A STUDY OF THE CARBOXY-TERMINAL Aα CHAIN POLYPEPTIDES OF HUMAN FIBRINOGEN

by

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SUMMARY

1. Human fibrin(ogen), the clottable protein of plasma, is the final substrate in both the coagulation and the fibrinolytic enzyme systems. The subunit structure of this protein consists of three pairs of polypeptide chains, Aα, Bβ and γ chains, which are joined together by disulphide bonds. Using antisera to the individual polypeptide chains of fibrinogen, immunological investigation should provide more information about the degradation products of fibrinogen than if anti-fibrinogen antiserum alone was used.

2. The immunological behaviour of fibrinogen and its derivatives has been investigated largely by means of an antiserum to one of the individual polypeptide chains of fibrinogen - the Aα chain. Using the relatively simple and qualitatively informative, analytical techniques of immunoprecipitation in agarose gel and gel electrophoresis, significant observations have been made on the immunological reactions of anti-Aα antiserum.

3. For the preparation of immunogen, fibrinogen was precipitated from human plasma and purified to fraction I-4. Fibrinogen was reduced by dithiothreitol and alkylated with iodoacetic acid. Separation of the individual carboxymethylated Aα, Bβ and γ polypeptide chains was achieved by ion-exchange chromatography. Studies on the solubility properties of the carboxymethylated Aα polypeptide chains of fibrinogen in various buffers suggested alkaline barbitone (pH 8.6) as the buffer of choice for immunodiffusion experiments.

4. After further purification of the Aα chain by polyacrylamide gel electrophoresis, antiserum to the Aα chain was raised in rabbits. Anti-Aα antisera was heat-decomplemented and absorbed with Bβ polypeptide chains. On immunodiffusion, absorbed anti-Aα antiserum reacted with Aα, but not with Bβ or γ chains. In addition, the antiserum

reacted with purified fibrinogen and plasma fibrinogen, showing a reaction of identity with the Aα chain.

5. The progress of plasmin degradation of fibrinogen was examined by immunoelectrophoresis. When anti-Aα antiserum was used, the appearance of two intersecting precipitin arcs confirmed that early cleavage of the Aα chain of fibrinogen had occurred. These two precipitin arcs persisted throughout the digestion. A 26,000 molecular weight polypeptide, isolated by gel filtration from a late digest, corresponded in electrophoretic mobility to the more cathodal arc of the late digest of fibrinogen. This polypeptide was named Aα-related antigen (molecular weight 26,000), Aα-RA(26,000).

6. Examination of the various stages of plasmin degradation of fibrinogen by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate revealed the progressive appearance of six low molecular weight polypeptides as the digestion proceeded. One of these, Aα-RA(26,000), was isolated and its immunological behaviour confirmed its Aα chain origin. Two other polypeptides of slightly higher molecular weight were isolated in semi-purified form; it appeared likely that they also had an Aα chain origin and were precursors of Aα-RA(26,000). None of these polypeptides reacted with commercial anti-fibrinogen antiserum, and reacted only weakly with unabsorbed anti-fibrinogen antiserum. Anti-Aα antiserum did not react with purified fibrinogen fragments Y, D or E, but a faint reaction was obtained with fragment X.

7. Urokinase activation of the plasma fibrinolytic system in vitro resulted in a limited degree of fibrinogenolysis. The extent of such plasma fibrinogen degradation may not be readily detected by assays employing anti-fibrinogen antiserum, but qualitative examination of the serum from such activated plasma revealed an increase in Aα-related

antigen(s). The serum Aα-related antigen(s) were not immunologically identical to Aα-RA(26,000).

8. Activation of the plasma fibrinolytic system in vivo by venous occlusion of the arm also resulted in a limited degree of fibrinogenolysis. In the limited number of cases studied, an increase in the level of Aα-related antigen(s) was demonstrated in the post-occlusion serum samples by the techniques of single radial immunodiffusion and Laurell electrophoresis. Further refinement of these techniques may constitute the basis for an assay of in vivo fibrin(ogen)olysis.

9. The site of origin of the fragment Aα-RA(26,000) was located in a central region of the Aα polypeptide chain of fibrinogen; the antigenic determinant sites of the fragment were contained in the Aα remnant of the high solubility fibrinogen fraction I-9, but not in the COOH-terminal polypeptides released from the Aα chain of fibrinogen by ancrod cleavage.

10. From the results of immunodiffusion with anti-Bβ and anti-γ antisera, some cross-contamination of the Bβ and γ chain preparations was likely. Neither antisera reacted with the Aα chains, and only anti-Bβ antiserum reacted weakly with whole fibrinogen. A method for the immunological examination of the Aα, Bβ and γ chains of plasma fibrinogen, using small volumes of plasma, has been suggested.

ABBREVIATIONS

A/Aα	Anti-Aα antiserum.
CM-fibrinogen	Carboxymethylated fibrinogen.
DIC	Disseminated intravascular coagulation.
DTT	Dithiothreitol.
EACA	ε-Aminocaproic acid.
FDP	Fibrin(ogen) degradation products.
MW	Molecular weight.
Na_4EDTA	Tetrasodium ethylenediamine tetra-acetate.
N-DSK	NH_2-terminal disulphide knot.
PMSF	Phenylmethyl sulphonyl fluoride.
SBTI	Soybean trypsin inhibitor.
SDS	Sodium dodecyl sulphate.
-SH	Sulphydryl group.

CHAPTER 1: FIBRINOGEN - STRUCTURE AND FUNCTION

1.1 Discovery of fibrinogen

While the phenomenon of blood coagulation must have led men of imagination to speculate on its nature, the recognition of the final cause of coagulation as the conversion of a soluble blood component to an insoluble fibrous material appeared only about 120 years ago. Previously, a white fibrous material, which formed independently of the blood cells during clotting, had been described by Malpighi as early as 1686, but the true origin of this substance was concealed by a variety of erroneous views which persisted until well into the 19th century. Among these ideas were early postulates that the cooling of blood, and the exposure of blood to air, were responsible for its coagulation. Later ideas which also proved to be popular were that fibrin aggregated after its release by membrane disruption of the red blood cells in one case and from platelets in another case.

Around the middle of the 19th century, important experiments were performed by Buchanan, who found that certain body fluid exudates could be coagulated by fresh serum, thus demonstrating

(i) the presence of "soluble fibrin" in these fluids, and

(ii) the ability of some agent, a serum component, to cause the solidification of fibrin.

The concept and term "fibrinogen", as applied to a fibrin precursor, was first used by Denis in 1859, although Virchow had previously used the term to refer to an altered fibrin. Denis attempted to purify this clottable substance from plasma and this work was continued by Hammarsten, whose studies led him to propose that the formation of fibrin was the specific result of the interaction between fibrinogen and thrombin, an enzyme which was not present in the circulation, but

was itself formed from a precursor, prothrombin. In addition, the recovery of a protein-like material from the supernatant solution, after pure fibrinogen had been converted to fibrin, was taken as evidence of a hydrolytic reaction and provided the idea that the formation of fibrin resulted from cleavage of the fibrinogen molecule. Since the turn of the century up to the present day, this proteolytic cleavage, even though rather limited, has been thoroughly investigated in terms of both the gross and the molecular alterations induced in fibrinogen as a consequence of the specific action of thrombin.

(Taken from reviews by Beck (14) and Murano (252)).

1.2 Physical Properties

Human fibrinogen is a glycoprotein with a molecular weight of 340,000 ± 20,000. Agreement on the molecular size of fibrinogen has been obtained from various methods of determination, which include sedimentation, diffusion and light scattering studies, as well as summation of the values for its constituent polypeptide chains (assuming a dimeric structure of fibrinogen) (68, 252). The molecular weights of most other mammalian fibrinogens are also within the above range, although higher values are occasionally found (68).

The isoelectric point of fibrinogen is 5.5 (68), although both higher (4) and lower (310) values have been reported. Fibrinogen is soluble up to a value of 4 g/100 ml (68), but precipitates out of solution when heated at 58^{0}C (148).

The sedimentation coefficient is 7.9S, and the extinction coefficient ($E^{1\%}_{1cm}$) at 280 nm was found to be 15 - 16 (68). Other physicochemical parameters, such as the partial specific volume, the frictional ratio, the intrinsic viscosity and diffusion coefficient have been calculated and their values permit a limited interpretation

of the molecular shape and size of fibrinogen (68, 252). In particular, by use of the techniques of optical rotatory dispersion, circular dichroism and low-angle X-ray diffraction, the α - helix content of the molecule has been estimated at 35% (252). By such means, the pH range of stability of fibrinogen was found to be 5.5 - 10, and denaturation in 5M urea solutions was reversible, but irreversible in 5 - 10 M urea as indicated by the destruction of the α - helical segments (252).

1.3 The shape of the fibrinogen molecule: changes induced by fibrin formation

Only in the past few years has there been some agreement among various groups of workers that the fibrinogen molecule may be approximately spherical or globular in shape, but the controversy which has surrounded the geometric form of this molecule for more than 30 years is far from settled and further studies will be required before any firm conclusions may be drawn concerning its overall shape. The importance of such studies lies in our need to understand the basic mechanisms involved in the conversion of fibrinogen, a soluble protein, to its insoluble form, fibrin, during the coagulation of blood.

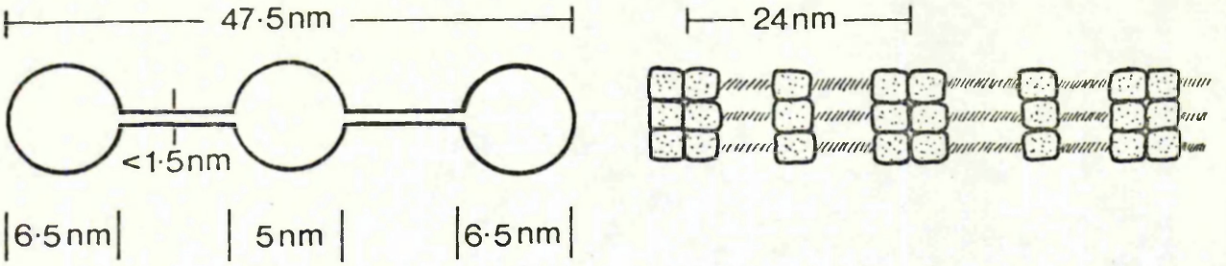
1.3.1 Physicochemical studies

For a number of years, the form of the fibrinogen molecule had been described as rod-shaped or as a compact prolate ellipsoid of revolution. This interpretation of its shape was derived from the hydrodynamic measurements of flow birefringence, sedimentation, viscosity and diffusion (68, 243) and was adhered to for more than a decade. As early as 1947, Ferry and Morrison (87), in their study of human fibrin formation, assumed the dimensions of the fibrinogen molecule to be 3.5 x 70 nm; moreover, they considered that no great

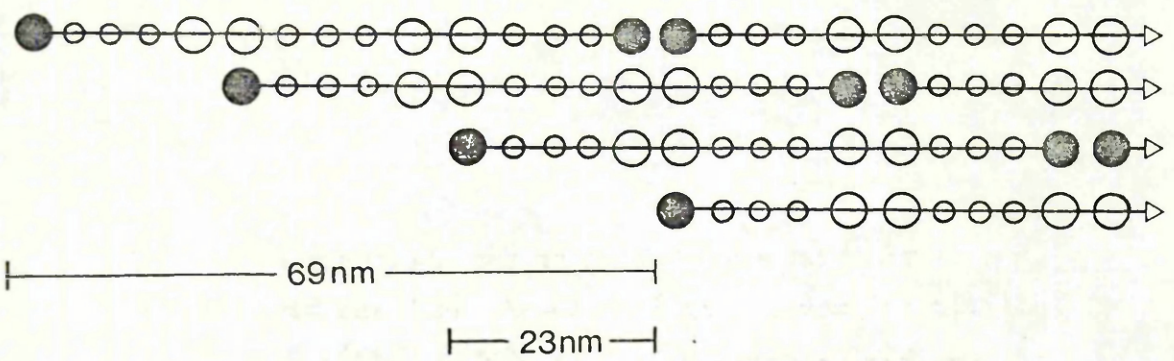
change of shape was involved when fibrinogen was converted to a fine fibrin clot, as only a small change of Rayleigh light scattering occurred during this process. Fine clots were thought to be constructed of single chains of fibrinogen molecules, joined end-to-end, while coarse clots were probably bundles of such chains. Flow birefringence studies on bovine fibrinogen (298) indicated a molecular length of 66 nm, and sedimentation and viscosity experiments (80) demonstrated that fibrin monomers in urea solution had the same gross size and shape as fibrinogen in the absence of urea. These studies (80, 298) indicated that intermediate polymers of fibrin consisted of 6 - 10 molecules of fibrin joined end-to-end, possibly with a twofold side-to-side degree of polymerisation. According to the review of Doolittle (68), early X-ray analysis of fibrin and fibrinogen gels yielded identical patterns and it was concluded that individual fibrinogen units maintained their size and shape during conversion to a fibrin gel.

Further evidence for an elongated fibrinogen molecule came from electric birefringence studies (141) which measured dipole moments and hence the electrical dissymmetry of species. These studies indicated that both fibrinogen and fibrin possessed a high degree of electrical symmetry. However, the observation that the removal by thrombin of one (of two) negatively charged fibrinopeptides A from fibrinogen led to a transient highly polar molecule was interpreted as an indication that the fibrinopeptide A species were located at positions near the opposite ends of the molecule. In addition, measurement of the dipole moment permitted calculation of the length of the molecule and this was found to be about 50 nm, a figure in agreement with a contemporary molecular model (136).

A.


47·5nm
24nm
<1·5nm
6·5nm
5nm
6·5nm

B.


69nm
23nm

C.


23nm

D.

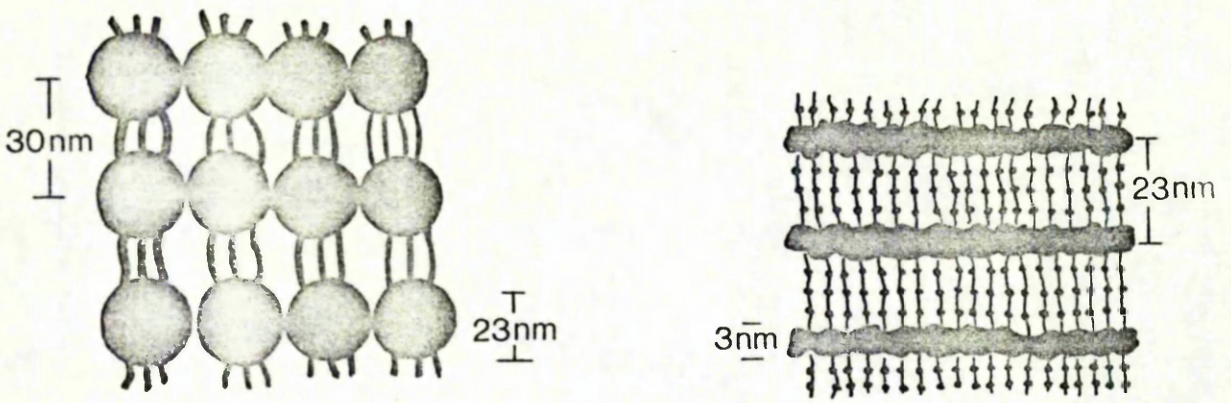

30nm
23nm
23nm
3nm

Fig. 1 Models of fibrinogen molecules and their arrangements in fibrin polymers, according to: A. Hall and Slayter (136); B. Kay and Cuddigan (172); C. Köppel (183); D. Pouit et al. (287).

1.3.2 Electron microscopy studies

Many of the models of the fibrinogen molecule have come from electron microscopy studies and observations in this field began in the 1940's when such a study (68) revealed the presence of transverse striations or bands on fibrin fibres, these bands repeating themselves at intervals of 23 nm. Along with these electron microscopic studies which have continued to the present day, a large number of biochemical and physicochemical investigations (such as those mentioned above and reviewed recently by Doolittle (68) and Mosesson and Finlayson (243)) were performed and provided data to furnish various molecular models of fibrinogen. One of constraints imposed upon these models of fibrinogen was that they should provide a satisfactory explanation of the cross-banding patterns of fibrin, as well as the characteristic periodicity of 23 nm.

1.3.2.1 Model of Hall and Slayter

One of the earliest and most durable of molecular models for fibrinogen was that of Hall and Slayter (136) whose micrographs of metal shadowcast preparations of bovine fibrinogen revealed the existence of elongated nodular structures, thus supporting the notion of rod-shaped fibrinogen molecules. A preponderance of these particles appeared as triads, 3 linearly attached globules (Fig. 1A), although some monads, dyads and tetrads were also seen. These triads (47.5 nm long) were believed to be intact fibrinogen molecules, their calculated molecular volume being close to that computed from hydrodynamic measurements (136), whereas the monads and dyads were evidence of disrupted molecular fragments, a suggestion refuted recently (243) from biochemical evidence. Hall and Slayter proposed that these molecules formed end-to-end polymers and shrank in length to about 24 nm during fibrin formation while side-to-side alignment of the linear polymers

accounted for the characteristic cross-banding pattern of fibrin fibres. A recent study (330) by electron microscopy and optical diffraction of microcrystals of fibrinogen, which had been partially degraded by a bacterial proteolytic enzyme, yielded evidence for a fibrinogen molecule of similar length as that of the previous workers, but suggested that the periodicity of fibrin might result from a staggering arrangement of fibrin monomers, a view similar to those of others (68).

1.3.2.2 Model of Kay and Cuddigan

While the major periodicity observed in fibrin fibres by numerous workers has been of similar dimensions, even when the fibrin was formed from different sources and by different enzymes (178), finer detail of the cross-banding pattern was observed by a negative staining technique (172) and cast doubts on the dimensions of the Hall and Slayter model. The fibrinogen molecule proposed was that of a linear array of nodular elements (Fig. 1B), with an observed width in the fibrin fibre of 3 nm, which was about half of the minimum possible width of the trinodular model. A two-thirds molecular overlap was proposed to account for the cross-banding pattern of fibrin, while the partial specific volume of fibrinogen required a molecular length of 69 nm.

1.3.2.3 Model of Köppel

Until the mid-1960's, much of the experimental data had pointed in the direction of an elongated fibrinogen molecule. However, Doolittle (68) has cautioned that, with hydrodynamic measurements, it was difficult to distinguish true dissymmetry of a molecular species from hydration effects. Attempts to do so employed the β-function, which gives information about the total effective volume of a protein by combining sedimentation, intrinsic viscosity and molecular weight parameters, and yielded axial ratios for fibrinogen of 1 to 5. This suggested that fibrinogen might be a highly swollen protein, less

elongated than had been proposed. However, if the shape of fibrinogen was approximately spherical, it would require to be hydrated by 8g of water per gram of protein, a value greatly in excess of the accepted upper limit of hydration (0.3 g/g of protein). In 1966, Köppel (183) observed particles of fibrinogen with a diameter 23 nm under the electron microscope. These individual nodules were not quite spherical, but appeared to be related to a pentagonal dodecahedron (Fig. 10), along the edges of which the polypeptide chains of fibrinogen were evenly distributed (184), thus forming a cage-like structure surrounding a relatively large volume of water. The majority of the water molecules were not trapped by this non-compact protein molecule, but rather were allowed to pass freely through the structures. Köppel proposed that the structure of fibrin could be explained on the basis of linear aggregates of the fibrinogen units, associated by vertex to vertex, edge to edge, or plane to plane. Support for the highly symmetrical fibrinogen molecule of Köppel came from Lederer and Finkelstein (198) whose experiments with aluminium scale models, similar to those of Köppel and Hall and Slayter, permitted calculation of a number of hydrodynamic parameters for each model. The values computed for the Köppel model were in much closer agreement to the actual experimental values than were those for the Hall and Slayter model. Moreover, small angle X-ray scattering measurements with dilute solutions of bovine fibrinogen yielded a scattering curve which was closer to the theoretical curve calculated for Köppel's model than to the curves calculated for four other models (197).

1.3.2.4 Model of Pouit and coworkers

In 1972, the electron microscopy study of Pouit et al. (287), using negatively stained preparations, showed that fibrinogen appeared as globules (average diameter 24 nm) with numerous filamentous

extensions (Fig. 1D). The presence of these filaments and the varying size of the globules were considered to represent the degree to which the fibrinogen molecule had "unrolled". During fibrin formation, the filaments from neighbouring molecules appeared to bind to each other, the globular particles became aligned through lateral association, and the periodic striations decreased from 30 nm to 23 nm. A decrease in the diameters of the globular elements from about 24 nm to 3 nm was also observed during such reorganisation of protein material in the fibrin fibre. (It is noteworthy that Ferry and Morrison (87) in 1947, considered the possibility of fibrinogen molecules opening to form extended polypeptide chains during fibrin formation, but these authors dismissed the idea as unlikely). Others have since observed spherical particles with filaments (15) and transformation of globular units to linear aggregates (18) during fibrin formation. In addition, Haschemeyer (142) in a reinvestigation of her previous work with a purer fibrinogen preparation, was unable to reproduce the electric birefringence results which had indicated the existence of a polar, elongated intermediate of fibrinogen; indeed, the later results were more compatible with the existence of a spherical molecule.

Recently, more detailed structural models of the fibrinogen molecule have been constructed by Suscillon and coworkers from a combination of their electron microscopy studies and current biochemical knowledge (157) and from neutron small-angle scattering studies (224), yielding spherical and banana-shaped molecular models, respectively.

