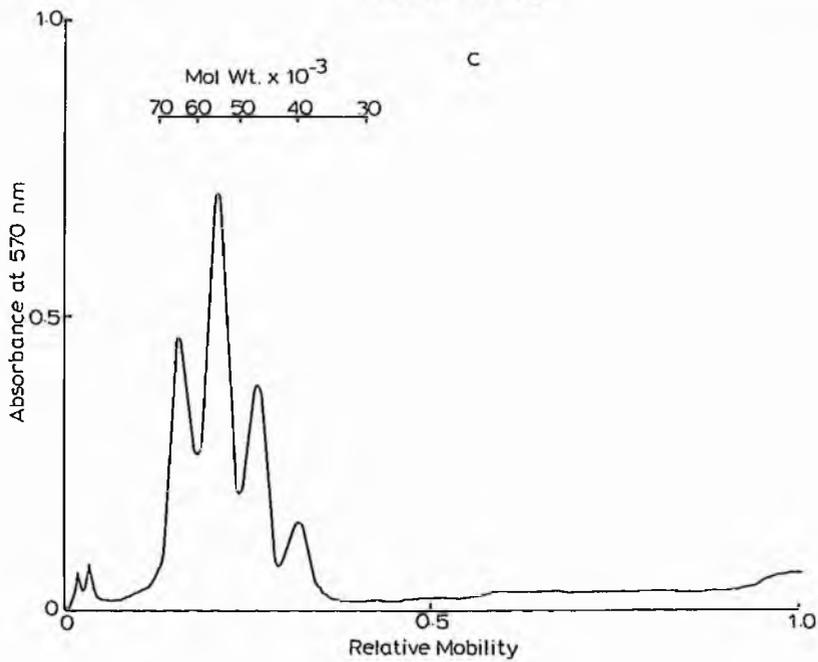
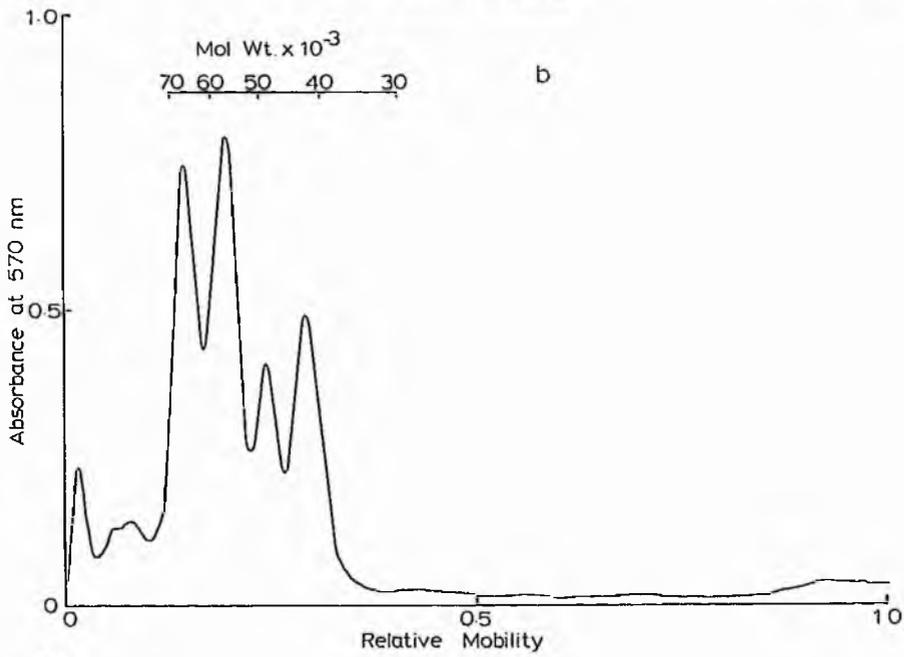
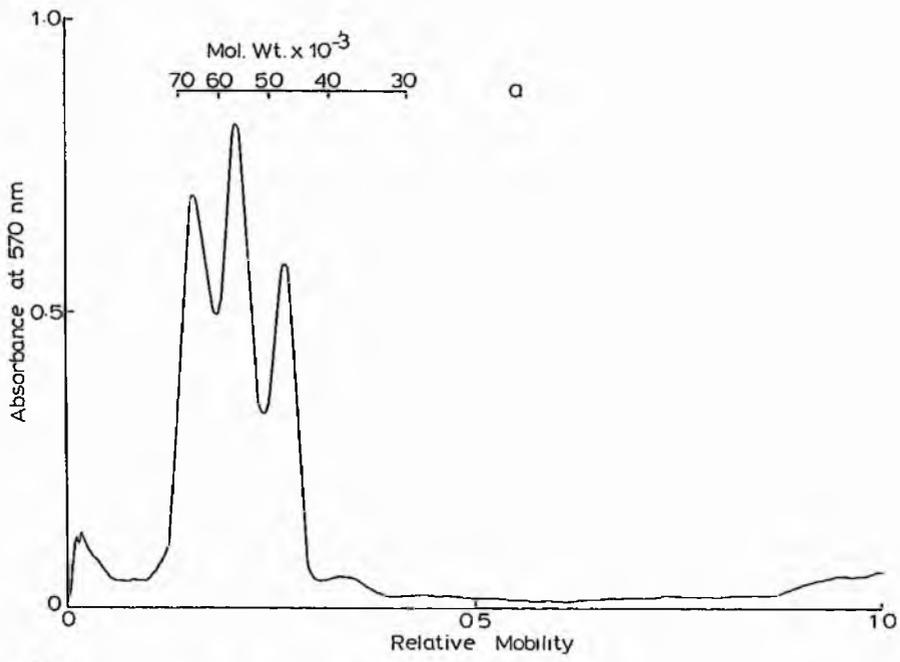