1.4 The molecular structure of fibrinogen

Although many of the structural details of fibrinogen have been derived from enzyme studies, using, for example, thrombin, plasmin and Factor XIII, the action of these enzymes will be considered

in later sections. This section deals largely with the subunit arrangement of fibrinogen obtained by chemical analysis; most of the information which has been obtained applies equally well to the subunit structure of fibrin (68).

1.4.1 Amino acid composition

Automated amino acid analysis has been performed on fibrinogens from different species by a number of workers (68, 252) and the differences between the species are small (252). All the common amino acids are present at a frequency not greatly different from their occurrence in other vertebrate proteins (68). No differences existed between the composition of fibrinogen and fibrin of human and bovine origin (152). Although a total of 56 - 58 half-cystine residues were found in fibrinogen and in fibrin (149), no free sulphydryl groups were detected, suggesting that all of the half-cystine residues were involved in the formation of 28 - 29 disulphide bridges.

1.4.2 Carbohydrate composition

Considerable variance has been found in the carbohydrate content of fibrinogen and fibrin from different species, but is probably related to the different methods of analysis used by the investigators (252). Blombäck (20) found that bovine fibrinogen contained 3.2% of hexoses (galactose and mannose), 1% of glucosamine and 0.8% of sialic acid. The role of these carbohydrate groups remains unclear at present (252).

1.4.3 Polypeptide chain structure of fibrinogen

The existence of a multiple chain structure of fibrinogen was demonstrated in 1951, as a result of NH_2-terminal amino acid analysis which revealed the presence of more than one residue each

of tyrosine and glutamic acid (7). These results were later confirmed by Blombäck and Yamashina (36) whose quantitative determinations demonstrated up to three pairs of NH_2-terminal end groups in fibrinogens and fibrins of various species, thus indicating the existence of six subunit polypeptide chains per molecule of fibrinogen. In the cases of human and bovine fibrinogens, only two pairs of NH_2-terminal residues have been found, since one pair of the polypeptide chains possessed a blocking NH_2-terminal pyroglutamic acid residue with no reactive α-amino end group (29, 36). In COOH-terminal amino acid analysis, up to three different residues have been found in fibrinogens of various species, although only valine and glutamine were demonstrated in human fibrinogen (63, 154, 266).

Further evidence for the polypeptide chain composition of fibrinogen was supplied by the preparative isolation of three different subunits after cleavage of the disulphide bonds by sodium sulphite (56, 146). Paper and starch gel electrophoresis of the sulphitolysed fibrinogen, S-sulpho-fibrinogen, in the presence of high concentrations of urea, revealed two or three bands, whereas intact fibrinogen showed only one band (56, 143, 148, 150, 151). Ultracentrifugal analysis of S-sulpho-fibrinogen yielded a molecular weight of 50,000 - 65,000, a value which was consistent with a six-chain structure of fibrinogen (143, 148), while later studies (212) on the isolated chains revealed the unique molecular weight values, 64,000, 56,000 and 47,000 for the three different chains. These values were in reasonable agreement with those obtained for the fibrinogen chains by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (234).

NH_2-terminal amino acid studies on bovine S-sulpho-fibrinogen and the isolated chains, have identified only those residues

which were demonstrable in the intact fibrinogen molecule (148, 150). In addition, thrombin has been shown to release fibrinopeptide A from the NH_2-terminal of one chain and fibrinopeptide B from another, but was without effect on the third chain (150). Accordingly, the three chains of fibrinogen have been designated as $A\alpha$, $B\beta$ and γ in order to identifiy those chains of fibrinogen from which the fibrinopeptides A and B are released by thrombin (252). The structural formula for intact fibrinogen may then be represented as $(A\alpha, B\beta, \gamma)_2$.

Besides sulphitolysis, fibrinogen has been dissociated into its polypeptide subunits by reduction of the disulphide bonds with the thiol reagents, mercaptoethanol, dithiothreitol and dithioerythritol, followed by alkylation of the half-cystine residues to prevent re-oxidation. However, demonstration of the multiple component system of S-carboxymethylated fibrinogen was not achieved by gel filtration or electrophoresis in the presence of dissociating agents (147, 151), until Murano et al. (255), followed by others (123, 153), isolated the individual chains by ion-exchange chromatography. The separation of the subunit chains of S-aminoethylated fibrinogen has also been described (118).

1.4.4 The disulphide knots of fibrinogen

Cyanogen bromide (CNBr) cleaves polypeptide chains at methionine residues and has been used to fragment the structure of fibrinogen in a considerable number of investigations, conducted mainly by Blombäck and coworkers. Since human fibrinogen contains 60 - 66 methionine residues per molecule (112), cleavage by CNBr gives rise to a large number of fragments (28), although it can be reasonably expected that a number of these peptide chains would remain linked to one another by disulphide bonds. Indeed, the isolation and

investigations of such disulphide-linked fragments have led to a greater understanding of the function and the conformation of the component polypeptide chains in human fibrinogen (112).

1.4.4.1 N-DSK: The monomeric model

The initial studies of Blombäck et al. (31, 32), on CNBr-cleaved fibrinogen, demonstrated the existence of a fragment which released the fibrinopeptides A and B on reaction with thrombin. The fragment contained about 40% of all the half-cystine residues of fibrinogen in the form of 11 disulphide bridges, with no free sulphydryl groups, but constituted only about 15% of the mass of fibrinogen (28). NH_2-terminal amino acid analysis of the fragment revealed the same residues as found for intact fibrinogen (31) and the amino acid sequence data indicated that the fragment consisted of the NH_2-terminal peptides of the $A\alpha$, $B\beta$ and γ chains (31, 32). This was directly confirmed by comparison with NH_2-terminal amino acid sequences of the $A\alpha$ and γ chains of intact fibrinogen (163). On the basis of these observations, the term "NH_2-terminal disulphide knot" (N-DSK) was used to describe this fragment (31).

Originally, a minimum molecular weight of about 26,000 was estimated for N-DSK by amino acid analysis (31). Since this value was in agreement with that from sedimentation equilibrium analysis, Blombäck et al. (31), concluded that N-DSK was a monomeric unit and that there were two identical N-DSK fragments located in the NH_2-terminal regions of each set of $A\alpha$, $B\beta$ and γ chains. Since the electric birefringence measurements of Haschemeyer (141) had previously located the A fibrinopeptides at the opposite ends of an elongated dimeric fibrinogen molecule, Blombäck et al. (22, 24, 27, 31) proposed that the N-DSK units of fibrinogen were situated accordingly at the opposite poles of an elongated fibrinogen molecule which lay

perpendicular to a two-fold axis of symmetry. This molecular model of fibrinogen was in accordance with that of Hall and Slayter (136), if it was assumed that each "half" of the fibrinogen molecule, i.e., each monomeric unit consisting of one Aα, one Bβ and one γ chain, was joined together by disulphide bonds in the COOH-terminal regions.

1.4.4.2 N-DSK: The dimeric model

The proposed molecular models for N-DSK and fibrinogen were later revised when further purification of N-DSK by countercurrent distribution led to the removal of hydrophobic impurities from the hydrophilic N-DSK (24), and subsequent studies indicated a molecular weight of 50,000 - 60,000 for the purified N-DSK, i.e. twice that of the monomer unit (23, 24, 26, 340). A similar value was also found for N-DSK by Marder and coworkers (217, 219). In addition, the discovery of symmetrically-linked pairs of Aα peptide fragments and γ peptide fragments - possible sites of disulphide linkages between the monomeric units - indicated that N-DSK was probably a dimeric structure (24). Therefore, it appeared that the NH_2-terminal regions of all six chains of fibrinogen were located in close proximity to each other and were linked together by disulphide bonds within the structure of N-DSK (23, 24, 26, 307), which may be represented by the molecular formula $(A\alpha\,1 - 51, B\beta\,1 - 115/118, \gamma\,1 - 78)_2$, with amino acid residues numbered from the NH_2-terminal ends (188). Quantitative immunological assay has confirmed the existence of only one N-DSK unit per molecule of fibrinogen (112).

Studies on the arrangement of the 11 disulphide bridges in N-DSK have shown that they may be classed as "stable", in which a single arrangement of the individual bridge has been found, and "labile", in which there is more than one possible arrangement of the bridges (28). The phenomenon of disulphide interchange, involving the

labile half-cystine residues, has accounted for the appearance of polymers of N-DSK (28), although other studies have suggested that the disulphide linkages of fibrinogen are stable (278).

In non-denaturing conditions, reductive cleavage of the disulphide bonds of intact fibrinogen broke only a few such bonds, and these were probably surface-orientated (30, 45). Of those bonds broken, three of them were shown to be symmetrical disulphide bridges joining the two"halves" of N-DSK (30), although it has been suggested, from immunochemical data (188), that N-DSK is largely hidden within the fibrinogen molecule.

The immunological behaviour of N-DSK of human fibrinogen has been extensively studied and it has been shown that the most immunogenic structures are the $B\beta$ and γ fragments (28, 33, 186, 188). On the other hand, conformational rather than sequential determinant structures might be expected to predominate in the rigid, highly-crosslinked N-DSK (188). Indeed, Gollwitzer and coworkers have shown that some of the antigenic determinants of F-CB1, a NH_2-terminal fragment from CNBr-cleaved bovine fibrinogen (120, 328), were destroyed by reduction of the disulphide bonds.

In addition to F-CB1, another analagous fragment to N-DSK has been reported in bovine fibrinogen, $F_{CNBr}I$ (179).

1.4.4.3 Other disulphide knots of fibrinogen

Since N-DSK accounted for only 40% of the disulphide bonds of human fibrinogen, fragmentation of fibrinogen by CNBr was expected to give rise to a number of disulphide-containing polypeptides. Recent studies employing gel filtration and countercurrent distribution have confirmed the existence of another four "disulphide knots" (112). Three of these fragments have been classified as hydrophobic (Ho1-DSK, Ho2-DSK and Ho3-DSK) (113), and one as hydrophilic

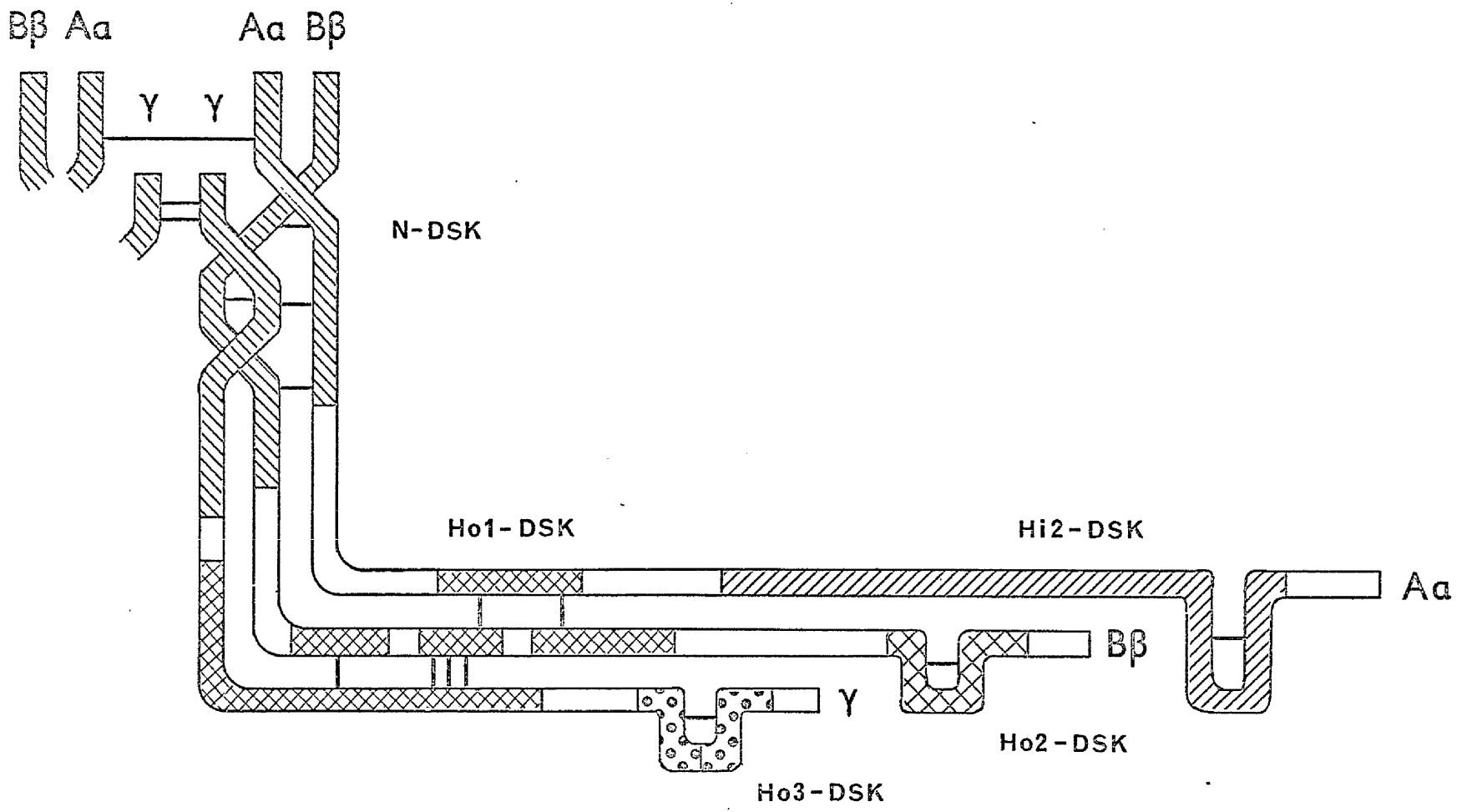


Fig. 2 Diagrammatic representation of the polypeptide chain structure of one half of fibrinogen, with one of several possible arrangements of the disulphide knots (according to Gårdlund et al. (112)).

(Hi2-DSK) (30). All of them were monomeric and there were two fragments of each per molecule of fibrinogen; moreover, the sum of their disulphide bridges, including those of N-DSK, appeared to account for all the disulphides of intact fibrinogen (112).

Ho1-DSK (molecular weight 43,000) appeared to consist of five peptide chains (1γ, 1Aα and 3 Bβ fragments) bonded together by six disulphide bridges (112). This fragment corresponded to F-CB2 (328) and/or $F_{CNBr}II$ (179) which had been isolated from CNBr-treated bovine fibrinogen.

Ho2-DSK was a small, single-chain fragment from the Bβ chain and contained one disulphide bond, while Ho3-DSK consisted of two small γ chain fragments, joined by a single disulphide bond (112). F-CB4, a small disulphide-containing fragment of bovine fibrinogen (329) might correspond to one of the latter two human fragments.

Hi2-DSK (molecular weight 27,000) was derived exclusively from the Aα chain and contained a single intrachain disulphide bond (30, 112, 155). This fragment corresponded closely in molecular size, amino acid composition and immunochemical properties to F-CB3, a polypeptide from bovine (120, 328, 329) and human (122) fibrinogen.

Immunochemical studies on the disulphide knots indicated that the hydrophobic fragments and N-DSK were largely buried within the parent molecule while Hi2-DSK and F-CB3 appeared to be located on the surface of fibrinogen (203, 252, 329). Fig. 2 shows a diagrammatic representation of the polypeptide chain structure of human fibrinogen with the proposed locations of the disulphide knots.

1.4.5 Primary structure of human fibrinogen

Amino acid analysis of the isolated chains of S-sulfo- and S-carboxymethyl-fibrinogen indicated that the compositions of the

individual chains differed significantly from each other (123, 148, 153, 212, 255).

Each of the three chains of fibrinogen has been reported to contain carbohydrate (150, 252), although Gaffney (102) and Pizzo et al. (272) were unable to stain the Aα chain of fibrinogen for carbohydrate after electrophoresis on polyacrylamide gels. In the γ chain of fibrinogen, a single oligosaccharide chain appears to be attached to an aspartic acid residue (number 52 in the γ chain) (33, 236), while a single point of attachment for carbohydrate (aspartic acid or asparagine) has been described in the COOH-terminal region of the Bβ chain (154, 331).

Amino acid sequence analysis of the small NH_2-terminal human fibrinopeptides A and B showed that they consisted of 16 and 14 amino acid residues, respectively (29). The sequences of the Aα- and Bβ chains were further extended by studies on NH_2-terminal fragments from plasmin digests of S-sulpho-fibrinogen (165), and on the isolated chains of N-DSK (22, 28, 31, 32, 33, 34, 186). These studies demonstrated the isologous segments, Cys-Pro-X-X-Cys, found in each of the polypeptide chain fragments and the repeating sequence, Arg-Pro-X, of the Bβ fragment (28, 252). In addition, partial sequence analyses of the hydrophobic disulphide knots (112) and the hydrophilic Hi2-DSK (112, 155) has revealed the primary structures of small segments of all three chains.

Sequence analysis of the Aα, Bβ and γ chain fragments of the plasmin degradation products D (59, 71, 319) and E (186, 319, 321) has also contributed to the knowledge of the primary structure of fibrinogen, particularly by providing recognisable amino acid sequences which overlapped with those from the CNBr fragments. A number of workers have also sequenced the NH_2-terminal end of a small plasmin-

produced fragment from the Aα chain of fibrinogen (112, 137, 155, 320), thus demonstrating its close relationship with a peptide isolated from CNBr cleavage of the Aα chain (70, 112, 155).

The most recent amino acid sequence studies have been performed on the CNBr-derived fragments of the isolated chains of fibrinogen. More than half of the 600 amino acids in the Aα chain have been sequenced and Doolittle et al. (70, 71) have demonstrated not only internal homology in the Aα chain, but also certain similarities to the Bβ and γ chains in the distribution of amino acids over a 110-residue stretch. The sequence of the Bβ and γ chains of fibrinogen have been almost completely elucidated by Henschen and Lottspeich (154), who established that 31% of the amino acid residues over the lengths of the aligned Bβ (462 residues) and γ (410 residues) chains were identical. In addition, several internal sequence homologies have been found in these chains. These findings strongly support the hypothesis that the Aα, Bβ and γ polypeptide chains of fibrinogen have evolved from a common molecular ancestor (68, 71, 154).

1.4.6 Heterogeneity of fibrinogen

The heterogeneity of human fibrinogen has been established on the basis of its solubility characteristics (239, 248), electrophoretic behaviour (200, 239) and isoelectric focusing (4, 101, 310) in polyacrylamide gels, and chromatography on ion-exchange resins (90, 314).

Chromatographic, electrophoretic and NH_2-terminal amino acid sequence analyses on fibrinopeptide A (29) and on the Aα fragment of N-DSK (34) have shown the existence of several chain variants. Besides fibrinopeptide A, smaller amounts of fibrinopeptides AP (a phosphorylated fibrinopeptide A), AY (fibrinopeptide A with NH_2-

terminal alanine missing) and possibly AYP (a phosphorylated fibrinopeptide AY) have been detected as NH_2-terminal components of the Aα chain. Blombäck *et al.* (34) have suggested that these Aα chain variants may represent derivatives produced *in vivo* by the action of exopeptidase and phophatase enzymes.

Heterogeneity of the Aα chains of human or bovine fibrinogen has been demonstrated by isoelectric focusing (101), ion-exchange chromatography (253, 255, 303) and polyacrylamide gel electrophoresis (42, 208, 212, 234, 246, 253, 255, 303). At least part of the observed heterogeneity is a consequence of Aα chain shortening at the COOH-terminal end (63). In addition, the heterogeneity of fibrinogen, identified by its solubility (246) and electrophoretic (200) behaviour, has been attributed largely to the degree of intactness of the Aα chain.

The Bβ chains of human fibrinogen have been shown to be heterogenous by isoelectric focusing (101) and ion-exchange chromatography (114).

Heterogeneity of the γ chains of human or bovine fibrinogen has been demonstrated by isoelectric focusing (101), ion-exchange chromatography (114, 153, 245), and paper and polyacrylamide gel electrophoresis (115, 153, 245, 250). The heterogeneity of intact fibrinogen, observed on ion-exchange chromatography (245, 250), has been attributed to γ chain heterogeneity.

1.5 Action of thrombin on fibrinogen: fibrin polymerisation

The enzyme responsible for the formation of fibrin from fibrinogen is thrombin (Factor IIa), which is a serine protease derived from an inactive precursor, prothrombin. In the presence of activated Factor X, phospholipids, calcium ions and Factor V, activation of

prothrombin (Factor II), a single chain polypeptide, occurs by proteolytic removal of a large peptide from the NH_2-terminal end; internal cleavage of the remaining polypeptide without further loss of peptide material gives rise to the active enzyme, thrombin (molecular weight 38,000), which consists of a small polypeptide A chain and a large polypeptide B chain linked together by disulphide bonds (302).

1.5.1 Cleavage of fibrinopeptides by thrombin

Direct evidence for the proteolytic action of thrombin during fibrin formation was obtained when the cleavage of small peptides from the NH_2-terminal end of fibrinogen resulted in a difference in the NH_2-terminal amino acid analysis of fibrinogen and fibrin (7). Quantitative analysis indicated that two A and two B fibrinopeptides were released from each molecule of fibrinogen (36), fibrinopeptide A being released firstly and at a faster rate than fibrinopeptide B (21). The lag phase before the release of fibrinopeptide B probably represents a local conformational change in fibrinogen, without which fibrinopeptide B would not be susceptible to cleavage by thrombin (26). The release of fibrinopeptide B does not appear to be essential for fibrin formation since

(i) the release of fibrinopeptide A, but not fibrinopeptide B, was correlated with amount of formed fibrin (35),

(ii) reptilase, which cleaved only fibrinopeptide A from fibrinogen, caused coagulation of fibrinogen (21), and

(iii) a venom factor from the Southern Copperhead snake released fibrinopeptide B much faster than fibrinopeptide A, but no clot was formed until adequate amounts of fibrinopeptide A were released (307).

1.5.2 Specificity of thrombin

Since only two pairs of fibrinopeptides were released by thrombin from fibrinogen, this enzyme with trypsin-like specificity was remarkable in that it cleaved only 4 arginyl peptide bonds out of 260 arginyl and lysyl peptide bonds in fibrinogen (165). It appeared that fibrinogen itself contained the structural features responsible for the narrow specificity of thrombin action. NH_2-terminal amino acid analysis on fibrin, together with amino acid sequence data on the fibrinopeptides and whole fibrinogen, have indicated that thrombin cleaved only arginyl-glycine bonds when releasing fibrinopeptides A and B from human and all other species of fibrinogen so far studied (29, 36, 163, 252). Fibrinopeptides A and B were released by thrombin digestion also from the isolated Aα and Bβ chains of S-sulpho-fibrinogen (150), from intact N-DSK and from the isolated Aα and Bβ chain fragments of N-DSK (32) and from the NH_2-terminal plasmin-cleaved Aα and Bβ fragments of S-sulpho-fibrinogen (165). Besides the rapid release of fibrinopeptide A from these degraded Aα remnants, the slow release of a tripeptide, Gly-Pro-Arg, from the newly-exposed NH_2-terminus of the α remnant was also observed (32), but it could not be cleaved from fibrinogen under physiological conditions (34).

Since the specificity of thrombin was unchanged whether the substrate was whole fibrinogen or chain fragments from N-DSK, the structures responsible for the binding and interaction with thrombin were confined to these NH_2-terminal chain fragments (307). In the case of fibrinopeptide A, the amino acid sequence at the COOH-terminal part of the peptide has been highly conserved throughout the mammalian series (23, 307). Nearly all species had a phenylalanyl residue in this segment at a distance of nine amino acid residues from the bond split by thrombin (252). Like this segment which

existed in α-helical conformation, helical conformation and inter-species similarities were found in short peptide segments on the COOH-terminal side of the susceptible Arg-Gly bond (23, 307). On the basis of these results, it appeared that a specific binding site for thrombin on the Aα chain of fibrinogen might reside in this region (307).

1.5.3 Measurement of fibrinopeptides

As a means of studying intravascular coagulation, methods for measuring the levels of fibrinopeptides A and B by radioimmunoassay have been developed (17, 263). After coupling to albumin, antisera to these fibrinopeptides were raised in rabbits. However, iodination of the fibrinopeptides required their prior coupling to tyrosine, and physical separation of the fibrinopeptides from fibrinogen was necessary before the assay could be performed (17, 116, 263). In the case of fibrinopeptide A, normal individuals had mean levels of 1 ng/ml and a half-life of only a few minutes was demonstrated for the peptide (116, 262). Increased levels of fibrinopeptide A have been found in patients with thrombotic diseases and during defibrination therapy (43, 116, 262).

1.5.4 Biological properties of fibrinopeptides

Both fibrinopeptides A and B have an antithrombin anti-coagulant activity (125). The haemostatic effect of fibrinopeptide B on rat arterioles has been attributed to its vasoconstrictor action (267). Furthermore, fibrinopeptide B potentiated bradykinin-induced contraction of the uterus (171) and exhibited chemotaxis for human monocytes and neutrophils (171, 295).

1.5.5 Fibrin monomer polymerisation

In the second stage of the fibrinogen-to-fibrin

conversion, the fibrin monomers aggregate to form soluble polymers and subsequently form a gel. The polymerisation reaction is independent of thrombin (193) and calcium ions appear to increase the rate of gelation as well as the final rigidity of the fibrin gel (41, 305). A light scattering study of thrombin-induced fibrin (devoid of fibrinopeptides A and B) and reptilase-induced fibrin (devoid of fibrinopeptide A only) indicated a predominance of end-to-end polymers in the reptilase-induced fibrin (195). Moreover, the viscosities of the two fibrins were different. In view of these results, it appeared that a set of binding domains became operational after release of fibrinopeptide A, while a different set was in force after removal of fibrinopeptide B (28). The notion that fibrinopeptide B removal was necessary for lateral aggregation of fibrin has been questioned by Doolittle (68).

Kudryk et al. (187) have demonstrated that insolubilised fibrin displayed a degree of affinity for the high molecular weight species of fibrinogen fragment D, indicating that fragment D contained sites of importance for the polymerisation of fibrin. Since intact fibrinogen displayed a similar affinity for insolubilised fibrin, the binding domains of fragment D might always be exposed on the fibrinogen molecule. It has been proposed that acceptor groups for fibrin polymerisation are exposed on removal of the electronegative fibrinopeptides, allowing spontaneous polymerisation of fibrin since donor groups are permanently available in other regions of the fibrinogen molecule (191).

The nature of the binding forces involved in fibrin polymerisation remains unknown, although thermodynamic data implicate the involvement of hydrogen bonds (68). In particular, histidine is believed to behave as a hydrogen bond acceptor (68),

an idea which is consistent with the recent studies of Plow (278) and Inada et al. (162). Tyrosine may function as a hydrogen bond donor, while lysine may also participate in fibrin polymerisation (68).

1.5.6 Soluble fibrin monomer complexes

Although the transient existence of soluble intermediate polymers of fibrin was recognised from early studies on fibrin formation, a number of reports indicated the persistence of soluble fibrin complexes in a variety of thrombotic states (9). In 1960, Shainoff and Page (304) examined the plasma of endotoxin-treated rabbits and isolated cryoprofibrin, a cold-precipitable fibrin complex, which appeared to have arisen from limited thrombin attack on plasma fibrinogen since a reduced amount of fibrinopeptide A was recoverable from cryoprofibrin compared with fibrinogen. Others have shown that soluble unclottable complexes may form during fibrinogen-to-fibrin conversion in the presence of fibrinogen degradation products (202).

Recent in vitro studies have employed the techniques of polyacrylamide gel electrophoresis (159, 160, 161, 176, 205), NH_2-terminal amino acid analysis (9, 176), ultracentrifugation (220) and agarose gel filtration (50, 159, 160, 161, 166, 205), as well as the ethanol gelation and protamine sulphate precipitation tests (9, 133), for the detection of soluble fibrin complexes. In addition, these complexes have also been studied by selective adsorption of fibrinogen derivatives on insolubilised fibrin and fibrinogen. By such investigations of the adsorption phenomenon, which was considered to be related to complex formation between fibrinogen and its derivatives (227), insolubilised fibrin monomer was shown to bind to fibrinogen (145), fibrinogen fragment D (187) and fibrinogen degradation products X, Y, D and E (228). On the other hand, others

have suggested that only fibrin fragments D and E, but not fibrinogen fragments D and E, were involved in complex formation (50).

In the case of fibrinogen-fibrin monomer complexes, von Hugo et al. (161) have shown that fibrinogen and fibrin monomer occurred in a ratio of 1 : 1. Investigation of the nature of the bonds in soluble fibrin complexes revealed the presence of a few γ-γ chain dimers (176), although other studies indicated that the bonds in the complexes were non-covalent in character (166). In vivo studies in the plasmas of patients with soluble fibrin complexes have shown similarities in the nature of these complexes to those produced in vitro (1, 128, 135, 177, 213). However, viscosity studies have indicated that the formation of fibrinogen-fibrin complexes might represent an in vitro phenomenon, which was dependent on reduced temperatures for its manifestation (19).

1.6 Effect of Factor XIII on fibrin

In the final stage of the fibrinogen-to-fibrin conversion, interchain crosslinks are introduced into the fibrin molecule, an event which is unique in the coagulation cascade, since it is the only reaction which involves the formation of covalent bonds (89). With the presence of these crosslinks, fibrin becomes stabilised against dissolution in denaturing solutions, such as 5M urea or 1% monochloroacetic acid (89).

The crosslinking enzyme, Factor XIIIa (fibrin stabilising factor, plasma transglutaminase) exists in plasma as a proenzyme, Factor XIII, and has a molecular weight 300,000 - 340,000 (89). Factor XIII is a tetramer, consisting of two a chains and two b chains of similar molecular weights (ca. 80,000), which are bound together by non-covalent bonds; the molecule is thus represented by

the formula a_2b_2 (11, 89, 299, 323). During activation of the enzyme by thrombin, a peptide of 36 residues is released from the NH_2-terminal end of the a chain, which contains the catalytically active site, and Factor XIIIa may be represented by the formula a'_2b_2 (89, 299). Although calcium ions are not required for the activation of the enzyme they are required for its cross-linking activity (89).

Factor XIII from platelets and the placenta has a molecular weight of 150,000 and consists of a_2 dimers, which are identical in all respects to the a chains of plasma Factor XIII (11, 89, 299, 311). The function of the b chains in plasma is unknown, but may serve to stabilise the a chains (89). Patients with congenital Factor XIII deficiency appear to lack the active a chains, but possess a-chain binding capacity, i.e. b chains, (11, 12, 89).

Besides thrombin, coagulase thrombin (316), Factor Xa (207) and trypsin (207) appear to activate Factor XIII. Activation of Factor XIII by the fibrin-forming enzymes from snake venom, ancrod and reptilase, may be due to contaminating enzymes in these preparations (207, 273).

1.6.1 The crosslinking of fibrin

Disulphide bridges have been proposed as likely candidates for the role of fibrin crosslinks and glycine residues have also been implicated (89). However, the crosslinking bonds were identified as ϵ-(γ-glutamyl) lysine residues after total enzymic digestion of crosslinked fibrin (89, 271). Moreover, separation of the crosslinked chains of fibrin by sulphitolysis and chromatography revealed that two γ chains were joined by reciprocal ϵ-(γ-glutamyl) lysine bridges (52, 53, 322). Each γ chain contained one donor unit (ϵ-amino group of lysine) and one acceptor unit (glutamine) in

close proximity at the COOH-terminal ends of the polypeptide chains and the reciprocal arrangement necessitated an antiparallel orientation. The crosslinking between γ chains was intermolecular, not intramolecular, since hybrid crosslinked units were formed by clotting a mixture of human and bovine fibrinogens (68).

Using the technique of SDS-polyacrylamide gel electrophoresis, other investigators confirmed that, not only did Factor XIIIa cause dimerisation of the γ chains, but polymerisation of the α chains of fibrin also occurred, while the β chain remained uncrosslinked (209, 211, 300). The α chain crosslinking occurred more slowly than the γ chain crosslinking (211, 300). While the γ chains accounted for two crosslinks per molecule of fibrin, it was estimated that α crosslinking would produce four crosslinks per molecule (89). A proportion of these crosslinking sites appeared to be situated at the COOH-terminal end of the Aα chain (92) while at least one potential site was located in a mid-section stretch of the Aα chain (91). Consistent with these observations, others (85, 96, 320) have demonstrated the existence of at least two crosslinking acceptor sites in the COOH-terminal aspect of the α chain of fibrin, using radioactive and fluorescent substitute donors. In particular, Takagi and Doolittle (320) characterised, by amino acid sequence analysis, the mid-section piece of the α chain which appeared to contain a glutamine crosslinking acceptor unit, but doubts were cast on the authenticity of this site by later work on the characterisation of two linked α chain CNBr fragments from crosslinked fibrin (69).

Concerning crosslinking of the α chain of fibrin, it is noteworthy that these chains may also be crosslinked to the subunit polypeptides of cold-insoluble globulin, a protein which shows

remarkable similarities to the clottable proteins of lobster blood (249). Moreover, on the basis of further similarities between a fibroblast membrane protein and cold-insoluble globulin, Mosher (249) suggested that Factor XIIIa may be involved in the covalent association of fibroblast membrane protein and fibrin.

1.7 Effect of plasmin on fibrinogen and fibrin

Induction of fibrinolytic activity in the blood appears to be due to the conversion of the proenzyme, plasminogen, to the active protease, plasmin. This enzyme has also been employed in the majority of the *in vitro* degradation studies on fibrinogen.

The conversion of the single chain plasminogen (molecular weight 93,000) to the disulphide-bonded two-chain plasmin (molecular weight 85,000) requires the action of the so-called plasminogen activators, and occurs in two steps. In the case of urokinase activation, the first step consists of the proteolytic cleavage of the NH_2-terminal 68 amino acid residues (possibly as two activation peptides) from the intact plasminogen (Glu-plasminogen). As the inactive intermediate product has NH_2-terminal methionine, it seems that the so-called Lys-plasminogen intermediate may represent a more advanced digestion intermediate. In the second step of the activation of plasminogen, the intermediate is hydrolysed by urokinase at a single arginyl-valine bond to form the active plasmin molecule, consisting of a heavy chain and a light chain which contains the active site.

Trypsin and tissue activator from pig heart activated Lys-plasminogen similarly to urokinase by cleaving a single arginyl-valine bond. Activation of Glu-plasminogen by catalytic amounts of streptokinase proceeds in the same manner as for urokinase, but with

stoichiometric amounts of streptokinase, leading to the formation of the plasminogen-streptokinase complex, the order of bond cleavage appears to be in reverse order (296).

1.7.1 Degradation of fibrinogen by plasmin

Early investigation of terminal plasmin digests of fibrinogen revealed the presence of five degradation products, A-E (265). Of these products, fragments A, B and C were small peptides, probably derived from the Aα chain of fibrinogen (265, 272), and did not react with anti-fibrinogen antiserum (264), whereas fragments D and E had molecular weights 83,000 and 35,000, respectively, and reacted with anti-fibrinogen antiserum, although these two products had no antigenic determinants in common (264, 265). Related observations on the terminal digestion products were made by others (168, 261), who also found that the D preparation was heterogenous on electrophoresis, exhibiting up to eight components.

During the degradation of fibrinogen, transient intermediate products were noted in the digestion mixture by Nussenzweig et al. (265) and investigated by others (46, 221). These fragments (X and Y) possessed potent anticoagulant activity and were characterised by Marder et al. (222) with respect to their physicochemical behaviour and immunological properties. From these studies, a scheme for describing the molecular fragmentation of fibrinogen by plasmin was formulated and remains today the most popular choice of various schemes for fibrinogen degradation, even though its postulates have received close scrutiny from numerous other workers. In outline, fibrinogen is largely converted by plasmin to fragment X, a clottable derivative of molecular weight 240,000, by the loss of the minor peptides A and B and C; asymmetric cleavage of fragment X leads to the simultaneous appearance

of the non-clottable fragment Y (molecular weight 155,000) and fragment D (molecular weight 83,000); fragment Y is then split into a second fragment D and a single fragment E species (molecular weight 50,000). From the molecular weights and yields of the various degradation products, Marder et al. (222) concluded that fibrinogen, during proteolytic cleavage by plasmin, could generate two fragment D species and one fragment E species, thus supporting a previous notion (265).

1.7.2 Polypeptide chain structures of the fibrinogen degradation products (FDP)

The elucidation of the subunit chain structures of the fibrinogen degradation products and the discovery of their sites of origin within the intact fibrinogen molecule were complementary pieces of information in the search for the structure of fibrinogen. One such important finding established that fragment E constituted a major junction of the NH_2-terminal ends of the fibrinogen chains and of the monomeric units of the fibrinogen molecule. In spite of a report that two monomeric N-DSK were overlapped by the fragments D (192), Wallén (335) suggested that the dimeric N-DSK and fragment E were structurally related. Immunological support for this notion was provided by the reaction of identity between N-DSK and fragment E, using anti-fibrinogen antiserum (217, 219). In addition, immunological analysis with anti-N-DSK antiserum confirmed that N-DSK and fragment E possessed some common antigenic structures (186, 188). However, some additional elements present on fragment E appeared to mask antigenic sites which were exposed on N-DSK; these sites were fully exposed on a NH_2-terminal CNBr fragment from fragment E, the E-knot, which was immunologically identical to N-DSK (186, 188). Physicochemical studies revealed that N-DSK and fragment E were similar regarding their molecular weights.

extinction coefficients, half-cystine contents and NH_2-terminal amino acids (219), although fragment E appeared to lack the NH_2-terminal portion of the Bβ chain fragment (186, 219). In addition, fragment E, as well as fragments X and Y, but not fragment D, contained at least a proportion of the theoretical amount of fibrinopeptide A (44, 186, 218, 219, 235, 244, 270). Final and conclusive proof that the NH_2-terminal portion of fibrinogen remained intact during plasmin degradation and was largely contained in fragment E was obtained from the amino acid sequence analysis of the E-knot (186); from such data, the parent fragment E appeared to have the structure (Aα 1-86, Bβ 54-119, γ 1-58)$_2$, and was thus closely related to N-DSK (188).

Studies on fragment D, the other terminal digestion product of fibrinogen, have not yet revealed its full structure, although the essential features of this fragment have now been discovered. It was derived from the COOH-terminal aspect of fibrinogen (219), its heterogeneity, at least in part, was related to the extent of plasmin digestion of the γ chain (59, 85, 103, 272, 319), it gave rise to three CNBr-derived fragments similar to Ho1-DSK, Ho2-DSK and Ho3-DSK (112, 113), and it was a monomeric structure, containing disulphide-bonded fragments of each of the chains of fibrinogen (59, 85, 99, 103, 319). The latter observation was based largely on quantitative NH_2-terminal amino acid analyses and molecular weight measurements of the polypeptide chain components of fragment D. The NH_2-terminal amino acid sequence has been described for all three chain fragments (59, 319), while the complete primary sequence for the Aα fragment was elucidated by Doolittle *et al.* (71). The difference in structure between two of the early fragment D species, D_1 and D_2, was shown to be a 23-residue segment at the NH_2-terminal end of the γ chain fragment (59, 319). Analysis of this peptide section demonstrated that the γ fragment of

N-DSK and that of fragment D had sequences in common, thus accounting for the weak immunological reaction of anti-N-DSK antiserum and fragment D (59, 113). These results also indicated that the NH_2-terminal ends of fragment D and the COOH-terminal ends of fragment E (for the γ chain at least) lay in close proximity in the intact parent fibrinogen molecule (59, 319).

Besides these intensive investigations on individual degradation products, the sequence of bond cleavage in fibrinogen during plasmin digestion has been conveniently examined by means of SDS-polyacrylamide gel electrophoresis (98, 106, 234, 235, 272). These studies generally supported the degradation scheme of Marder et al. (222) and demonstrated that the Aα chains of fibrinogen were cleaved first; further removal of a small Bβ peptide produced fragment X (272). Assymmetric cleavage of fragment X in at least three positions separated the fragments Y and D, while further plasmin attack on fragment Y resulted in the generation of another fragment D and fragment E (272). Occasionally, small degradation products, related to fragment D, were detected on SDS-polyacrylamide gel electrophoresis (99, 244, 293).

Recently, early cleavage fragments of the Aα chain of fibrinogen have been isolated and characterised; these fragments have been called variously Hi2-Ala and Hi2-Met (112, 155), fragment A (317, 320) and fragment H (137) and were related to the hydrophilic disulphide knot, Hi2-DSK (112, 155).

1.7.3 Degradation of fibrin by plasmin

In the case of non-crosslinked fibrin, plasmin degradation led to digestion products X, Y, D and E which were similar to those of fibrinogen (103, 274) except that fibrin fragments X, Y and E no longer possessed fibrinopeptides. However, crosslinking of fibrin by

Factor XIIIa altered the pattern of plasmin digestion in that one of the degradation products contained crosslinked polypeptide chains and the order of chain degradation appeared to be changed (103, 274). Although X-dimer and Y-dimer have not been observed during the course of digestion, examination of a terminal plasmin digest of highly crosslinked fibrin showed that the major components were fibrin fragment D-dimer and fragment E (85, 103, 274). The molecular weight of the D-dimer was estimated at 160,000 - 200,000 (85, 103, 182, 274) and was shown to consist of two α fragments (molecular weight 12,000 - 15,000), two β fragments (molecular weight 42,000 - 44,000) and a crosslinked dimer of a γ chain fragment (molecular weight 76,000 - 81,000) (85, 275).

Whereas the size of the γ chain fragment varied with the stage of monomeric fragment D degradation, only one type of γ subunit was observed in D-dimer, indicating that crosslinking of the γ chain of fibrin, as well as incorporation of a fluorescent substitute donor into the γ chain crosslink acceptor site, prevented its COOH-terminal degradation by plasmin (85, 274). In addition, incubation of Factor XIIIa with fragment D produced D-dimer, but electrophoretic analyses revealed that the crosslinking capacity was restricted to the earliest fragment D, indicating that the COOH-terminal crosslinking sites had remained intact only in the γ' chain of fragment D_1 (85, 100, 275). Therefore, while recent findings (59, 100, 319) have indicated that the initial plasmin attack on monomeric fragment D occurred at the NH_2-terminal end of the γ chain fragment, it seems likely that a simultaneous COOH-terminal cleavage at the crosslinking sites occurred on the same polypeptide.

In the case of the α chains of fibrin, neither crosslinking nor incorporation of fluorescent substitute donors appeared to influence

strongly the pattern of plasmin cleavage of these chains (85, 274). No satisfactory explanation has yet been given for the appearance from a totally crosslinked fibrin digest of fragments A or H (137, 274), even though these fragments possessed crosslinking acceptor sites (85, 96, 320). However, Pizzo et al.(274) have reported the occurrence of small crosslinked peptides of the α polymer after plasmin digestion.

A number of reports have suggested that crosslinking of the α chains endowed fibrin or its α polymers with an increased resistance to lysis by plasmin (103, 210, 274). However, these results could not be confirmed by others (243, 290).