Fig. 2. SDS polyacrylamide gel electrophoresis of fibrinogen digested by plasmin in the presence of 2 mM CaCl_2 . (a) Digest, not reduced (5% gel); (b) digest, not reduced, but made 5 mM with respect to EDTA (5% gel); (c) digest reduced at 25°C for 30 min (10% gel); (d) digest, reduced at 25°C for 30 min and then made 5 mM with respect to EDTA (10% gel).

Fig. 1. Densitometric scans of SDS polyacrylamide gel electrophoresis separations of fibrinogen. (a) Reduced at 37°C for 30 min after dialysis against Tris buffer, (b) reduced at 20°C for 30 min after dialysis against Tris buffer containing 2 mM CaCl_2 , (c) reduced at 37°C for 30 min after dialysis against Tris buffer containing 5 mM EDTA, followed by dialysis against Tris buffer containing 2 mM CaCl_2 (all gels, 10%).



volume of buffer and both were maintained at 25°C before applying to the gel. After staining and destaining [10] the gels were scanned on a Vitatron densitometer or photographed. Molecular weights were estimated with the aid of the following protein markers purchased from Sigma (London, U.K.). Phosphorylase *a* (1.4- α -D-glucan:orthophosphate α -glucosyltransferase; E.C. 2.4.1.1) from rabbit muscle; bovine serum albumin; ovalbumin; myoglobin. Standards were reduced for 5 min at 100°C in the presence of 1.5% mercaptoethanol, 1.5% SDS, 4 M urea.

Results

The mobility of the γ chain of fibrinogen during SDS polyacrylamide gel electrophoresis was affected by the presence of Ca^{2+} . Fibrinogen, previously dialysed against Tris buffer was reduced at 37°C. The pattern obtained after electrophoresis was as expected, with the typical distribution of (A) α , (B) β and γ chains apparent (Fig. 1a). A similar examination of fibrinogen, previously dialysed against Tris buffer containing Ca^{2+} and reduced at 20°C, resulted in the pattern shown in Fig. 1b. In this case there were four components. The relative mobilities of three of these gave the values expected for the (A) α , (B) β and γ chains, but the size of the γ chain peak was smaller than in Fig. 1a. The fourth peak had a relative mobility greater than that of the γ chain. Addition of EDTA to this sample after reduction and immediately before electrophoresis caused this additional band to disappear. There was a corresponding increase in the size of the γ chain peak giving the pattern typical of fibrinogen.