1.7.4 D-E complexes of fibrinogen and fibrin

Difficulty has been encountered in separating fibrinogen fragments D and E by gel filtration in physiological buffers (46, 261) although fractionation was readily performed in acetic acid solution (113). Suggestions that fragments D and E formed a non-covalent complex (46, 106) were more fully investigated by Plow et al. (279, 280) who demonstrated a D:E complex, in a probable ratio of 1:1, in plasmin digests of fibrinogen. The bonding forces appeared to be hydrophobic and hydrogen bonding in nature.

An analagous non-covalent complex has also been demonstrated in plasmin digests of crosslinked fibrin (107, 158); immunological and electrophoretic investigations indicated the association of D-dimer with fragment E to form a D_2E complex.

1.7.5 Biological properties of the fibrin(ogen) degradation products

Fibrinogen and fibrin degradation products interfere with the thrombin-fibrinogen reaction in an inhibitory manner. Fragment D acts as an inhibitor of fibrin monomer polymerisation, probably as a result of competition for fibrin monomer polymerisation sites (190). The

activity of fragment D species in this respect is dependent on their structure and origin, since D-dimer from totally crosslinked fibrin exhibited a greater inhibitory effect on fibrin monomer polymerisation and a greater affinity for insolubilised fibrin monomer than fragment D from fibrinogen or non-crosslinked fibrin (73). The anticoagulant activity of fragment E appears to come from its competition with receptor sites on the fibrinogen molecule for the active enzyme centre of thrombin (190). The intermediate fragments X and Y are more potent anticoagulants than fragments D and E. Fragment Y is the most potent derivative (220), while the inhibiting effect of fragment X appears to be inversely related to its clottability (190). In vivo, the anticoagulant effects of these fragments may be of short duration, on account of their greatly decreased half-lives, compared with fibrinogen (190).

Anticoagulant activity has also been attributed to a decapeptide from a plasmin digest of bovine fibrinogen (325), while its synthetic equivalent possessed a bradykinin-like activity (324). Fragments D and E exhibited chemotactic activity for monocytes, and a lower molecular weight polypeptide from a plasmin digest of fibrinogen was chemotactic for neutrophils (171, 295).

1.7.6 Alternative schemes for plasmin degradation of fibrinogen

Besides the popular scheme of Marder et al. (222), by which plasmin degradation of fibrinogen ultimately yields two molecules of fragment D and one molecule of fragment E, two other schemes of degradation have been proposed. In the first, Mosesson et al. (244) have postulated that degradation of fibrinogen produced one molecule of fragment E and one molecule of fragment D, the latter fragment being a dimeric structure consisting of disulphide-bonded remnants of all six

polypeptide chains. This argument was based mainly on molecular weight values, identification of polypeptide chain components and NH_2-terminal amino acid analysis of fragment D species.

Crucial to the dimeric fragment D model was the experimental observation of the early plasmin release of a large β polypeptide fragment from the fibrinogen core (244). However, others (85) have shown that this fragment originated in the Aα chain. In addition, other studies (59, 85, 99), identifying the polypeptide chains of fragment D by electrophoresis, presence of carbohydrate, quantitative NH_2-terminal analysis and tryptic peptide mapping, have produced results different from those of Mosesson. The finding of D-dimer, and not polymeric D, on digestion of crosslinked fibrin, appeared to give exclusive support to Marder's molecular model (85), but Mosesson and Finlayson (242, 243) have refuted this claim by demonstrating that all crosslinked D fragments were ultimately degraded to species indistinguishable from those of non-crosslinked fibrin. However, the balance of arguments favour Marder's molecular scheme, particularly since amino acid sequence analysis has positively identified an important γ chain section in fragment D (59, 319).

Another degradation scheme for fibrinogen, based on quantitative immunochemical measurement of antigenic expression of D:E complexes and the semi-quantitative assessment of relative yields of fragments D and E in plasmin digests of fibrinogen, has been proposed (281). In this scheme, fibrinogen can give rise to two monomeric D and two monomeric E fragments.

1.7.7 Neoantigenic expression in fibrinogen degradation products

The emergence of neoantigenic expressions following plasmin cleavage of fibrinogen was described by Plow *et al.* (286). The

cleavage-specific neoantigens were localised in fragment D and were distinct from those native D antigens expressed in intact fibrinogen. Studies on the emergence of the D neoantigen indicated that it was present in the fibrinogen molecule at a sterically hindered site, but was exposed after proteolytic cleavage of fibrinogen (276). As in the case of fragment D, two distinct sets of antigenic expressions were also demonstrated for fragment E; one major set of antigens was expressed by the parent molecule, fibrinogen, and a minor set of cleavage-associated neoantigens, located on the γ chain fragment, which was not expressed by fibrinogen (282). While equimolar expression of the D neoantigen was found for fragments X, Y and D, but not for fragment E (277), the fragments X, Y and E revealed an increase in the quantitative expression of the E neoantigen, indicating that the sequential cleavage process led to progressive exposure of the neoantigenic site (77).

By the use of radioimmunoassay procedures with appropriate antisera, immunochemical differences between the fibrinogen and fibrin neoantigens have been demonstrated for each of the D and E neoantigens, thus illustrating conformational or structural differences imposed by the fibrinogen-to-fibrin transition, but which were independent of the presence of fibrinopeptides (51, 76, 77). In vivo studies have revealed the presence of increased levels of D neoantigens in patients with thrombotic disease (277) and after surgery (124). In addition, normal human sera was shown to contain humoral antibodies specific for a cleavage-associated neoantigenic site, located in fragment D (284). This antigenic determinant was similar to, but not identical with the D neoantigen described above. No antibody to fragment E was detected in human sera.

1.7.8 Effect of some other proteolytic enzymes on fibrin(ogen)

Besides plasmin, several other proteolytic enzymes have been employed during structural studies on fibrinogen and investigations into the nature of the plasmin-resistance of the terminal digest fragments D and E. Comparison of the initial sequence of chain degradation by plasmin with those of other serine proteases, trypsin, chymotrypsin and elastase, showed that, in all cases, the Aα and Bβ chains were degraded at the same time, but that the Aα chains were degraded more rapidly than the Bβ chains (257). Electrophoretic and immunological analyses of the later stages of non-crosslinked and crosslinked fibrin digestion by trypsin, chymotrypsin and brinase revealed the formation of D- and E- antigenic fragments with properties similar to those of plasmin-cleaved fragments (126); continued digestion by these enzymes destroyed all D-antigenic fragments and produced E fragments which were antigenically deficient. Studies of trypsin and plasmin digestion of bovine fibrinogen yielded similar results, whereby both enzymes degraded fragment D to lower molecular weight species (81).

Apart from their proteolytic action in removing fibrinopeptide A from fibrinogen, the snake venom enzymes, ancrod and reptilase, have been shown to cleave the Aα chains of non-crosslinked and crosslinked fibrin (75, 96, 230, 341), although some differences were reported by Pizzo *et al.* (273). In addition, Aα chains of fibrinogen were readily cleaved by a number of bacterial proteases, indicating substrate susceptibility rather than enzyme specificity, while further digestion caused Bβ and then γ degradation (257).

Among the enzymes considered as candidates for involvement in physiological fibrin(ogen)olysis are leucocyte proteases and thrombin. Observations on the digestion of fibrinogen by leucocyte proteases

indicated the presence in leucocyte granules of a separate fibrinolytic system (283), while thrombin has been implicated in the proteolysis of the ∝ chains of fibrin (234) and in the formation of FDP in serum (232). Furthermore, thrombin, on prolonged incubation with non-crosslinked and crosslinked fibrin, exhibited extensive fibrinolysis with the formation of fragments analogous to the fragments X, Y, D and E of plasmin digestion (170).

1.8 Fibrinogen in the circulation

1.8.1 Biosynthesis of fibrinogen

Almost all the fibrinogen is synthesised in liver hepatocytes, but little is known of the regulation of its synthesis or the processes of polypeptide assembly (292). Fragment D has been shown to induce an increase in fibrinogen biosynthesis in rabbits (39), and low molecular weight materials in human urine may be separated into a stimulator and an inhibitor of fibrinogen synthesis (292). Whereas Doolittle (68) has suggested that the three polypeptide chains of fibrinogen might be derived from a single long polypeptide chain after at least two cleavages, others (2, 40) have suggested that the three chains are synthesised separately.

1.8.2 Catabolism of fibrinogen

Despite a great number of investigations in man and animals, little is yet known about the sites of fibrinogen catabolism and considerable disagreement exists concerning the mechanism of such catabolism. It has been suggested that under physiological conditions there is a dynamic equilibrium between fibrin formation by the coagulation system and fibrin clearance by the fibrinolytic system (54). In favour of this theory is the rapid turnover of coagulation

factors compared with other plasma proteins and the half-life of fibrinogen has been estimated in normal individuals and in afibrinogenaemic subjects as 2-4 days (62, 117, 326), although later estimates have been higher, 4-5 days (94). However, the failure of heparin or ϵ-aminocaproic acid administration to influence the rate of decay of labelled fibrinogen has cast doubts on the theory of continuous *in vivo* coagulation and fibrinolysis (62). In a similar study in rabbits, Regoeczi (294) found no significant amounts of degradation products in the circulation and concluded that normal fibrinogen catabolism was probably an intracellular process. An alternative degradation pathway involving direct plasminolytic attack on fibrinogen has been proposed by Mossesson and coworkers (140, 240), but was criticised by Collen *et al.* (61), who, however, suggested that such a fibrinogenolytic scheme might be operative during physical exercise (60).

Experimental studies in rabbits have shown that labelled soluble fibrin was cleared from the circulation more rapidly than labelled fibrinogen (251). However, inhibition of the fibrinolytic system by aprotinin had no influence on the elimination characteristics of fibrinogen or soluble fibrin, demonstrating that the clearance of fibrin in this case (251) was not caused by activation of the fibrinolytic enzyme system. On the other hand, aprotinin delayed the elimination of fibrin deposits in the lungs of rats, compared with those rats which received no aprotinin (67).

In clinical studies, a shortened fibrinogen half-life has been reported in patients with microangiopathic haemolytic anaemia (8), haemophilia A (332) and maturity onset diabetes mellitus (10). Heparin was shown to correct the increased fibrinogen turnover in patients with polycythaemia (332), thrombocytosis (332) and venous thrombosis (139), but not in a patient with acute infectious hepatitis (256). Normal

catabolism of fibrinogen was reported in patients with arterial thrombembolism and arteriosclerosis (139).

1.9 Platelet, foetal and abnormal fibrinogens

1.9.1 Platelet fibrinogen

Fibrinogen associated with platelets has been differentiated into a membrane-bound fraction, which may simply be adsorbed plasma fibrinogen, and an intracellular fraction, located in the α-granules (48, 167). Isotope studies indicated that no exchange occurred between plasma fibrinogen and the intra-platelet component, platelet fibrinogen (48). Furthermore, platelet fibrinogen was shown to be intact in defibrinogenated dogs (169).

In order to establish the relationship between platelet and plasma fibrinogen, a number of comparative studies have been performed on these proteins. While both species were clottable, the sedimentation coefficient and intrinsic viscosity of platelet fibrinogen was lower than that of its plasma counterpart, indicating a lower molecular weight for the platelet protein (110). The presence of a smaller molecule of platelet fibrinogen was confirmed by SDS-polyacrylamide gel electrophoresis of unreduced fibrinogen samples (167) and of reduced fibrinogen samples (110, 169); in the latter studies, slightly smaller molecular weights were indicated for each of the three polypeptide chains components of platelet fibrinogen. The rate of plasmin degradation of platelet fibrinogen was found to be relatively slow, and indicated greater resistance to plasmin, especially since no inhibitors could be demonstrated (167). Minor differences in the electrophoretic patterns of digestion products were also observed (110, 167, 169).

Immunological comparisons between plasma and platelet fibrinogen have revealed antigenic identity on immunodiffusion (110), but different mobilities on agar immunoelectrophoresis (167) and differences in the antigenic expressions of whole fibrinogen, as well as those of the individual polypeptide chains (285). Since some of the immunological differences increased progressively on storage of the platelet lysates, intracellular proteolysis of platelet fibrinogen was suggested, but the nature of the alterations indicated that the responsible enzyme was not plasmin-like (285).

In a case of congenital dysfibrinogenaemia (Fibrinogen Metz, in which the Aα chain displayed an abnormal electrophoretic mobility), Soria et al. (312) have shown that the polypeptides of the corresponding platelet fibrinogen displayed electrophoretic mobilities resembling more closely those of normal platelet fibrinogen than those of plasma Fibrinogen Metz. On this basis, these authors concluded that the origin of platelet and plasma fibrinogen was different. However, Doolittle et al. (72) were unable to distinguish platelet and plasma fibrinogens by a variety of biochemical and functional criteria, and deduced that they were identical gene products.

1.9.2 Foetal fibrinogen

Prolonged clotting times of umbilical cord plasma, in the absence of FDP, has been taken as a possible indication of the presence of foetal fibrinogen (83). Analytical studies on purified cord fibrinogen have demonstrated the same amino acid composition (339), hexose content (338) and subunit chain structure (327) as for purified adult fibrinogen. However, different phosphorus contents (338) and different chromatographic behaviour (339) have also been reported. Agreement has not yet been reached on whether or not the prolonged

clotting times seen with cord plasma were also observed with purified cord fibrinogen; contradictory results have also been reported for tryptic peptide mapping of these two fibrinogens (327, 339). The most convincing evidence for the existence of a foetal form of fibrinogen has been the reduced rate of fibrin polymerisation (130, 327). Furthermore, polymerisation similarities have been demonstrated in cord monomers and fibrin monomers isolated from the abnormal Fibrinogen Paris I (130).

1.9.3 Acquired dysfibrinogenaemia

The occurrence of abnormal fibrin polymerisation in liver disease is more common than previously suspected and largely accounts for the prolonged thrombin times frequently observed in these patients (129). In 1969, the first case of an acquired abnormality of fibrin monomer polymerisation in a patient with primary hepatoma was described (84). The occurrence of inhibitors was excluded and the defect was partially corrected by reducing the ionic strength of the fibrin monomer solution or by the addition of calcium ions.

Prolonged thrombin, ancrod and reptilase clotting times were observed in patients with acute and chronic liver disease, in some cases the defects in the rate and extent of polymerisation correlating with the prolongation in clotting times (129, 189). SDS-polyacrylamide gel electrophoresis of fibrin from these patients indicated that the subunit chain structure, crosslinking ability and plasmin digestion products were the same as for normal fibrin (189). The plasma from one patient (189), as well as the hepatoma patient (84), was able to inhibit the clotting time of normal plasma.

In one case of liver cirrhosis with suspected congenital dysfibrinogenaemia (127), the abnormal fibrin polymerisation in plasma

was shown to be due to a specific polymerisation inhibitor.

1.9.4 Congenital dysfibrinogenaemia

To date, more than 40 cases of congenitally abnormal fibrinogens have been described, the inheritance following an autosomal pattern (94). In a considerable number of these cases, there was no associated clinical disorder and the abnormality was discovered during routine laboratory testing, whereas other patients suffered bleeding and/or thrombotic episodes. In the majority of patients, thrombin and reptilase clotting times were prolonged, and discrepant values were obtained for plasma fibrinogen levels by fibrin polymerisation time tests and immunological methods. In one family with recurrent venous thromboembolism, a shortened thrombin clotting time has been observed (79).

Since the abnormal fibrinogens have been shown to affect one or more of the three stages of the fibrinogen-to-fibrin conversion, they may be classified according to disorders of

(i) fibrinopeptide release,

(ii) polymerisation of fibrin monomers, and

(iii) crosslinking of the γ and α polypeptide chains.

More than half of the known abnormal fibrinogens exhibited delayed fibrin monomer polymerisation (94). Additional classification of dysfibrinogenaemias is possible, since the variants have demonstrated normal, anodal or cathodal mobilities (with respect to normal fibrinogen) on immunoelectrophoresis and many of them were able to inhibit the thrombin clotting time of normal plasma (94, 231). In a few cases, abnormal electrophoretic mobilities have been demonstrated for the individual polypeptide chains of fibrinogen; in other cases, the existence of two populations of fibrinogen molecules in plasma has been suggested (94).

Only in the case of Fibrinogen Detroit (26) has amino acid analysis demonstrated an altered structure in the fibrinogen molecule. Close to the thrombin-susceptible arginyl bond at position 16 in the Aα chain, a serine residue has replaced the normal arginine-19. It has been suggested that, after the release of fibrinopeptide A (normal release in Fibrinogen Detroit), this amino acid substitution prevented the conformational rearrangement and unfolding of the polymerisation site in the N-DSK region, which was necessary for the release of fibrinopeptide B (no release in Fibrinogen Detroit) (26, 94).

CHAPTER 2: PREPARATION OF FIBRINOGEN AND ITS POLYPEPTIDE CHAINS

2.1 Introduction

Since immunological techniques were used in this study of fibrinogen and its subunits, materials of the highest purity were required and the preparations are described in this chapter.

2.1.1 Preparation of human fibrinogen

Methods for the purification of plasma fibrinogen have been available from the end of the last century. The earliest procedures consisted of precipitating fibrinogen from plasma with high concentrations of neutral salts and were further improved by the prior adsorption of prothrombin on insoluble calcium and barium salts (25). The low solubility of fibrinogen at reduced temperatures has been employed to prepare fibrinogen by freezing it out of plasma (25) and ammonium sulphate solutions have also been used for precipitating this protein from plasma (6, 25).

Fractionation schemes for the preparation of fibrinogen using organic solvents have been devised, the operations being conducted at low temperatures to avoid denaturation of the products. The method of Kekwick et al. (174) used diethyl ether, while Cohn et al. (58) precipitated fibrinogen with ethanol solutions to give fraction I. This fraction has been further purified by Blombäck and Blombäck (25) to fraction I-4 with a clottability of 98-100%, using ethanol-glycine solutions. Others have employed the amino acids, glycine (173) and β-alanine (315), to precipitate fibrinogen directly from plasma. Recent observations (145) that fibrinogen may be adsorbed on fibrin-agarose columns have led to an affinity chromatography method for isolating plasma fibrinogen (61). Plasminogen, an important contaminant of many fibrinogen preparations, may be removed by affinity

chromatography with lysine-agarose (226).

For this study, the method of choice for the preparation of human fibrinogen was that of Blombäck and Blombäck (25), since their fraction I-4 has been accepted as the fraction most representative of native fibrinogen (240). However, it must be borne in mind that this preparation of fibrinogen is considerably contaminated with plasminogen and the proactivator of plasminogen (25), as well as with factor XIII (209).

2.1.2 Preparation of the polypeptide chains of fibrinogen

A number of reagents are known which are able to break or reduce the disulphide bonds holding together the polypeptide chains of a protein molecule such as fibrinogen. The isolated chains of fibrinogen or fibrin were first obtained after treatment of the parent molecule with sodium sulphite, yielding the three S-sulpho derivative chains, Aα, Bβ and γ (56, 143, 146, 148, 212). However, it has been reported that sulphitolysis of fibrinogen may result in incomplete cleavage of the fibrinogen chains or re-oxidation of the formed sulphydryl groups to form polymeric chains (118). The latter problem may be avoided by the careful use of a sulphydryl blocking agent, such as iodoacetic acid (156), as in the studies of Henschen (147), Henschen and Edman (153) and Murano et al. (255). As one of these studies (255) employed a solid, and therefore convenient, reducing agent (dithiothreitol) (57), that method for the preparation and isolation of fibrinogen chains was followed in the present investigation.

Recently, the use of ethylenimine as a sulphydryl blocking group during the isolation of the chains of fibrinogen has been described (118). This reagent appeared to have a greater selectivity for -SH groups than iodoacetic acid and reduced the dangers of inter-

ference from the side reactions associated with this reagent (156).

2.2 Materials and methods

2.2.1 Human fibrinogen

Fibrinogen with clottabilities ranging from 80-98% was prepared on several occasions from

(a) frozen, aged plasma, and

(b) fresh blood from single donors.

In addition, Kabi fibrinogen (Grade L, AB Kabi, Sweden) was also further purified.

Plasma fractionation at low temperatures was performed in an Ultra-Kryomat (Messgeräte-Werk Lauda) and pH was measured on a Model 290 pH Meter (Pye Unicam).

2.2.1.1 Cohn fraction I

When the starting material was blood, fraction I was prepared according to Cohn *et al.* (58). 6-7 volumes of blood were collected into a sterile bag containing 1 volume of acid-citrate-dextrose anticoagulant and centrifuged for 45 min at 10°C (1000g). The plasma was removed and spun again. The plasma was then cooled to 0°C with stirring and the pH brought to 7.0 with 0.8M sodium acetate buffer (pH 4.0). Chilled 53.3% v/v aqueous solution of ethanol was added slowly through a peristaltic pump to the plasma, while stirring and reducing the temperature to -3°C, until the final ethanol concentration was 8% v/v. After the suspension had been stirred for 1 hour, fraction I was obtained by centrifugation for 20 min at -3°C (1000g). Cohn fraction I, 60% of which is fibrinogen (58), was further purified by the method of Blombäck and Blombäck (25) with slight modifications (37). The method consisted of several stages.

2.2.1.2 Fraction I-0

In the first stage, the majority of contaminating prothrombin and other plasma components of low solubility were removed by extraction to yield fraction I-0. Use was made of the finding that glycine at concentrations greater than 0.5M has a salting-out effect on fibrinogen, but a large salting-in effect on the contaminating proteins (25). Therefore, the extraction was performed with the buffer, 1M glycine/6.5% v/v ethanol/0.055M trisodium citrate (pH 6.8). The buffer was added slowly to 1/4 of the original plasma volume with stirring to achieve a fine suspension of fraction I at -3°C. After stirring for 1 hour, the suspension was centrifuged for 10 min at -3°C (1000g) and the supernatant discarded. The extraction and centrifugation were repeated once more.

2.2.1.3 Fraction I-2

Fraction I-0 was dissolved in 1/8 original plasma volume of 0.055M citrate buffer (pH 6.8) at 30°C and protein and clottable fibrinogen assays were performed. The solution was diluted to 0.75% w/v protein with the same buffer and cooled to 0°C. Chilled 0.45M glycine (twice the volume of the protein solution) was added, followed by the dropwise addition of 10% v/v ethanol to a final concentration of 0.5% v/v. After stirring for ½-1 hour at 0°C, the precipitate, fraction I-1 (cold-insoluble globulins), was removed by centrifugation for 20 min at 0°C (1000g). (The final precipitation conditions were: temperature 0°C, ethanol 0.5% v/v, glycine 0.29M, protein 0.24% w/v, pH 6.8 and ionic strength 0.1.) Fibrinogen fraction I-2 was obtained by adding sufficient chilled 53.3% v/v ethanol to the supernatant to bring the concentration to 6.5% v/v, while reducing the temperature from 0°C to -4°C. After stirring for 1 hour, fraction I-2 was obtained by centrifuging for 20 min at -4°C (1000g).

2.2.1.4 Fraction I-4

Fraction I-2 was dissolved in 1/10 original plasma volume of

0.055M citrate buffer (pH 6.35) at 30°C and protein and clottable fibrinogen assays were performed. The solution was diluted to 0.7% w/v protein with same buffer and cooled to 0°C. Chilled 0.75M glycine solution (twice the volume of the protein solution) was added, followed by the dropwise addition of 1/10 volume (of glycine-protein solution) of 0.5M glycine, containing 8.25% v/v ethanol. Fraction I-3 (cold-insoluble globulins) formed after stirring for at least 2 hours and was removed by centrifugation for 30 min at 0°C (1000g). (The final precipitation conditions were: temperature 0°C, ethanol 0.75% v/v, glycine 0.5M, protein 0.21% w/v, pH 6.5 and ionic strength 0.09.) Fibrinogen fraction I-4 was obtained, as for fraction I-2, by precipitation at 6.5% v/v ethanol concentration at -4°C. Protein and clottable fibrinogen assays were performed on fraction I-4, dissolved in citrate buffer, pH 6.35.

Solutions of fibrinogen fraction I-4 were dialysed overnight at 4°C against regular changes of distilled water and freeze-dried, a process which has been shown to have little effect on fibrinogen clottability (20, 174).

The purification scheme for Kabi fibrinogen was the same as for plasma, except that, as starting material, it was regarded as fraction I-0 if its stated minimum clottability was 90%, and fraction I-2 if its stated minimum clottability was 95%.

The purity of the fibrinogen fractions were assessed by clottability determinations, immunodiffusion, immunoelectrophoresis, and polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulphate.

2.2.1.5 Estimation of fibrinogen

Protein concentration and clottability of purified fibrinogen fractions were performed according to the syneresis method of Blombäck

and Blombäck (25), except that the fibrin clots were collected on glass cloth instead of silk cloth.

The accuracy of this method had been confirmed by the comparative performance of a micro-biuret assay (164) on solutions of fibrinogen.

2.2.2 Reduction and S-carboxymethylation of fibrinogen

The method used was that of Murano et al. (255) and a typical experiment is described. 184 mg of fibrinogen fraction I-4 was dissolved in 9 ml of 8.4M guanidinium chloride (pH 8.5) and the pH was readjusted to 8.5 with 1M NaOH. The solution was incubated at 40°C for 1 hour. Using 0.82 μmoles dithiothreitol (DTT)/mg fibrinogen, reduction was performed by adding 0.5 ml of 47 mg/ml of DTT (0.15 mmole) to the fibrinogen solution, and the pH was adjusted to 8.8. The bottle was flushed with nitrogen, sealed and incubated at room temperature for 1½ hours.

Carboxymethylation of the reduced fibrinogen was carried out by adding sufficient 1M iodoacetic acid (recrystallised five times from hot carbon tetrachloride (255)) to provide a 5 molar excess over DTT. Thus, 140 mg iodoacetic acid (0.75 mmole), dissolved in 0.8 ml of 0.1M NaOH, were added to the reaction bottle, which had been protected from light by wrapping in aluminium foil. The pH fell, but was held at pH 8.3 by titration with 0.5M NaOH for a reaction time of 15 min. The reaction was stopped by adding 1.1 ml glacial acetic acid to produce a final concentration of 10% v/v.

In the early alkylation experiments, the carboxymethylated chains were desalted by gel filtration of the reaction mixture on a column (6.3 x 45 cm) of Sephadex G-25. The column was wrapped in aluminium foil to prevent generation by light of iodine from iodide. The eluent

was 10% v/v acetic acid and the flow rate was 190 ml/hr. Elution of the protein was monitored by absorbance at 280 nm, using a LKB Uvicord II photometer and LKB Chopper Bar Recorder. The separated protein peak was collected by a LKB UltroRac fraction collector and freeze-dried.

In the later alkylation experiments, the carboxymethylated chains were dialysed in an amber-coloured bottle at 4°C against regular changes of distilled water, before freeze-drying.

2.2.3 Separation of the carboxymethylated chains of fibrinogen

2.2.3.1 Method 1 (255)

Separation of the chains of fibrinogen was achieved by means of the cation exchanger, Whatman CM 52, packed in glass columns, 12 x 3.8 cm or 24 x 1.5 cm. Before use, the resin was precycled in alkali and acid, and equilibrated in the starting buffer, 0.025M sodium acetate/8M urea (pH 4.80), both before and after packing in the column. Using a flow rate of 10 ml/hr, about 100 mg of the carboxymethylated (CM) fibrinogen were dissolved in a few millilitres of the starting buffer and applied to the top of the column. The chains of fibrinogen were sequentially eluted from the resin by the application of a combined pH and ionic strength gradient, which was formed by a multi-chamber gradient elution device (the Autograd, Technicon Corporation). Chambers 1, 2 and 3 contained 400 ml of the starting buffer and chambers 4, 5 and 6 contained 400 ml of the final buffer, 0.175M sodium acetate/8M urea (pH 5.30). Both buffers were prepared freshly. Elution of protein was monitored at 280 nm by the LKB Uvicord II and fractions were collected every 20-30 min.

Protein peaks were pooled, dialysed against distilled water for 2 days at 4°C and freeze-dried. The identity of the chains was checked by their relative positions of elution from the column, according to

Murano et al. (255), by their mobility on polyacrylamide gel electrophoresis, and by their molecular weights, determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.

2.2.3.2 Method 2 (253)

The alkylated chains of fibrinogen were separated using the same gradient buffer system and ion-exchange resin as in Method I. However, use was made of a LKB Ultragrad Gradient Mixer in conjunction with an optical density level-sensing device, which provided an automatically-regulated gradient, and the eluted proteins were monitored by the LKB Uvicord III photometer at 206 nm and 280 nm. Using a concave-shaped gradient, a complete separation of the chains was achieved.

2.2.4 Solubility studies of the carboxymethylated chains of fibrinogen

Since dissolution of the polypeptide chains of fibrinogen in a biological buffer was essential for an immunological study of these chains, their solubility in various buffers was examined. Generally, the dissolution of the chains in the buffer was aided by the use of concentrated (8M) urea solutions, which was later removed to different degrees by dialysis of the polypeptide-urea solutions. Solubility of the chains in buffers was determined by

(i) visual inspection of the protein solutions,

(ii) photometric measurement at 280 nm of soluble protein after centrifugation of the solution, and on some occasions,

(iii) polyacrylamide gel electrophoresis of the supernatant solutions and precipitated materials.

2.2.5 Gel filtration studies of the carboxymethylated chains of fibrinogen

The void volumes, Vo, of 2 columns (27 x 1.5 cm) of Sephadex

G-200 were determined from the volumes of elution of Blue Dextran solutions. One column was eluted with 0.06M barbitone buffer (pH 8.6) and the other with 0.06M barbitone buffer (pH 8.6), containing 0.5% w/v sodium dodecyl sulphate. Elution volumes, Ve, for CM-fibrinogen, dissolved in the corresponding buffers, were determined, and the ratio, Ve/Vo, calculated for each column.

2.2.6 Electrophoresis

2.2.6.1 Analytical polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out in a Quickfit P.A.G.E. apparatus, using 5% v/v acetic acid/2M urea buffer system (42). 7.5% w/v acrylamide gels were used, the current was 3 mA/gel and the time of electrophoresis was 70 min.

2.2.6.2 Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS, was performed at 7 mA/gel in a Quickfit P.A.G.E. apparatus, according to McDonagh *et al.* (208), except that urea was used in the gels at a final concentration of 1.25M in order to increase the gel density and facilitate overlayering of buffer. Standard curves for molecular weight determinations were each obtained for 5, 7 and 10% w/v acrylamide gel concentrations (Fig. 3), using the single polypeptide chain proteins (336), listed in Table I. Relative electrophoretic mobilities of the standards and unknowns were obtained by electrophoresis of a mixture of the sample and pepsin on the same gel (74), thus avoiding small gel-to-gel variations. The anomalous electrophoretic behaviour of ribonuclease on SDS-polyacrylamide gels and the standard curve inflections when the protein molecular weights fall below a critical size (Fig. 3) has been described elsewhere (74). Furthermore, sigmoidal curves for standard proteins have been described

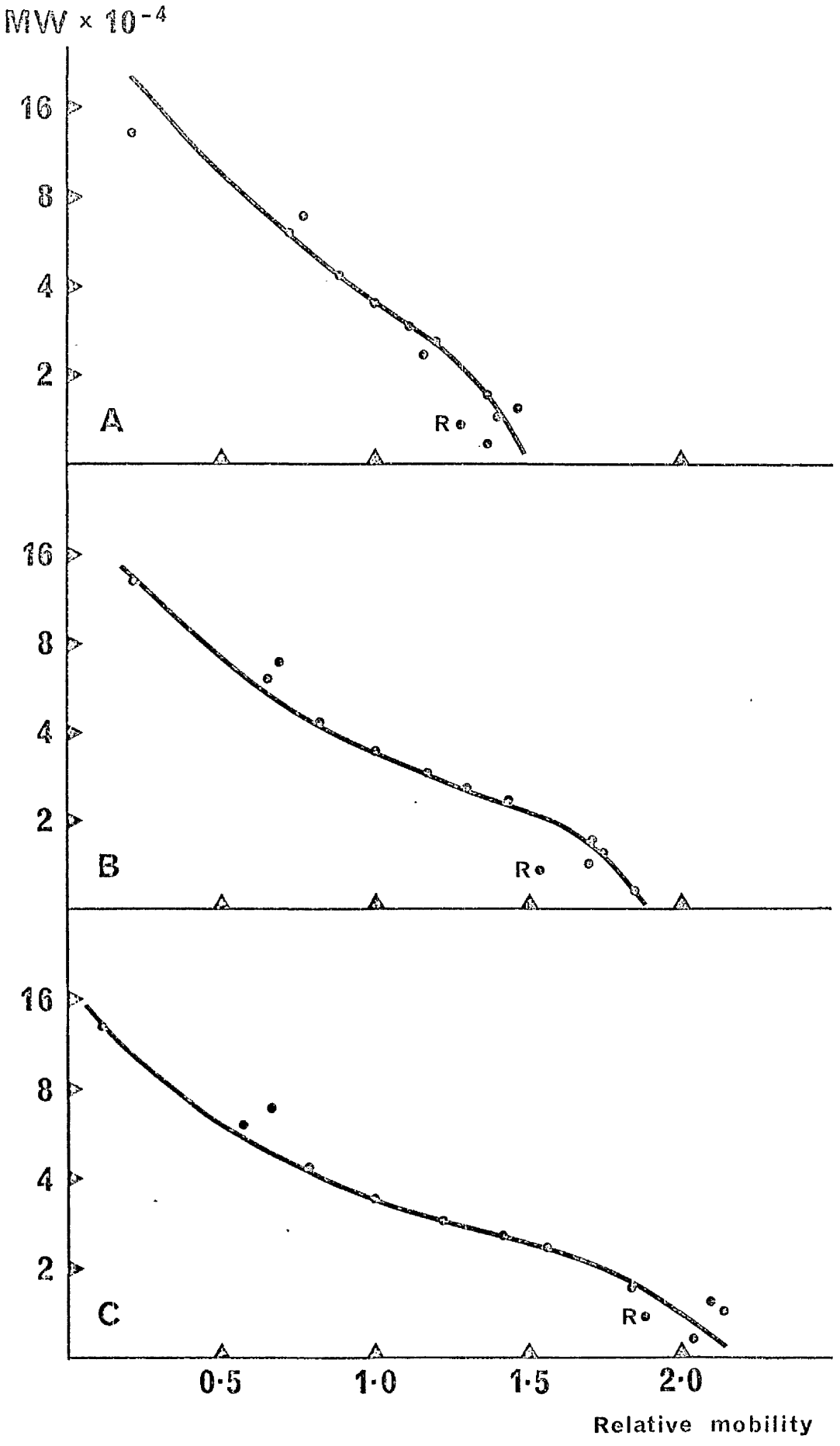

MW x 10⁻⁴
16
8
4
2
A
R
16
8
4
2
B
R
16
8
4
2
C
R
0·5
1·0
1·5
2·0
Relative mobility

Fig. 3 Plot of molecular weight of standard proteins in Table I against relative mobility on: A. 5%, B. 7% and C. 10% w/v acrylamide gels in sodium dodecyl sulphate. The anomalous electrophoretic mobility of ribonuclease (R) is indicated.

Table I

Molecular weights (MW) of protein standards and relative mobilities used in SDS-polyacrylamide gel electrophoresis

Protein[1]	MW of polypeptide chain[2]	Relative mobility[3] on:		
		5%	7%	10% gels
Cytochrome C	11,700	1.37	1.85	2.05
Ribonuclease	13,700	1.28	1.54	1.88
Lysozyme	14,300	1.40	1.70	2.14
Haemoglobin	15,500	1.47	1.75	2.10
Myoglobin	17,200	1.37	1.71	1.79
Trypsin	23,300	1.16	1.44	1.56
Chymotrypsinogen A	25,700	1.20	1.30	1.42
Carbonic anhydrase[4]	29,000	1.11	1.17	1.22
Pepsin	35,000	1.00	1.00	1.00
Egg albumin	43,000	0.88	0.82	0.78
Catalase	60,000	0.72	0.65	0.57
Albumin	68,000	0.77	0.69	0.67
β-Galactosidase[4]	130,000	0.21	0.22	0.11

[1] All proteins were obtained from B.D.H., except cytochrome C and β-galactosidase (Calbiochem) and pepsin (Sigma).

[2] Taken from Weber and Osborn (336).

[3] Ratio of distances moved by standard and pepsin.

[4] Although these molecular weights were used, the species of origin of β-galactosidase (Aspergillus) and carbonic anhydrase (bovine erythrocytes) differed from Ref. 336.

for continuous (95) and discontinuous (259) polyacrylamide gel systems. Although Weber and Osborn (336) achieved linearity when the protein mobilities were plotted against the logarithms of molecular weight over the range 10,000-70,000, they demonstrated that the hyperbolic curve obtained with higher molecular weight standards yielded molecular weights of a similar accuracy to that obtained from the standard straight line.

2.2.6.3 Other electrophoretic techniques

Preparative polyacrylamide gel electrophoresis was performed on the Prep. P.A.G.E. apparatus (Quickfit) and on a thick rectangular polyacrylamide slab, using the same buffer and gel compositions as employed by Brummel and Montgomery (42). In some cases, a stacking gel of 3.5% w/v acrylamide concentration was employed.

In order to achieve electrophoretic separation of the alkylated chains of fibrinogen in more physiological buffers than those described above, the following systems were also examined.

(1) Analytical polyacrylamide gel electrophoresis was performed, using the barbitone, tris and bicarbonate buffers described in the Results Section (2.3.4.2).

(2) Electrophoresis was performed on a preformed polyacrylamide gradient gel (4-26%) (Gradipore, Universal Scientific Limited), using tris/EDTA/boric acid buffer (pH 9.2).

(3) Electrophoresis on cellulose acetate (Cellogel, Reeve Angel Scientific Ltd.) was performed, using barbitone buffer, pH 8.6, ionic strength 0.05.

2.2.7 Immunodiffusion

Using a Gelman Immunodiffusion kit, immunodiffusion was performed essentially as described (268).

2.2.8 Protein concentrations

When protein solutions were reconstituted from freeze-dried products, the concentrations were calculated from the weights of material used. Except in the case of Kabi fibrinogen, no account was taken of the small ash and moisture content (20). Where pure protein preparations had not been freeze-dried, the concentrations of the solutions were calculated from the appropriate extinction coefficients at 280 nm (222).

2.2.9 Reagents

Bovine thrombin was obtained from Parke-Davis or Leo Pharmaceuticals, CM 52 cellulose from W. & R. Balston, Ltd., Blue Dextran and Sephadex products from Pharmacia, and antisera to human serum, albumin, antithrombin III, factor VIII, plasminogen and fibrinogen from Hoechst Pharmaceuticals.

All other reagents were supplied by B.D.H. and were of analytical grade if possible.

2.3 Results

2.3.1 Preparation of human fibrinogen

11 batches of human fibrinogen were prepared and the range of clottabilities obtained at different stages of purification are shown in Table II. Only those preparations with clottabilities greater than 95% were used for further preparative and analytical procedures.

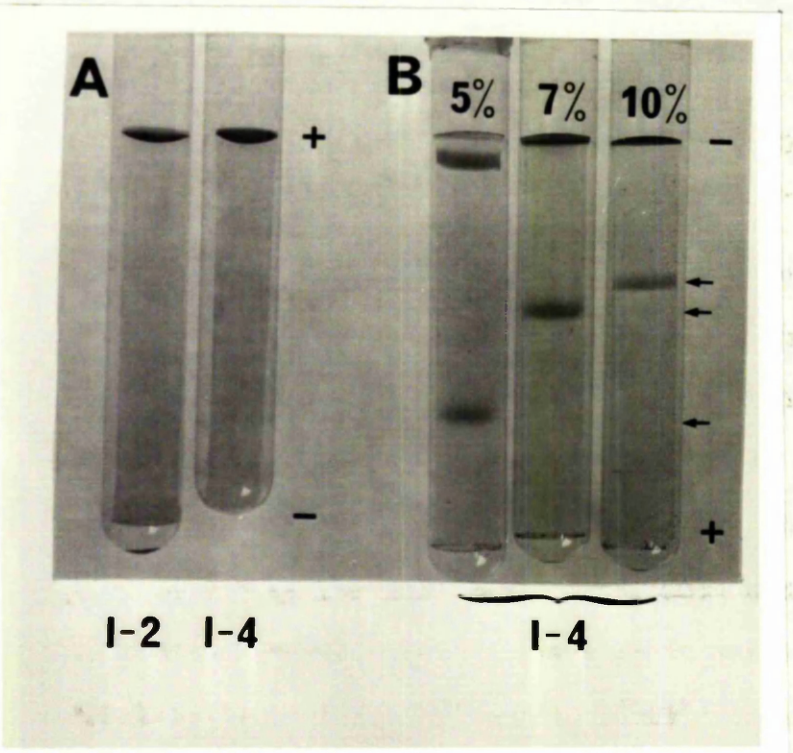


Fig. 4 A. Electrophoresis of fibrinogen fractions I-2 (23 μg) and I-4 (35 μg) on 7.5% w/v acrylamide gels at acidic pH. B. Electrophoresis of fibrinogen fraction I-4 (10 μg) on 5%, 7% and 10% w/v acrylamide gels with SDS. The fibrinogen bands are situated near the tops of the gels and the lower bands (arrowed) are due to the molecular weight standard, pepsin.

Table II

Clottabilities of fibrinogen fractions

Fraction	I-0	I-2	I-4
Clottability	83-96%	88-100%	81-99%

The purity of the fibrinogen preparations were assessed by polyacrylamide gel electrophoresis. At acidic pH (Fig. 4A), fractions I-2 and I-4 appeared as a single band which barely entered the gel, although a faint band of higher mobility was occasionally seen in the I-2 preparation. Fig. 4B shows the single band, except for pepsin, obtained when fraction I-4 was examined by SDS-polyacrylamide gel electrophoresis at 5, 7 and 10% acrylamide concentrations.

Immunodiffusion of fibrinogen fraction I-4 did not reveal a reaction with antiserum to human serum, nor with specific antisera to albumin, antithrombin III, factor VIII or plasminogen, but only with anti-fibrinogen antiserum.