When fibrinogen, dialysed against Tris buffer containing EDTA, was reduced for 30 min at 37°C, a pattern typical of fibrinogen and indistinguishable from Fig. 1a resulted. When this EDTA treated fibrinogen was dialysed against 2 mM CaCl_2 before reduction at 37°C, the additional high mobility γ chain component reappeared (Fig. 1c).

These results suggest that Ca^{2+} binds to the γ chain of fibrinogen altering its conformation and therefore its mobility during SDS polyacrylamide gel electrophoresis. This Ca^{2+} could be removed by EDTA and subsequently replaced by dialysis against Ca^{2+} . The Ca^{2+} could also be removed under denaturing conditions since the amount of the high mobility γ chain decreased as the temperature of reduction was increased (Fig. 1b and 1c) until none was visible after reduction at 100°C for 5 min.

During SDS polyacrylamide gel electrophoresis the mobilities of other fibrinogen derivatives were affected by Ca^{2+} . In the presence of Ca^{2+} , fragment D(Ca^{2+}) migrated as a double band (Fig. 2a). Addition of EDTA immediately before electrophoresis removed the faster moving band and caused an increase in the intensity of the band of lower mobility. (Fig. 2b). A faint band, presumably from a high mobility fragment D, also appeared as a consequence of EDTA addition. The mobility of fragment E was not affected by the addition of EDTA, nor were the mobilities of the fragments D produced by plasmin action in the absence of Ca^{2+} .

The mobility of the γ chain remnant of fragment D(Ca^{2+}) was also affected by the presence of Ca^{2+} . Reduction of fragment D(Ca^{2+}) at 25°C produced a γ chain remnant with an apparent molecular weight of 29 000. (Fig. 2c). When

a B(β) chain remnant of molecular weight 43 000 and a γ chain remnant of molecular weight 38 000. Further degradation of this fragment did not occur, even in the presence of 2 M urea. In contrast, the fragment E produced in the presence of Ca^{2+} was identical to that produced in their absence.

Purves et al. [5] confirmed the conclusion of Haverkate and Timan [3] that Ca^{2+} protects fragment D(Ca^{2+}) against attack by plasmin, and suggested that this stabilisation might be brought about by calcium binding to fragment D.

In a recent preliminary report [6] we presented evidence that the fragment D of molecular weight 93 000 did indeed contain a bound Ca^{2+} . The results presented here provide additional evidence for this, and indicate that the Ca^{2+} is located within the γ chain remnant, forming an intrachain bridge near the C-terminus of that chain.

Methods

Fibrinogen. Human Fibrinogen (Grade L, KABI Pharmaceuticals, London, U.K.), was dissolved at a concentration of 5 mg/ml, freed from contaminating plasminogen by the affinity chromatography procedure of Deutsch and Mertz [7] and dialysed at 4°C for 18 h against 0.05 M Tris-HCl buffer, pH 7.5, alone or this buffer containing either 2 mM CaCl_2 or 5 mM EDTA.

Plasminogen was prepared and activated to plasmin as previously described [8], using 170 units of streptokinase (Hoechst Pharmaceuticals, Hounslow, U.K.) per C.T.A. (Committee on Thrombolytic Agents) unit of plasminogen. Assay was by the caseinolytic procedure of Johnson et al. [9].

Fibrinogen digestion. Plasmin was added in a ratio of 0.17 C.T.A. units per mg fibrinogen and the digestion carried out at 37°C for 2 h. Where digestions were carried out in the presence of CaCl_2 or EDTA the concentrations of these reagents were maintained at 2 mM and 5 mM, respectively. The digestions were stopped by the addition of 5500 kallikrein inactivator units of Trasylol (Bayer Pharmaceuticals, Surbiton, Surrey, U.K.) per C.T.A. unit of plasminogen.