2.3.2 Chromatographic preparation of the carboxymethylated chains of fibrinogen

2.3.2.1 Method 1

Fig. 5A shows the elution profile of the alkylated chains of fibrinogen from the ion-exchange column as the pH increased from 4.8 to 5.3 and the ionic strength increased from 0.025 to 0.175. Three incompletely separated protein peaks were observed, which were pooled as indicated. Material from each of the 6 fractions was lyophilised and subjected to polyacrylamide gel electrophoresis at acidic pH. Comparison with the electrophoretic pattern of carboxymethylated (CM) fibrinogen permitted identification of the major

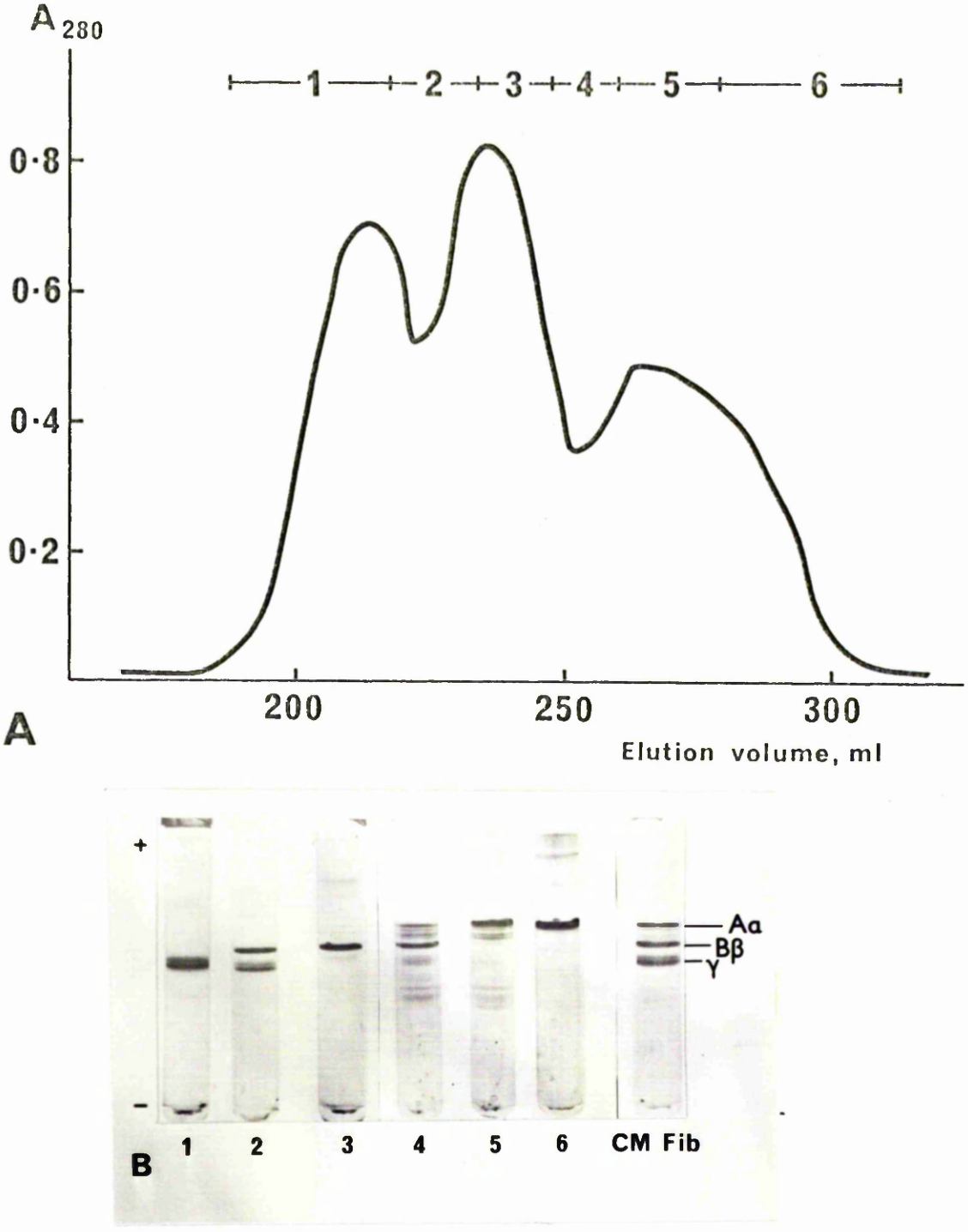
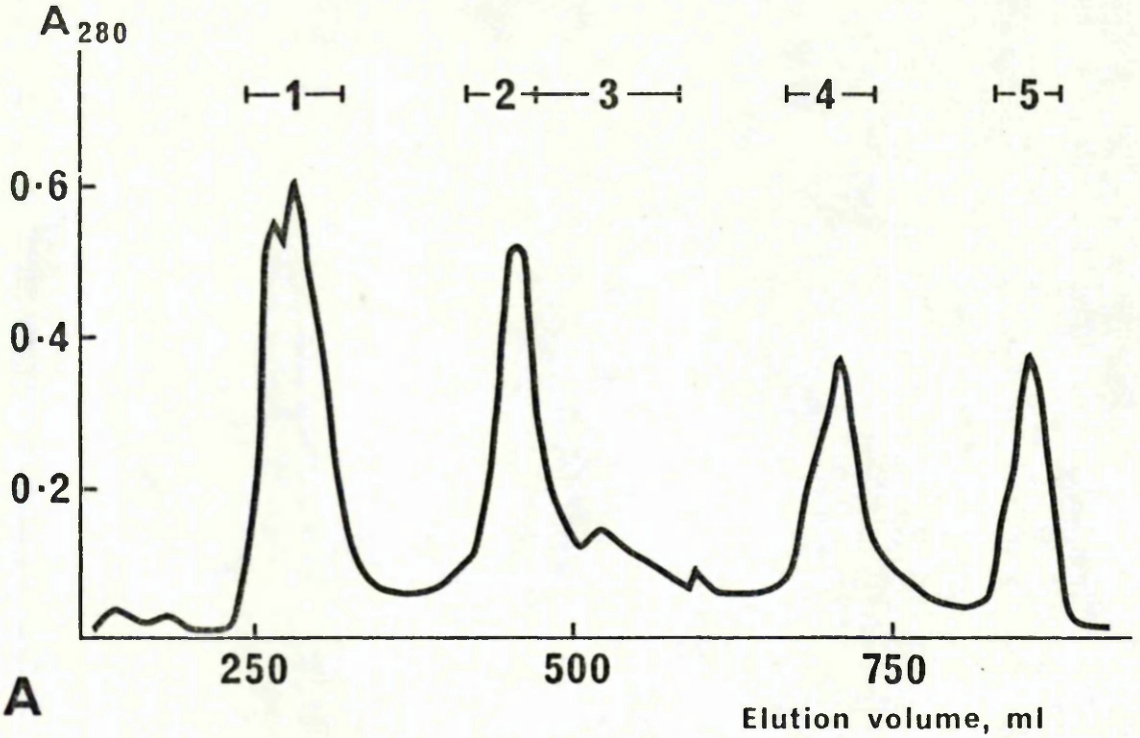


Fig. 5 A. Elution profile of the carboxymethylated chains of fibrinogen on ion-exchange chromatography on CM 52 cellulose. The optical density was measured at 280 nm and the pH and ionic strength gradient was formed by the Technicon Autograd.

B. Electrophoresis of the pooled fractions (numbered in A) on 7.5% w/v acrylamide gels at acidic pH. As reference, the Aα, Bβ and γ bands of unfractionated carboxymethylated fibrinogen (CM Fib) are indicated. Approximately 25 μg of fractions 1-6 and 45 μg of CM Fib were applied to the gel rods.



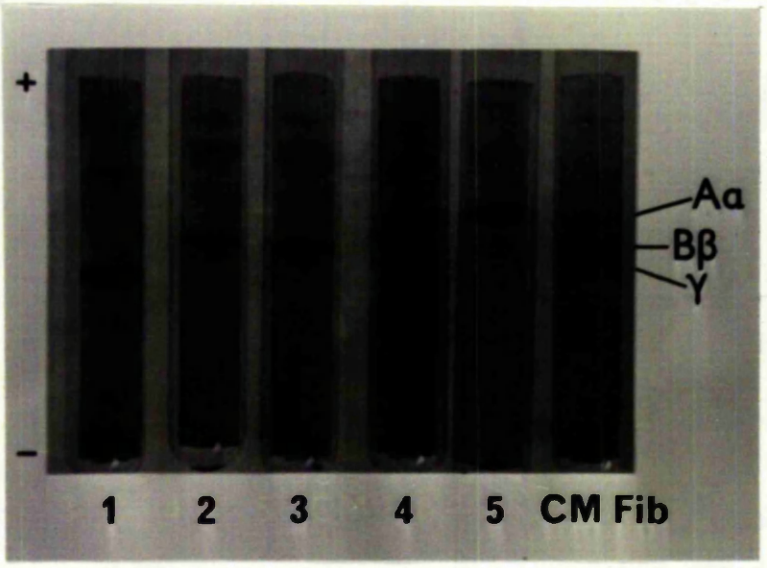


Fig. 6 A. Elution profile of carboxymethylated fibrinogen on ion-exchange chromatography on CM 52 cellulose. The optical density was measured at 206 nm (not shown) and 280 nm, and the pH and ionic strength gradient was formed by the LKB Ultragrad.

B. Electrophoresis of the pooled fractions (numbered in A) on 7.5% w/v acrylamide gels at acidic pH. As reference, Aα, Bβ and γ bands of unfractionated carboxymethylated fibrinogen (CM Fib) are indicated. Approximately 25 μg of fractions 1-5 and 60 μg of CM Fib were applied to the gel rods.

polypeptide species in each protein peak (Fig. 5A and B) and the alkylated chains were found to elute in the order γ, Bβ and Aα. Virtually pure γ chain was found in fraction 1, Bβ in fraction 3 and Aα in fraction 6. Fraction 5, the major part of the 3rd peak, revealed a band corresponding to the Aα chain, as well as a series of bands with mobilities greater than that of the Aα chain.

2.3.2.2. Method 2

Using an automatically-regulated buffer gradient, 4 separated major protein peaks were observed (Fig. 6A) and were identified as γ chain (fraction 1), Bβ chain (fraction 2), a series of bands with mobilities $\geqslant$ Aα (fraction 4) and Aα chain (fraction 5) by polyacrylamide gel electrophoresis at acidic pH (Fig. 6B). Small peaks eluting before the γ chain contained material of low electrophoretic mobility and were not further investigated. Fraction 3 generally appeared as a trailing shoulder of fraction 2 (Bβ chain), but both fractions yielded identical electrophoretic band patterns. Although the chains appeared to be separated from each other, electrophoresis showed other minor bands (usually of lower electrophoretic mobility) besides the main band.

2.3.3 Preparative polyacrylamide gel electrophoresis of the carboxymethylated chains of fibrinogen

Several attempts to obtain high purity carboxymethylated fibrinogen chains by electrophoretic separation on a 4 cm diameter cylindrical column of polyacrylamide or on a 1 cm thick polyacrylamide slab were unsuccessful. The system of Brummel and Montgomery (42), which was successful on an analytical scale, was used. Stained slices of gel revealed large amounts of protein which had not entered the gel but had precipitated on top of the gel, possibly due to heat

denaturation.

2.3.4 Some physical properties of the carboxymethylated chains of fibrinogen

2.3.4.1 Solubility

CM-fibrinogen, the mixture of alkylated fibrinogen chains, was found to be soluble in the following buffers: 0.03 and 0.06M barbitone (pH 8.6), 0.2M ammonium bicarbonate/triethylamine (pH 8.5), 0.15M tris/HCl (pH 8.5) and 0.05M tris/0.4M glycine (pH 8.3). Polyacrylamide gel electrophoresis of the barbitone and tris/HCl solutions of CM-fibrinogen, after centrifugation, revealed the presence of Aα, Bβ and γ chains and confirmed their solubility. Further photometric examination of the barbitone solution revealed that the fibrinogen chains remained soluble in this solution over the temperature range -20°C to 37°C, for at least 5 days.

The fibrinogen chains were found to be insoluble in: 0.05-0.5M phosphate buffer (pH 7.4), 0.1M Sorensen's phosphate buffers and phosphate-buffered saline (pH 7.0-8.0), and Owren's 0.1M barbitone/0.13M NaCl buffer (pH 7.3), although low concentrations of urea (0.25M) maintained the fibrinogen chains in solution of some of these buffers.

2.3.4.2 Electrophoretic separation

(1) Polyacrylamide gel electrophoresis in 7.5% w/v acrylamide gels, using 0.03M barbitone buffer (pH 8.6) gave only a poor separation of the alkylated polypeptide chains of CM-fibrinogen, but was considerably improved by the inclusion of 2M or 3M urea and further improved by reduction of the acrylamide concentration to 4.5% w/v. However, besides the 3 main bands of CM-fibrinogen, slower-moving bands were usually seen, even with the isolated chain preparations, and may have been due to non-covalently associated multimers of the

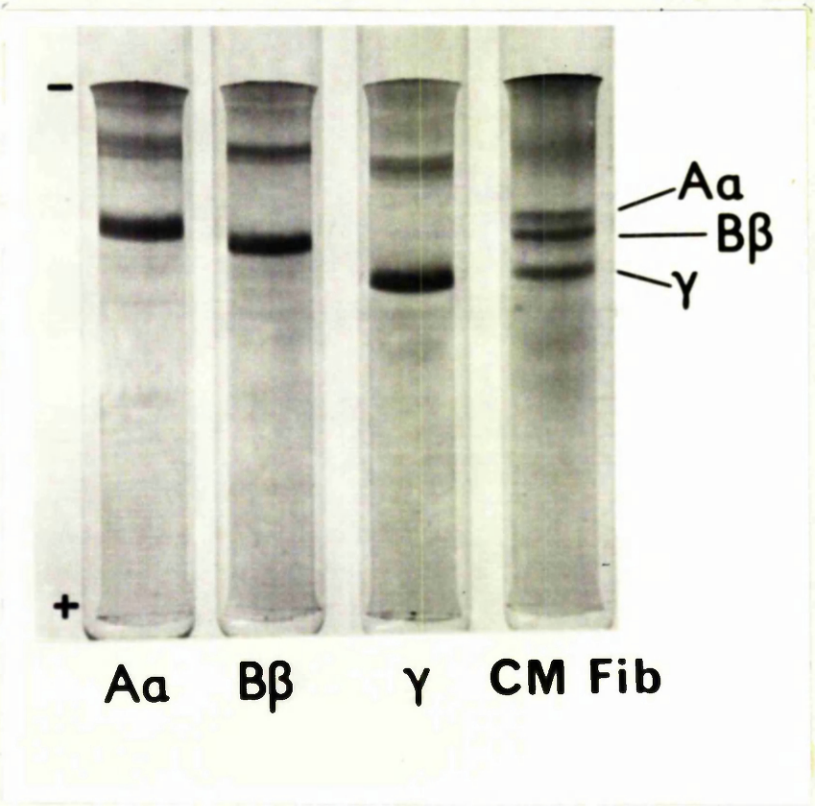


Fig. 7 SDS-polyacrylamide electrophoresis (7% w/v gels) of carboxymethylated Aα chains (12 μg), Bβ chains (10 μg) γ chains (14 μg), with carboxymethylated fibrinogen (CM Fib) (15 μg) as reference.

Is Fig 7 SDS PA on

chains. These low-mobility bands were also observed in a 4.5% w/v gel system, using 0.08M tris/EDTA/borate buffer (pH 9.2) with 8M urea, which gave a good separation of the monomeric fibrinogen chains, otherwise. However, no electrophoretic separation was obtained when the urea concentration is this system was reduced to 2M, nor with 7.5% w/v gels using 0.2M ammonium bicarbonate buffer (pH 8.8) or 0.15M tris buffer (pH 8.5).

(2) Using a Gradipore gradient gel (4-26%), poor separation of the chains of CM-fibrinogen was obtained after a 17 hour electrophoretic run. Staining of the gel revealed only "streaks" of protein.

(3) No separation of the chains of CM-fibrinogen was achieved by electrophoresis on cellulose acetate (Cellogel), using 0.06M barbitone buffer (pH 8.6) with and without 2M urea.

2.3.4.3 Gel filtration

On columns of Sephadex G-200, the elution volume/void volume ratio, Ve/Vo, for CM-fibrinogen was 1.2 in barbitone buffer (pH 8.6), containing 0.5% w/v SDS. In barbitone buffer alone, Ve/Vo = 1.0, indicating the presence of large molecular weight aggregates of alkylated chains in this buffer, but not in the presence of the dis-aggregating agent, SDS.

2.3.4.4 Molecular weights

Fig. 7 shows that SDS-polyacrylamide gel electrophoresis clearly resolved the major polypeptides of CM-fibrinogen and confirmed the purity of the isolated, carboxymethylated $A\alpha$, $B\beta$ and γ chains of fibrinogen, as also observed by polyacrylamide gel electrophoresis at acidic pH in Figs. 5B and 6B. However, fainter bands of twice the molecular weight of the individual chains were also observed on each gel and were probably due to dimeric aggregates of the alkylated chains.

The molecular weights of the individual chains were found to

be: Aα, 67,000; Bβ, 56,000; γ, 47,000.

2.4 Discussion

While the purification procedure of Blombäck and Blombäck (25) is rather lengthy, the resulting fibrinogen appeared to be pure. This fraction, I-4, has been acknowledged as the fraction which is most representative of native fibrinogen (240). The fibrinogen preparations used in these studies had high clottabilities (95-99%) and did not precipitate with antisera to human serum and selected serum proteins on immunodiffusion. Polyacrylamide gel electrophoresis at acidic pH and in the presence of SDS revealed only a single protein band. Furthermore, the subunit structure of the molecule appeared to be intact, as shown by the electrophoretic patterns of the 3 chains in CM-fibrinogen (Figs. 5B, 6B and 7).

The reduction and carboxymethylation of fibrinogen was performed exactly according to Murano et al. (255) in order to avoid the unwanted side reactions between iodoacetic acid and methionine, histidine, lysine and tyrosine residues, as outlined by Hirs (156). The separation of the alkylated Aα, Bβ and γ chains by ion-exchange chromatography using a continuous pH and ionic strength gradient was successful, as judged by the chromatographic profile and electrophoresis of the separated fragments. However, the yields of the 3 chains were improved considerably by the use of the fully-automated buffer gradient provided by the LKB Ultragrad, since the need to pool the fractions sharply was avoided.

On polyacrylamide gel electrophoresis, the γ chain usually appeared as a homogeneous band, but occasionally, a fainter, adjacent, second band was visible, which might correspond to one of the γ chain variants reported elsewhere (42, 153, 245, 250). Besides the major

band in the gels of the Bβ preparations, there were other bands of lower mobility and one faint band of slightly greater mobility. The latter band might correspond to a γ contaminant described by others (253), but in the gels of Fig. 6B, it appeared to have lower mobility than the γ chain. The minor heterogeneity of the γ and Bβ peaks on ion-exchange chromatography (Fig. 6A) has recently been attributed to differences in the content of sialic acid (114). In addition, the trailing fraction on ion-exchange chromatography of the Bβ chain (Fig. 6A, fraction 3) has also been previously described (253). The series of polypeptides with mobilities faster than the Aα chain (Fig. 5B, gel 5 and Fig. 6B, gel 4) were designated Aα- and have been described as structural variants of the Aα chain, arising from Aα chain degradation in vivo or in vitro (253, 255). The intact Aα chain was eluted last from the column in a relatively small peak and polyacrylamide gel electrophoresis sometimes revealed a double band, indicating the heterogeneity of the Aα chain of human fibrinogen (208, 211, 234, 272). Examination of the polyacrylamide gels of CM-fibrinogen and the isolated carboxymethylated chains in this study (Figs. 5B, 6B and 7) and those of others (111, 253, 255) revealed the existence of fainter bands of low mobility. Gårdlund (111) has shown that these bands represented non-covalently bound aggregates of the alkylated chains, although they appeared to be firmly associated as they were evident on SDS-polyacrylamide gels which were run under normal conditions.

The alkylated chains of fibrinogen were found to be soluble in mildly alkaline buffers (pH 8.3-8.6), but insoluble in neutral buffers (pH 7.0-8.0), unless urea or SDS was used in these solutions. Other workers have remarked on the insolubility of the carboxymethylated chains of fibrinogen (123, 147, 254, 255) and, in particular,

the results here support those of Gollwitzer et al. (123) who found that the polypeptide chains were soluble in buffers of pH 8.5, but not of pH 3.6 or 5.3. Although the alkylated chains were soluble in barbitone buffer (pH 8.6), the gel filtration and polyacrylamide gel electrophoretic studies suggested the existence of soluble, non-covalent aggregates in this buffer.

The results of the analytical electrophoretic experiments show that the chains of fibrinogen are poorly separated in those systems which did not include urea or SDS. Similar results have been found for CM-fibrinogen by Gollwitzer et al. (123). Henschen (147, 151) experienced initial difficulty in separating the chains of CM-fibrinogen by paper and starch gel electrophoresis, even in the presence of urea or SDS, although later attempts were successful (153). Henschen (148, 151) has also shown that high concentrations of urea were required for the electrophoretic separation of the chains of S-sulpho-fibrinogen.

Approximate molecular weights for the carboxymethylated chains of fibrinogen were obtained by SDS-polyacrylamide gel electrophoresis and were: $A\alpha$, 67,000; $B\beta$, 56,000; γ, 47,000. These figures are in close agreement with those of McDonagh et al. (208) among others, and provide a molecular weight for fibrinogen of 340,000, using the formula $(A\alpha, B\beta, \gamma)_2$.

CHAPTER 3: PREPARATION AND EVALUATION OF AN ANTISERUM TO THE CARBOXYMETHYLATED Aα CHAIN OF HUMAN FIBRINOGEN

3.1 Introduction

The investigation of the structure of the fibrinogen molecule and its proteolytic derivatives has been greatly facilitated by the use of specific antisera. Much of the work on this coagulation factor has involved the use of antisera against the whole fibrinogen molecule (222, 244, 264) or against its final plasmin degradation products, fragments D and E (107, 158, 168, 261). Further information about the immunology of fibrinogen was obtained with the development of antisera against fibrinopeptides A and B (17, 263) and with the discovery of neoantigens on fragments D and E (282, 286). Recently, cyanogen bromide has been used to cleave the fibrinogen molecule and the resulting fragments have been purified and used as immunogens, so that the subunit structure of fibrinogen might be more closely studied by means of such antisera (30, 112, 113, 120, 155, 186, 203, 289). However, until a few years ago, there had been only brief mentions concerning the use of antisera which had been raised against the intact polypeptide chains of human or bovine fibrinogen, i.e. Aα, Bβ and γ (28, 155, 186, 255). Very recently, some studies on the antigenicities and cross-reactions of these polypeptide chains and their antisera have been performed (111, 121, 199, 285, 309).

In the present study, a pilot experiment to raise antisera in rabbits to the three chains of fibrinogen revealed that success had been achieved only in the case of the Aα chain, negative or anomalous results being obtained with the Bβ and γ chains. Accordingly, further investigations were channelled in the direction of Aα chain antigenicity and the results obtained constitute the greater part of

this thesis. This chapter describes the immunological characterisation of antiserum raised to the carboxymethylated Aα chain of human fibrinogen, using the relatively simple techniques of immunoprecipitation in agarose gels. More successful attempts to raise antisera to the Bβ and γ chains of fibrinogen are described in a later chapter.

3.2 Materials and methods

3.2.1 Preparation of human fibrinogen and fibrinogen chains

Fibrinogen fraction I-4 and the carboxymethylated chains of fibrinogen, Aα, Bβ and γ, were prepared as described in Chapter 2. In addition, the Aα+ chains, the series of polypeptides which were eluted from the ion-exchange column close to the intact Aα chain, but with electrophoretic mobilities equal to or greater than that of the intact Aα chain, were prepared (Fig. 8).

3.2.2 Human plasma

9 volumes of blood + 1 volume of 0.11M trisodium citrate were mixed and centrifuged at 1000g for 30 min (4°C). Plasma was obtained from normal donors, an umbilical cord and patients with disseminated intravascular coagulation (DIC), and were stored at -20°C.

Cryofibrinogen. Blood was obtained from patients whose plasma exhibited a cryoprecipitate, subsequently identified as cryofibrinogen, at low temperature. The blood, anticoagulated with 0.11M citrate or 0.11M citrate/0.01M EACA (ε-aminocaproic acid), was collected at 37°C and immediately centrifuged. The supernatant plasma was left overnight at 4°C and the precipitate collected by centrifugation, washed twice with 0.15M NaCl (4°C) and reconstituted in a small volume of 0.15M NaCl (37°C). These samples were examined immediately.

Suspected abnormal fibrinogen. Plasma was obtained from a patient with a suspected acquired abnormal fibrinogen (liver disease, prolonged thrombin time, inhibition of thrombin time of normal plasma, reduced fibrinogen level with fibrin polymerisation time test (333) compared with total coagulable fibrinogen (25), abnormal plasma fibrin polymerisation curve (308) which was partially corrected at low ionic strength).

3.2.3 Human serum

Serum was generally collected by clotting blood for several hours at room temperature in the presence of 0.01M EACA or in sample tubes, containing soya bean trypsin inhibitor (3,600 NF units) and bovine thrombin (20 NIH units), obtained from Wellcome Reagents Ltd. (sample tubes for FDP assays). The clotted samples were centrifuged and the sera were withdrawn and stored at -20°C.

3.2.4 Immunodiffusion

Immunodiffusion was performed in 1% agarose, essentially as described (268), in the following buffer systems: 0.03M barbitone, with or without urea, 0.06M barbitone, 0.03M barbitone/0.15M NaCl, (all pH 8.6), 0.2M ammonium bicarbonate (pH 8.5), 0.067M phosphate/ 0.15M NaCl (pH 7.3).

Generally, the carboxymethylated chains of fibrinogen were applied to the agarose wells in 0.06M barbitone buffer (pH 8.6, ionic strength 0.05), along with 8-10M urea or else the urea was removed from the solutions by dialysis before application.

3.2.5 Immunoelectrophoresis

Immunoelectrophoresis was performed essentially as described (297).

3.2.6 Polyacrylamide gel electrophoresis

Electrophoresis in 5% w/v acetic acid/2M urea was performed as described in Chapter 2. As a preparative tool, each gel rod was loaded with 50 μg of Aα chain in a volume of 5 μl.

3.2.7 Preparation of antiserum to the Aα chain of fibrinogen

In a pilot study, one rabbit was immunised at 4 intramuscular sites with 5 mg of carboxymethylated Aα chain in a 1 ml suspension with Freund's Complete Adjuvant at intervals of 3 weeks. The resulting antiserum revealed precipitation reactions in agarose gel with Aα chains, fibrinogen fraction I-4 and normal plasma, but not with human serum.

In the subsequent study, the Aα chain preparation was purified further by polyacrylamide gel electrophoresis, as described above. 2 out of 16 gel rods were stained with Coomassie Brilliant Blue to ascertain the location of the Aα band and the corresponding bands were cut from the other unstained gels in a 2 mm section. These gel sections were emulsified with 2 ml 0.15M NaCl and 1 ml 15% Tween 80 and then mixed with 6 ml Freund's Complete Adjuvant. Initial subcutaneous and intramuscular injections were given to each of 6 rabbits, so that each rabbit received 50-100 μg Aα chain. Booster injections were given after 6 weeks and at 10-day intervals thereafter. 2 hours after bleeding, the rabbit serum was separated from the clot and stored at -20°C after the addition of sodium azide.

3.2.8 Preparation of immunoglobulins from rabbit serum

A modification (88) of the method of Steinbuch and Audran (313) was used for 2 rabbit sera. The method involved a preliminary precipitation with ammonium sulphate before obtaining the immunoglobulins with octanoic acid. The last step, involving ion-exchange

chromatography, was omitted.

3.2.9 Absorption of the antiserum

Antiserum to the Aα chain of human fibrinogen was absorbed by incubating with doubling dilutions of the material of interest at room temperature for several hours, and then at 4°C overnight. The absorbed antiserum was obtained by centrifugation of the fine precipitate. Only the top portion of the supernatant serum was used.

3.2.10 Commercial antisera

Specific antisera to the human proteins, albumin, antithrombin III, factor VIII, and fibrinogen fragments D and E, and polyvalent anti-human serum were obtained from Hoechst Pharmaceuticals.

Rabbit anti-fibrinogen antiserum was obtained from Hoechst Pharmaceuticals, Dakopatts A/S, ImCo Corporation, Calbiochem and Nordic Diagnostics; goat anti-fibrinogen antiserum was obtained from Dade Division.

Anti-Aα antiserum was obtained from ImCo Corporation.

3.2.11 Normal rabbit serum

Normal rabbit serum was obtained from an untreated rabbit.

3.2.12 Reagents

Agarose was obtained from Miles-Seravac, Freund's Complete Adjuvant from Difco Laboratories and Coomassie Brilliant Blue from Sigma. All other reagents were obtained from B.D.H.

3.3 Results

3.3.1 Purity of the Aα polypeptide chain

Before immunisation of rabbits, the purity of the Aα chain preparation was assessed by immunodiffusion. No reactions were

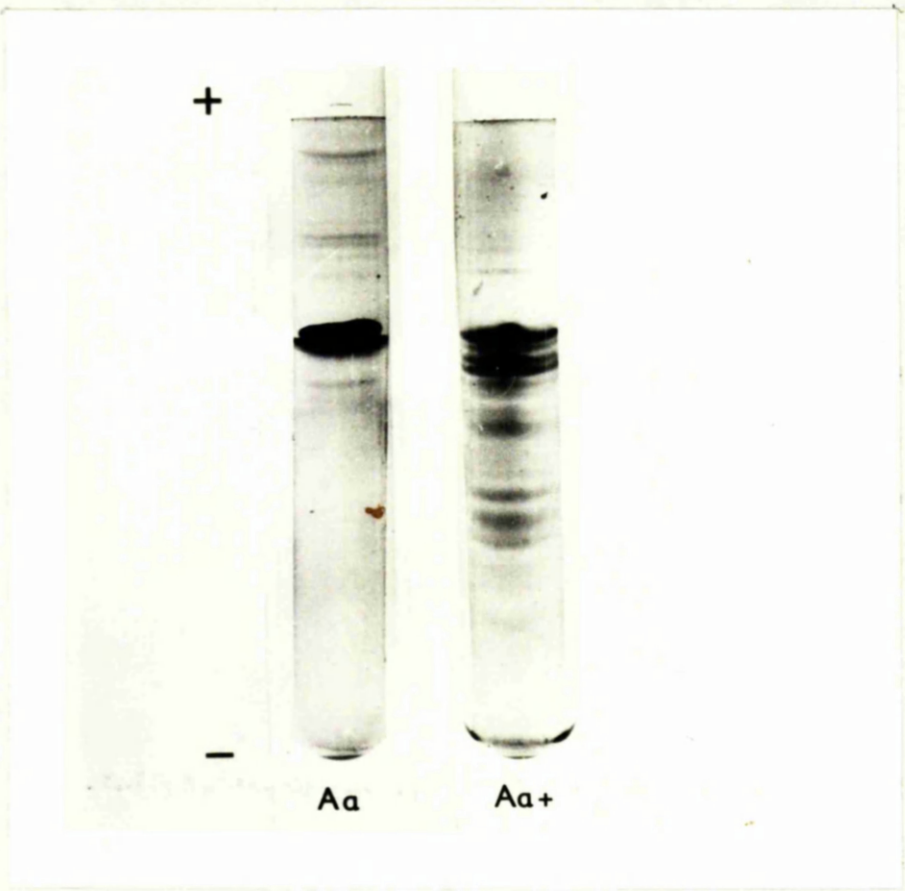


Fig. 8 Electrophoresis of intact Aα chains (30 μg) and Aα+ chains (35 μg) of human fibrinogen on 7.5% w/v acrylamide gels at acidic pH.

observed with anti-human serum, nor with specific antisera to albumin, antithrombin III, factor VIII or fibrinogen fragments D and E.

In spite of the apparent high purity of the Aα polypeptide chain, as assessed in this way, polyacrylamide gel electrophoresis of the preparation revealed the existence of other fainter bands besides the main Aα band (Fig. 8). Therefore, for the purposes of raising Aα chain antiserum, the major band sections of the gels were excised and used as immunogen, as described in Materials and Methods.

3.3.2 Immunodiffusion conditions

During the study of the precipitation reactions of anti-Aα antiserum (A/Aα) by immunodiffusion, the great insolubility of the carboxymethylated chains of fibrinogen presented a problem. At first, the chains were dissolved in buffers (0.03 and 0.06M barbitone (pH 8.6), 0.03M barbitone/0.15M NaCl (pH 8.6) and 0.2M ammonium bicarbonate (pH 8.5)) containing 8-10M urea, which was removed by dialysis, leaving the polypeptide chains in buffer solution. However, to avoid the time-consuming dialysis, the carboxymethylated chains in 8M urea buffers were applied directly to the agarose gel. Comparison of the precipitin patterns revealed no difference whether the urea had been removed by prior dialysis or not, a finding consistent with the observation that plasma proteins precipitated with their corresponding antisera in concentrations of 3M urea. Indeed, due to the relatively rapid diffusion of a small volume (5 µl) of urea on a 3 ml agarose slide, it was unlikely that urea concentrations as high as 3M would be encountered, except immediately after sample application.

In order to avoid the danger of carboxymethylated chains precipitating out of solution due to the neutral pH of the buffer, the buffer used hereafter for immunodiffusion was slightly alkaline - 0.06M barbitone, pH 8.6, ionic strength 0.05; nevertheless, similar precipitation patterns of the Aα chain antiserum could be observed in 0.067M phosphate/0.15M NaCl, pH 7.3.

3.3.3 Reactions of anti-Aα antiserum: general observations

The sera of all 6 rabbits showed precipitation reactions against the Aα chain, purified fibrinogen, CM-fibrinogen and normal plasma on immunodiffusion and immunoelectrophoresis. In addition, some antisera revealed a very faint precipitin line when reacted with normal sera. No reaction was observed when the Aα chain was diffused against normal rabbit serum. The relative titres of the antisera were estimated by their ability to precipitate doubling dilutions of the antigens; the two highest-titred antisera, A/Aα(4) and A/Aα(6), were thereafter employed for the greatest part of the study.

Using ImCo anti-Aα antiserum, very faint, multiple precipitin lines were observed with Aα chains and normal plasma on immunodiffusion. This antiserum was not further studied, especially since ImCo Corporation confirmed the presence of these multiple lines on immunodiffusion, but were unable to explain their origin.

3.3.3.1 Absorption Studies

Absorption of anti-Aα antiserum with fibrinogen fraction I-4 prevented any further reactions of the antiserum with the Aα chain, purified fibrinogen or plasma. Similar results were obtained when anti-Aα antiserum was absorbed with Aα+ chains.

Unexpectedly, absorption of anti-Aα antiserum with $\frac{1}{5}$ volume

of normal serum also destroyed the ability of the antiserum to react with the Aα chain, purified fibrinogen or plasma.

3.3.3.2 Observations on a double precipitin line

On reaction with the Aα chain, fibrinogen or plasma, the sera from several rabbits showed a distinct double precipitin line on gel diffusion and is shown for several Aα chain preparations in Fig. 9A. However, heating of the anti-serum at 56°C for 30 min - 1 hour prevented double line formation (Fig. 9B), only single precipitin lines being apparent. The non-antibody nature of the heat-dependent precipitin line was confirmed by the following observations:

(i) addition of an equal volume of normal rabbit serum to heated anti-Aα antiserum restored the ability of the antiserum to produce double precipitin lines on immunodiffusion.

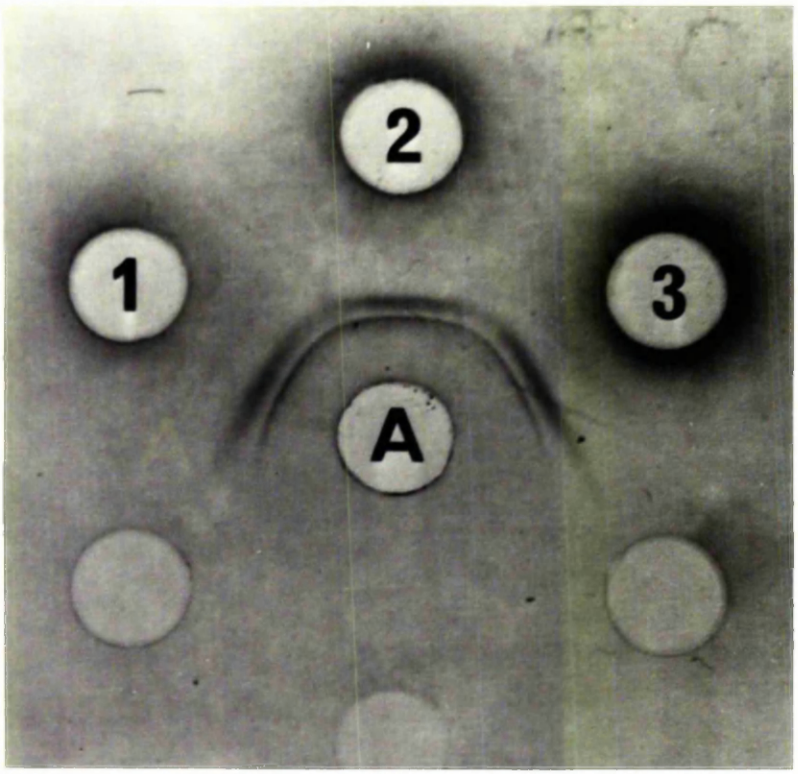
(ii) addition of an equal volume of heated normal rabbit serum to heated anti-Aα antiserum did not restore this ability and only single precipitin lines were observed on immunodiffusion.

(iii) the immunoglobulins of anti-Aα antiserum were prepared and the precipitin patterns of these antibodies with various antigens revealed only single precipitin lines.

Consequently, these rabbit antisera, which produced double precipitin lines on gel diffusion, were routinely heated at 56°C before use.

3.3.4 Immunodiffusion of the carboxymethylated Aα, Bβ and γ chains

Using the double diffusion system, anti-Aα antiserum showed a strong reaction with the Aα chain preparations. No reaction was observed with the γ chain, but a faintly diffuse precipitate could be seen around the Bβ chain sample well (Fig. 10A). Absorption of anti-Aα antiserum with varying quantities of Bβ chain (1-32 µg/ml antiserum) produced little modification of the Aα precipitin lines,



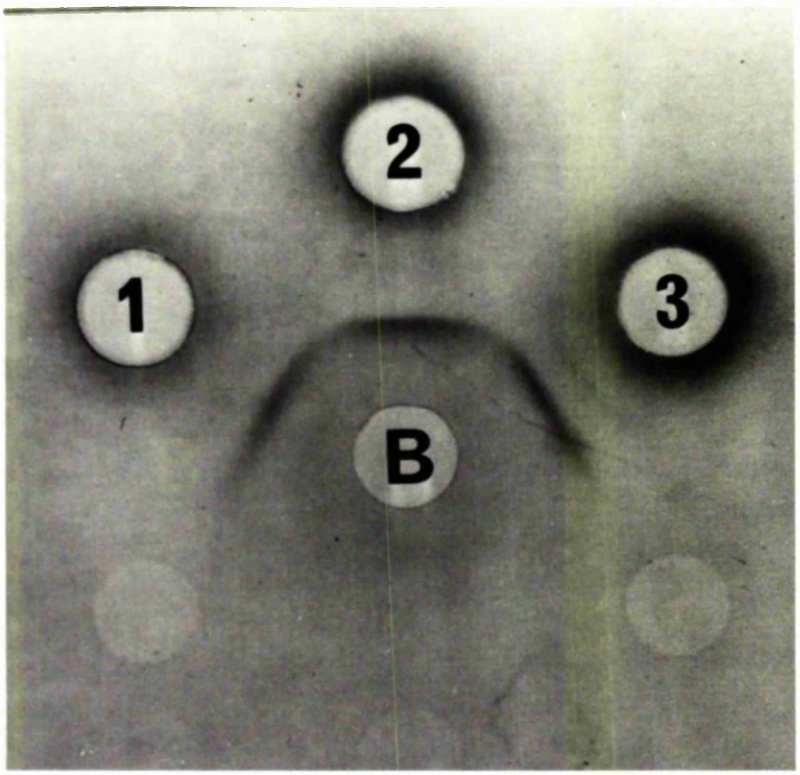


Fig. 9 Immunodiffusion of 3 lots of Aα chain preparations (1-3) with anti-Aα antiserum (centre wells).
A. Unheated antiserum. B. Heated (56°C) antiserum.

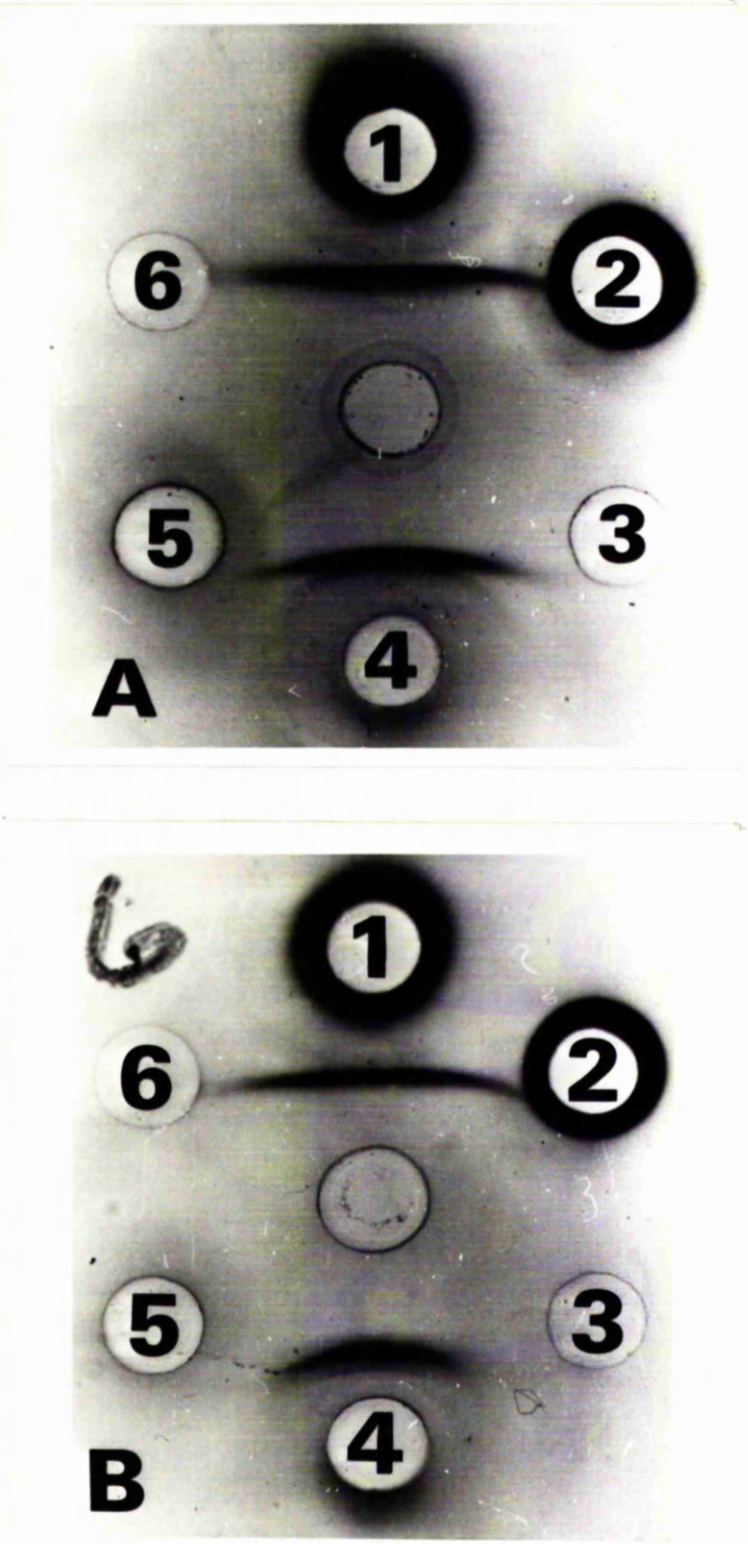


Fig. 10 Immunodiffusion of Aα chains (1) 5 mg/ml, (4) 1 mg/ml; Bβ chains (2) 5 mg/ml, (5) 1 mg/ml; and γ chains (3) 5 mg/ml, (6) 1 mg/ml; with antiserum in centre wells. A. Unabsorbed anti-Aα antiserum. B. Anti-Aα antiserum absorbed with Bβ chains (2 μg/ml antiserum).