Polyacrylamide gel electrophoresis. Acrylamide and methylenebisacrylamide (B.D.H., Poole, U.K.) were dissolved directly in 6 M urea, 0.1 M Tris-HCl, 0.2% sodium dodecyl sulphate (SDS) pH 7.5. The final concentration of acrylamide was either 5% (w/v) containing 5% (w/w) methylenebisacrylamide) or 10% (w/v) containing 3% (w/w) methylenebisacrylamide). For 5% acrylamide, gelling followed the addition of N,N,N',N' -tetramethylethylenediamine and ammonium persulphate to final concentrations of 0.125% and 0.06% respectively, while for 10% gels, the final concentrations were 0.1% and 0.05% respectively. The electrophoresis chamber buffer contained 0.2% SDS in 0.1 M Tris-HCl, pH 7.4. Samples were reduced by mixing with an equal volume of a 2% 2-mercaptoethanol, 3% SDS, 8 M urea solution. The times and temperatures of incubation with this mixture were varied and are detailed at the appropriate point in the text. Samples run without reduction were mixed with an equal volume of a 3% SDS, 8 M urea solution before application to the gel. Where EDTA was added immediately prior to electrophoresis, samples were first treated with urea/SDS or urea/SDS/mercaptoethanol solutions and then split into two parts. EDTA was added to one to give a final EDTA concentration of 5 mM (unless otherwise stated). As a control, the other part was diluted with an equivalent

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THE PRESENCE OF A Ca^{2+} BRIDGE WITHIN THE γ CHAIN OF HUMAN FIBRINOGEN

JAN S. LAWRIE and GRAHAM KEMP

Department of Biochemistry, University of St. Andrews, North Street, St. Andrews, KY16 9AL (U.K.)

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Summary

The presence of Ca^{2+} increased the mobility of fragment D, and the γ chain from fibrinogen on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, suggesting that a Ca^{2+} was associated with these fibrinogen derivatives. The mobilities of the constituent chains from fragment D produced under various conditions, indicate that Ca^{2+} bound to fibrinogen form an intrachain bridge towards the C-terminus of each γ chain.

Introduction

Several recent publications [1—5] have emphasised the importance of Ca^{2+} to the structure of fibrinogen. The results of Marguerie et al. [1] indicated that each molecule of native fibrinogen has three tightly bound Ca^{2+} , and Marguerie [2] suggested that at least one of the binding sites is located within the (A) α chain.

Haverkate and Timan [3] investigated the effect of physiological concentrations of Ca^{2+} on the digestion of fibrinogen by plasmin. The digestion proceeded more slowly in the presence of Ca^{2+} an observation also made by Marguerie [2]. The end point of digestion was a single fragment D of molecular weight 93 000, containing an (A) α chain remnant of molecular weight 12 000,

Abbreviations: SDS, sodium dodecyl sulphate. Fragment D(Ca^{2+}), the fragment D produced by plasmin action in the presence of Ca^{2+} , with constituent chains of molecular weight 42 000 (derived from the (B) β chain), 40 000 (derived from the γ chain) and 11 000 derived from the (A) α chain. This fragment is probably the same as the fragment D(cate) of Haverkate and Timan [3] and the fragment D 1 of Purves et al. [5].

The Degradation of Denatured Fragment D from Human Fibrinogen by Plasmin

JAN S. LAWRIE, WILLIAM S. GILMORE* and GRAHAM KEMP

Department of Biochemistry, University of St. Andrews,
St. Andrews KY16 9AL, Fife, Scotland, U.K.

Human fibrinogen is a large molecule (mol.wt. 340000) consisting of three pairs of polypeptide chains ($A\alpha$, $B\beta$ and γ) held together by disulphide bonds. The disulphide bonds are not uniformly distributed throughout the molecule, but appear to be located in particular regions, the so-called disulphide knots. A knowledge of the location, extent and structure of these knots is of prime importance in understanding fibrinogen structure and function.

Plasmin (EC 3.4.21.7) digests fibrinogen producing three major end products, fragment E and two identical fragments D (Marder *et al.*, 1969). Fragment E, which contains the *N*-terminal regions of the six polypeptide chains of fibrinogen, contains many disulphide bonds and is well characterized (Blomback *et al.*, 1976).

Fragment D preparations are normally heterogeneous, containing three distinct species with mol.wts. 94000, 88000 and 83000. It has been shown that this heterogeneity is due to the existence of three different sizes of γ -chain remnant (Furlan *et al.*, 1975). Plasmin will degrade denatured fragment D into a smaller species of mol.wt. 45000

* Present address: School of Life Sciences, Ulster College, The Northern Ireland Polytechnic, Jordanstown, Newtownabbey, Co. Antrim, N. Ireland, U.K.

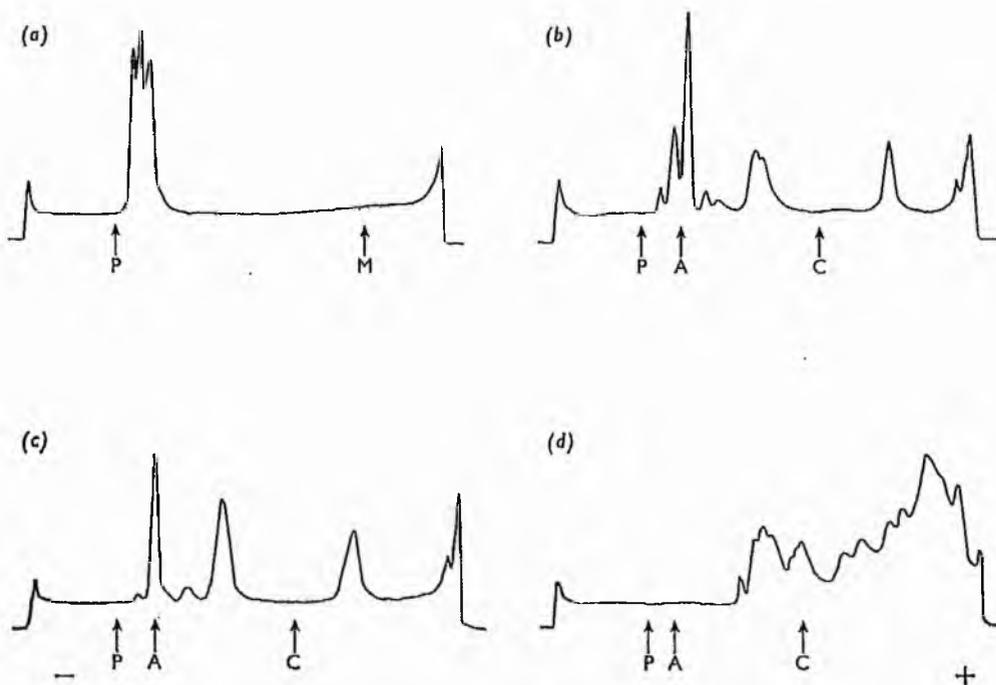


Fig. 1. Densitometric traces of the plasmin digests of denatured fragment D

The reaction was stopped with Trasylol after (a) 0, (b) 2 or (c) 22 h. (d) The 22 h digest was reduced with mercaptoethanol. The arrows indicate the positions of proteins of known molecular weight run on accompanying gels. P, phosphorylase *a*, mol.wt. 94000; A, bovine serum albumin, mol.wt. 68000; C, carbonic anhydrase, mol.wt. 29000; M, myoglobin, mol.wt. 17000.

(fragment d), which contains most of the disulphide bonds found in fragment D (Kemp *et al.*, 1973).

In an attempt to obtain more information on the location and extent of the disulphide knot within fragment D, we have made a closer study of the plasmin digestion of denatured fragment D.

Human fibrinogen (Grade L; KABI Pharmaceuticals, Stockholm, Sweden) was freed of contaminating plasminogen by the affinity-chromatography method of Deutsch & Mertz (1970). Plasminogen was prepared from human plasma by the same affinity-chromatography procedure, or obtained from KABI Pharmaceuticals. Plasminogen was assayed by the caseinolytic procedure described by Johnson *et al.* (1969). Plasminogen was activated to plasmin by incubation for 10 min at 37°C with streptokinase (Hoechst Pharmaceuticals, Frankfurt/Main, Germany) in a ratio of 1 C.T.A. (Committee on Thrombolytic Agents) unit of plasminogen to 150 units of streptokinase. Some 250 mg of fibrinogen in 0.05 M-phosphate buffer, pH 7.5, was digested at 37°C with 2.5 C.T.A. units of plasmin, and the reaction stopped after 3 h by the addition of 500 mg of lysine-Sepharose (Pharmacia, Uppsala, Sweden). The mixture of fragments D and E was isolated as described by Furlan & Beck (1972) and separated by chromatography on sulphopropyl-Sephadex C-25 (Pharmacia), the buffer system described by Hagenmaier & Foster (1971) being used. In this system, fragment E passes straight through the column, and fragment D is eluted by raising the pH of the eluting buffer to 5.5.

In a typical experiment, 1.5 mg of fragment D was denatured by incubation at 37°C in 0.02 M-phosphate, pH 7.5, and 2 M-urea for 30 min, and then digested with 0.15 C.T.A. unit of plasmin. The reaction was stopped after various time-intervals by the addition of Trasylol (Bayer Pharmaceuticals, Surbiton, Surrey, U.K.) to a final concentration of 6000 kallikrein-inactivator units per C.T.A. unit of plasmin. The digest was examined by polyacrylamide-gel electrophoresis (5% gels) in the presence of sodium dodecyl sulphate, by the procedure of Swank & Munkres (1971).

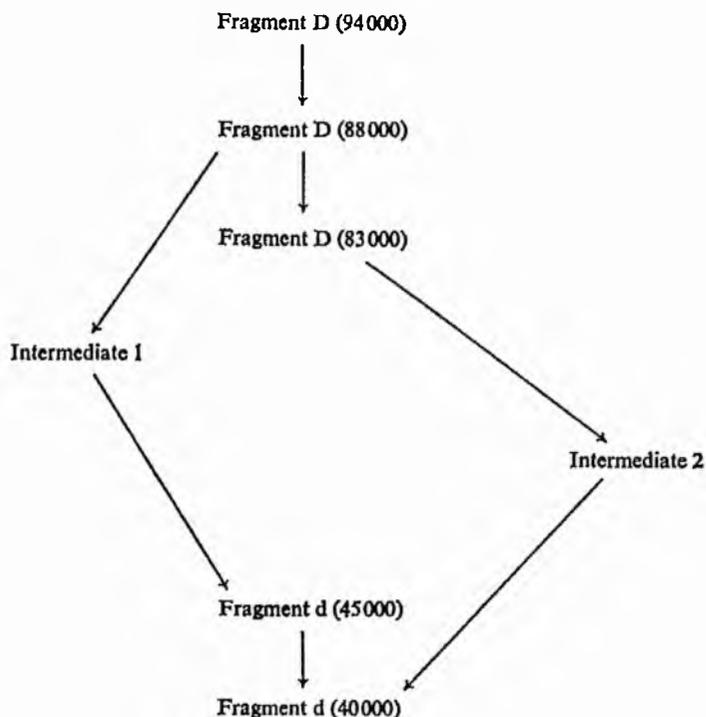


Fig. 2. Proposed pathways for the degradation of denatured fragment D by plasmin. Molecular weights are given in parentheses.

The results are summarized in Fig. 1. The two higher-molecular-weight species of fragment D disappeared rapidly and this coincided with the appearance of a band with the mobility expected for fragment d. In contrast, the lowest-molecular-weight fragment D appeared much more resistant and was still present after a digestion time of 66h. Reduction of a 22h digest with mercaptoethanol indicated that the β -chain of this fragment D had been split by plasmin in at least three places and the γ -chain remnant in at least two. The fact that the mobility of the unreduced fragment remained unchanged suggests that the pieces are still held together by disulphide bonds.

In conclusion, digestion of fragment D to fragment d seems to take place according to the scheme outlined in Fig. 2. These results are consistent with the model of fragment-D structure proposed by Furlan *et al.* (1975), but suggest a rather uniform distribution of disulphide bonds within fragment D.

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The Biosynthesis of Caerulein

DAVID S. JONES, M. ELIZABETH KIDSTON, GILLIAN A. SIMPSON
 and BARBARA P. FULLER

*Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool
 L69 3BX, U.K.*

Caerulein was first isolated from the skin of *Hyla caerulea*, an Australian tree bull frog, and its structure shown to be: <Glu*-Gln-Asp-Tyr(SO₃H)-Thr-Gly-Trp-Met-Asp-Phe-NH₂ (Anastasi *et al.*, 1967, 1968). Its C-terminal heptapeptide sequence is the same as that of cholecystokinin (Mutt & Jorpes, 1971), except that the fifth residue in caerulein, threonine, is replaced by a methionine residue, and its C-terminal pentapeptide sequence is identical with that of gastrin (Gregory *et al.*, 1964). It possesses all the biological actions of gastrin and cholecystokinin.

More recently caerulein was shown to be present in dermal granular glands of *Xenopus laevis* (South African clawed toad) (Dockray & Hopkins, 1975). On stimulation (by adrenaline injection into the dorsal lymph sac) caerulein is discharged from these glands, one animal yielding on average 15mg of caerulein in the secretion (Dockray & Hopkins, 1975). The large amount of caerulein secreted would suggest that it is a major product of the glands.

Since caerulein has biological activities similar to those of cholecystokinin and gastrin, it has been suggested that it might replace these hormones in the gastrointestinal tract and be synthesized there. It could then be transported to the skin glands as a method of excretion. Here, however, we demonstrate that caerulein is in fact synthesized in the skin of *X. laevis*.

Immediately after death, skin from the dorsal region of *X. laevis* was removed and washed with amphibian 'Ringers' solution (Paul, 1972). After soaking in various bactericide and fungicide solutions the skin was layered on to an adrenaline

* Abbreviation: <Glu, pyroglutamic acid.

solution, which prompted an immediate discharge from the cutaneous granular glands. The skin was then cut into small pieces and cultured in a nutrient/salts medium in a manner similar to that described by Baily (1971). One of the amino acids in the medium was replaced by the ^3H -labelled amino acid. The skin cultures were incubated for up to 7 days at 28°C .

After 3 and 7 days, samples of skin were first treated with an adrenaline solution, then dried *in vacuo* and extracted with methanol. The adrenaline solution, the methanol extract and the remaining medium were examined for the presence of ^3H -labelled caerulein. The samples were mixed with an authentic sample of caerulein and examined by paper electrophoresis or paper chromatography. Samples from the adrenaline solution and the methanol extracts after 3 or 7 days incubation gave no radioactive peaks corresponding to the caerulein marker. However, samples of the medium in which the skins had incubated for 3 days gave a small peak of radioactivity which co-migrated with caerulein. When this solution was kept at 37°C over a period of several hours it was found that the amount of radioactivity which co-migrated with caerulein increased considerably. This suggests that caerulein matures during this period from a precursor molecule.

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Membrane Lipid Metabolism in *Acholeplasma laidlawii* A during Normal and Temperature-Shift-Down Conditions

ANDERS CHRISTIANSSON and ÅKE WIESLANDER

Department of Microbiology, University of Lund, Sölvegatan 21, S-223 62 Lund, Sweden

Experiments with *Acholeplasma laidlawii* have contributed considerably to our understanding of membrane structure and function (Razin, 1975). The membrane-lipid metabolism of *Acholeplasma* cells is, however, not as well known as that for fatty acid auxotrophs of *Escherichia coli* used in similar membrane studies. Previous studies by us (Wieslander & Rilfors, 1977) have shown that in *A. laidlawii* A strain EF22 the membrane lipid content (and especially the glucolipid content) varies as a consequence of saturated or unsaturated fatty acid supplementation to a lipid-depleted growth medium. In the present investigation the temperature-shift-down technique was used to establish the impact of a factor that affects membrane viscosity (temperature) on the lipid metabolism and lipid relationships in *A. laidlawii*.

Acholeplasma laidlawii A strain EF22 was grown statically in a lipid-depleted tryptose/bovine serum albumin medium (Razin & Rottem, 1975). Each litre of medium was supplemented with either a mixture of palmitic acid ($75\ \mu\text{mol}$) plus oleic acid ($75\ \mu\text{mol}$), or oleic acid alone ($150\ \mu\text{mol}$). These were also given together with 8 mg of cholesterol per litre. Membrane lipids were labelled by adding ^{14}C - and/or ^3H -labelled fatty acids to the medium. Cultures were grown 12 h at 37°C , divided into two portions, one of which was rapidly cooled to 17°C and the other was maintained at 37°C . Samples for lipid and protein analysis were removed periodically and centrifuged.

The cells were extracted with chloroform/methanol (2:1, v/v) and the lipid extract was then purified from contaminants by Sephadex column chromatography (Wells & Dittmer, 1963).