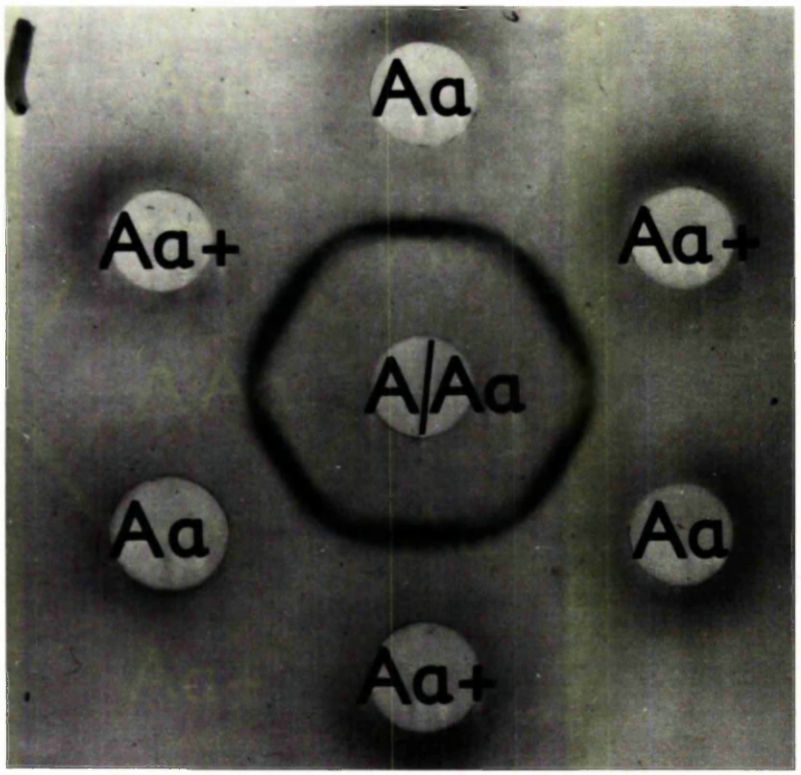


Fig. 11 Immunodiffusion of Aα chains (0.9 mg/ml) and Aα+ chains (1.5 mg/ml) with anti-Aα antiserum (A/Aα).

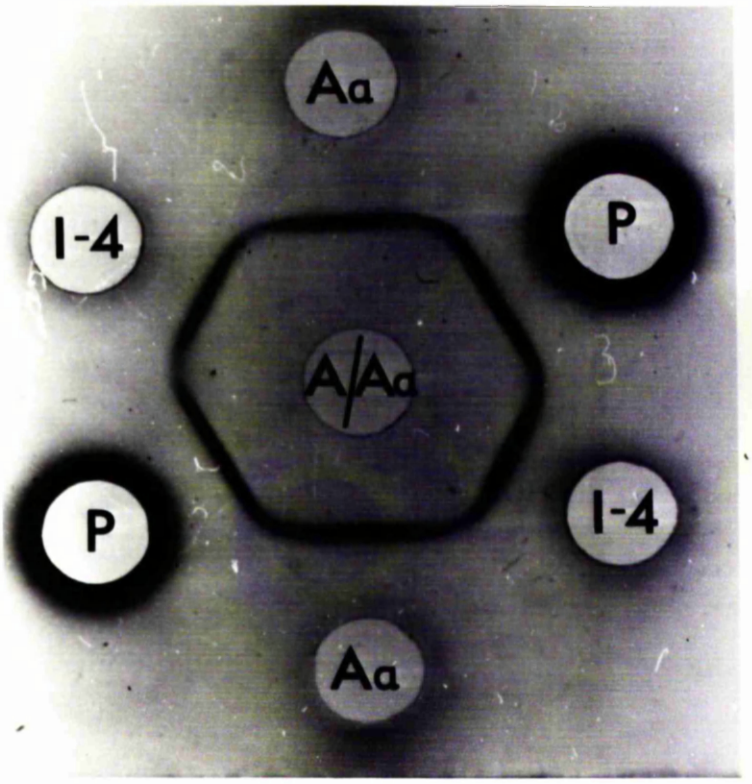


Fig. 12 Immunodiffusion of Aα chains (1 mg/ml) (Aα), fibrinogen I-4 (1 mg/ml) (I-4) and pooled normal plasma (P) with anti-Aα antiserum (A/Aα).

but it removed the faint Bβ chain precipitate (Fig. 10B). Analogous results were obtained on immunoelectrophoresis of the polypeptide chains.

Fig. 11 shows that antigenic identity existed between the intact Aα chain and Aα+ chains.

3.3.5 Immunodiffusion of human fibrinogen

When fibrinogen fraction I-4 reacted against anti-Aα antiserum by gel diffusion, a precipitin line was observed (Fig. 12). Moreover, this line exhibited antigenic identity with the carboxymethylated Aα chain and with normal human plasma.

Antigenic identity was always observed with the plasma of several normal donors, 9 DIC patients and the neonatal plasma, when either anti-fibrinogen or anti-Aα antiserum was used in immunodiffusion. Moreover, cryofibrinogen, obtained from 5 of the DIC patients also revealed antigenic identity with normal plasma fibrinogen; using SDS-polyacrylamide gel electrophoresis, the subunit polypeptides of this material appeared as 3 bands as in normal fibrinogen, along with a high molecular weight band. Similarly, plasma from the patient with the suspected abnormal fibrinogen showed antigenic identity with normal plasma when anti-Aα antiserum was used; SDS-polyacrylamide gel electrophoresis showed normal band patterns for non-crosslinked and crosslinked fibrin from this patient.

3.3.6 Reactivity of the Aα chain with anti-fibrinogen antiserum

By gel diffusion, no reaction was observed between CM-fibrinogen and anti-fibrinogen antiserum. Moreover, the Aα chain of fibrinogen revealed no reaction against commercial anti-fibrinogen antisera from 6 different sources.

3.4 Discussion

An Aα chain preparation, to be used for raising antiserum, was shown to be free from serum contaminants by immunodiffusion against anti-human serum. Nevertheless, on polyacrylamide gel electrophoresis, the presence of contaminating bands, possibly due to firmly associated Aα aggregates (111), was observed, and the excised, unstained bands were therefore used as immunogen, a technique which has also been used by Soria *et al.* (309) for raising antisera against the three polypeptide chains of fibrinogen.

Much of the present study employed immunological precipitation in agarose gel as an investigative tool, and the time required for sample preparation was considerably reduced by applying the carboxymethylated chain samples in the agarose wells in 8M urea buffers. In view of the extreme denaturing conditions already suffered by the polypeptide chains of fibrinogen during reduction, alkylation and separation, it was considered that temporary solution in an 8M urea buffer would cause little further damage to these chains. It has previously been shown by gel diffusion that the readily precipitated Tamm-Horsfall glycoprotein retained the ability to bind to its antibody after sample application in 8M urea (225).

During preliminary characterisation of anti-Aα antiserum, a double precipitin line was observed when various antigens reacted in agarose gel against several antisera. A serum component other than antibody was shown to be responsible for the development of one of the precipitin lines. Since C'1 complement component has been implicated in the precipitation of soluble antigen-antibody complexes (237, 337) and complement subcomponent, C1q, was precipitated after heat treatment at 56°C for 30 min (204), the observations on the nature of the double precipitin line might be explained by the

participation of rabbit C1q in antibody complex formation during the diffusion process. Indeed, a similar double-line phenomenon has been described in double diffusion analysis of hapten specific immune systems (269). Rabbit sera, which exhibited double immunoprecipitin line formation, were therefore routinely heat-decomplemented at 56°C.

The specificity of anti-Aα antiserum was assessed by gel diffusion of the 3 chains of fibrinogen and the antiserum, and showed that anti-Aα antiserum reacted with the immunogen, Aα chain, but not with the γ chain, thus confirming previous results (111, 199). In addition, Lefvert et al. (199) have found no evidence of an immunological reaction between unabsorbed anti-Aα antiserum and Bβ chains of human fibrinogen, using both gel diffusion and radioimmunoassay techniques. However, Gårdlund (111) found that his anti-Aα antiserum reacted with both Aα and Bβ chains on immunodiffusion, but that absorption of the antiserum with the Bβ preparation abolished the reactions of both the Aα and the Bβ chains. In this study, the slight precipitation observed on double diffusion of anti-Aα antiserum and the Bβ chain may have been due to the presence of a few anti-Bβ antibodies in the rabbit serum, since absorption of the antiserum with varying amounts of the Bβ chain (1-32 µg/ml antiserum) removed the diffuse precipitate which was associated with the Bβ chain, but did not noticeably affect the Aα-anti-Aα precipitin line. However, because of the insensitive nature of immunodiffusion techniques, these absorption results do not exclude the existence of some common antigenic sites on the Aα and Bβ chains. Indeed, Gollwitzer et al. (121, 123) have suggested the existence of considerable antigenic homology between the Aα and Bβ chains of bovine fibrinogen on the basis of evidence from precipitation and haemagglutination techniques, using both anti-bovine fibrinogen antiserum and specific chain antisera. However, using

a haemagglutination-inhibition assay, the same authors (121) showed that the cross-reaction between the carboxymethylated Aα and Bβ chains was not exhibited between the corresponding aminoethylated products, a result which may have arisen from the better separation of the aminoethylated Aα and Bβ chains on ion-exchange chromatography.

It has been suggested that the Aα+ chain preparation represents Aα chains which have been damaged at the COOH-terminal end of the polypeptide during fractionation procedures or by plasmin degradation (253). That being the case, the absorption and immunodiffusion results with antiserum to the Aα chain do not provide any evidence that the removal of small peptides from the COOH-terminal end of the chain is accompanied by a loss in antigenicity.

Several studies have provided evidence that at least part of the Aα chain of fibrinogen is located on the surface of the intact fibrinogen molecule. Blombäck et al. (30) have demonstrated the existence in human fibrinogen of a surface-oriented disulphide knot, Hi2-DSK, which is derived exclusively from the Aα chain, as is F-CB3, a fragment which is released readily from bovine fibrinogen (329) by treatment with cyanogen bromide, thus indicating its surface location. Other workers have shown the existence of a region on the bovine Aα chain which is susceptible to bacterial proteinases and is located on the surface of the fibrinogen molecule (257), while Matthias et al. (229) have shown that the covalent binding of fibrinogen to agarose took place almost exclusively at the Aα chain. The absorption and immunodiffusion results presented here have shown that anti-Aα antiserum reacts with human fibrinogen, both in a purified condition and in plasma. Moreover, a reaction of identity was established with the Aα chain, suggesting that most of the antigenic determinants of the Aα chain were exposed on the intact fibrinogen molecule. This result substantiates

and enlarges upon the observation of Lotter and Timpl (203) that the Aα fragment, F-CB3, and bovine fibrinogen were indistinguishable by radio-immunoassay, when using anti-bovine F-CB3 antiserum.

Some Aα chain antisera revealed a faint reaction line on immunodiffusion with normal human serum. Because of the elaborate purification procedure for the immunogen, Aα chain, this weak reaction cannot easily be dismissed as an indication of contaminating antibodies in the antiserum. On the other hand, since it has been established that the Aα chain is one of the first sites of plasmin attack on the fibrinogen molecule (106, 234, 272) and in view of the recent findings (38, 119, 138, 318) of the presence of early fibrin(ogen) breakdown products in the form of detached Aα fragments in normal human serum, the precipitin line obtained with serum in agarose gels, and also the serum absorption results, may represent the detection of an early degradation product of fibrinogen or fibrin. This hypothesis will be developed and discussed in later chapters.

Using a number of different anti-fibrinogen antisera, no precipitation reaction was observed with the Aα chain. This result is similar to that of Blombäck and Blombäck (28), but is at variance with the results of others (121, 123). These results will be discussed at length in the following chapter.

CHAPTER 4: ANTIGENIC DETERMINANTS OF THE Aα CHAIN OF HUMAN FIBRINOGEN - STUDIES ON PLASMIN-DERIVED DIGESTION PRODUCTS

4.1 Introduction

For several years, immunological investigations of the degradation of fibrinogen by plasmin to lower molecular weight products have utilised antisera against the whole fibrinogen molecule, fragment D and fragment E. Two major regions (D and E) of the fibrinogen molecule have thus been characterised as independent antigenic entities (222, 244, 261, 264). On a similar basis, anti-Aα antiserum has been employed in the present study to follow the fate of the antigenic determinants of the Aα chain of fibrinogen during plasmin digestion. The study of the precipitation reactions of this antiserum in Chapter 3 has revealed that it is specific for the Aα chain, whether in isolated form or as part of the intact fibrinogen molecule.

During plasmin degradation of human fibrinogen, each of the three polypeptide chains of fibrinogen, Aα, Bβ and γ, are eventually cleaved to several fragments; some of the fragments remain disulphide-bonded to each other, while other fragments are released from the parent molecule as free polypeptides. In particular, the initial plasmin attack on fibrinogen has been shown to release free polypeptides from the COOH-terminal end of the Aα chain (272). In this study, polyacrylamide gel electrophoresis has been used to examine more closely the release of several polypeptides from fibrinogen during plasmin degradation. In addition, three of the larger fragments (molecular weights 26,000, 33,000 and 44,000) have been prepared in a purified or a semi-purified state and their immunological properties have been studied.

4.2 Materials and Methods

4.2.1 Human fibrinogen

Kabi fibrinogen was used directly for the study of plasmin degradation. For other purposes, fibrinogen fraction I-4 was prepared as described in Chapter 2.

4.2.2 Immunological techniques

Immunodiffusion and immunoelectrophoresis were performed as described in Chapter 3. In one case, immunodiffusion was performed in the presence of 0.04M calcium chloride.

4.2.3 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis and the determination of molecular weights were described in Chapter 2.

After staining of the gels with Coomassie Brilliant Blue, densitometry was performed at 570 nm, using a Pye Unicam SP 1809 Scanning Densitometer. Areas under the incompletely separated densitometric peaks were estimated from overlapping isosceles triangles, extrapolated for each peak.

4.2.4 Antisera to fibrinogen and fibrinogen fragments

Antiserum to the carboxymethylated Aα chain of human fibrinogen was raised in rabbits as described in Chapter 3. Antisera to fragments D and E were obtained from Hoechst Pharmaceuticals. Antiserum to human fibrinogen was obtained from Hoechst Pharmaceuticals, Dakopatts A/S, Calbiochem and Nordic Diagnostics.

In addition, antiserum to fibrinogen fraction I-4 was raised in rabbits by immunisation of each rabbit with 1 mg fraction I-4 solution in an equal volume of Freund's Complete Adjuvant at 4 subcutaneous sites. At 10 day intervals, equal amounts of fibrinogen were injected as booster doses, but without Freund's Complete Adjuvant. After collection of rabbit blood, serum was stored at -20^{o}C in the presence of sodium azide.

Although the faint lines observed with antisera from some bleeds on immunodiffusion against normal human serum indicated the presence of contaminating antibodies, the antisera were not absorbed with human serum.

4.2.5 Plasmin degradation of fibrinogen

Kabi fibrinogen, at a final concentration of 20 mg/ml, was digested by plasmin after activation of the plasminogen present in the preparation, according to Marder et al. (222). The digestion reaction was initiated by adding streptokinase to a final concentration of 100 u/ml and stopped at timed intervals by adding aprotinin and ϵ-aminocaproic acid (EACA) to final concentrations of 200 KIU/ml and 0.2M, respectively.

The progress of the reaction was followed by immunoelectrophoresis and SDS-polyacrylamide gel electrophoresis.

4.2.6 Preparation of fibrinogen degradation products (FDP)

4.2.6.1 Fragment X

A plasmin digest of Kabi fibrinogen was stopped after 10 min and subjected to gel filtration on a column (88 x 2.5 cm) of Sephadex G-200, using the buffer system of Marder et al. (222): 1M NaCl/0.025M tris/0.025M trisodium citrate/0.2M EACA (pH 7.4), containing 0.1 mg/ml soybean trypsin inhibitor. The first eluted peak was subjected to rechromatography on the same column after concentration by ammonium sulphate precipitation (222). The final concentrated solution was stored at -20°C in the presence of 0.01M EACA.

4.2.6.2 Fragment Y

Fragment Y was prepared from a 40 min digest of fibrinogen as for fragment X (222), but was twice subjected to rechromatography on the G-200 column. The final concentrated solution was stored at -20°C in the presence of 0.01M EACA.

4.2.6.3 Fragment D

Fragment D was prepared from a 22 hour plasmin digest of fibrinogen by the method of Nussenzweig et al. (265). The digest mixture was subjected to ion-exchange chromatography on a column (24 x 1.5 cm) of Whatman DE 52 resin, using the automatically-regulated buffer gradient apparatus, described in Chapter 2. A linear ionic strength gradient was formed with 0.01M Na_2CO_3 (pH 8.9) as starting buffer and 0.01M Na_2CO_3/ 0.1M NaCl (pH 8.9) as final buffer. The material in the fragment D peak was dialysed against distilled water and freeze-dried. Several preparations of Fragment D from plasmin digests of fibrinogen were also obtained by the acetic acid method of Gårdlund et al. (113).

Immunological investigation established that both types of fragment D preparations were free from fragment E contamination. SDS-polyacrylamide gel electrophoresis of the unreduced fragments showed that both were similar in molecular size, while reduction of the samples indicated a similar polypeptide chain composition (Fig. 13). However, during immunodiffusion studies, the following differences in behaviour between these two preparations were noted:

(i) sample dissolution for gel diffusion studies required 2-4M urea for D(Gårdlund), while D(Nussenzweig) was readily soluble in physiological buffers,

(ii) D(Gårdlund) produced only a faint precipitin line with commercial anti-fibrinogen antiserum, whereas the precipitin line with D(Nussenzweig) was sharp and distinct, and

(iii) on reaction with anti-fragment D antiserum, D(Gårdlund) was antigenically deficient compared with D(Nussenzweig), fibrinogen or an unfractionated digest of fibrinogen (Fig. 14).

These findings are related to those of Kemp et al. (175) and indicate that, although both species of fragment D appeared to have an intact subunit

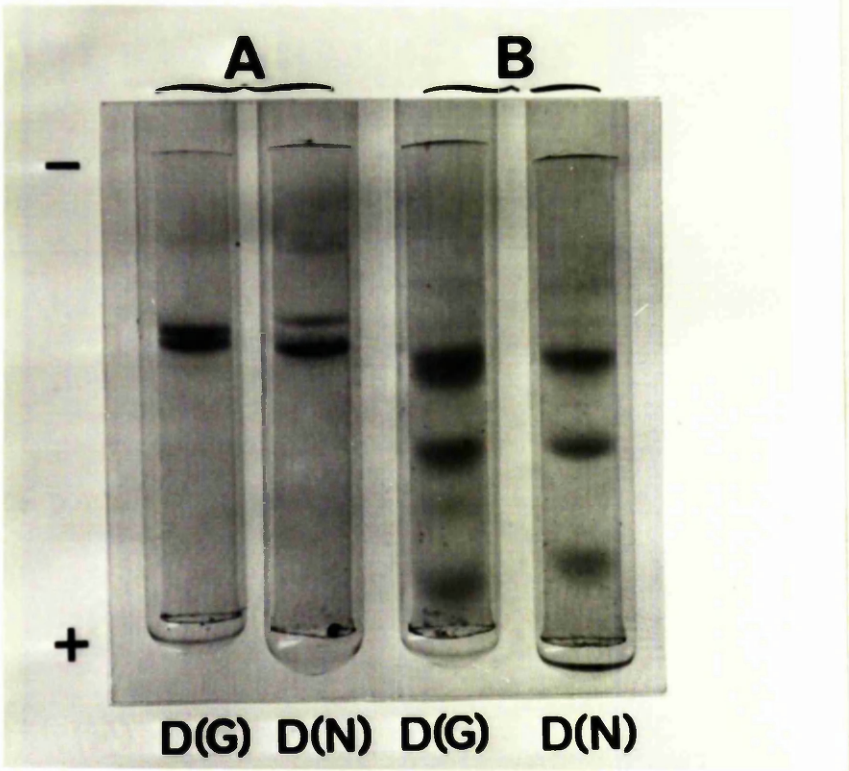


Fig. 13 SDS-polyacrylamide gel electrophoresis of fragment D prepared by the method of Gårdlund et al. (113) (D(G)) and fragment D prepared by the method of Nussenzweig et al. (265) (D(N)).

A. 5% w/v gels with non-reduced samples (7 μg).

B. 7% w/v gels with reduced samples (10-15 μg).

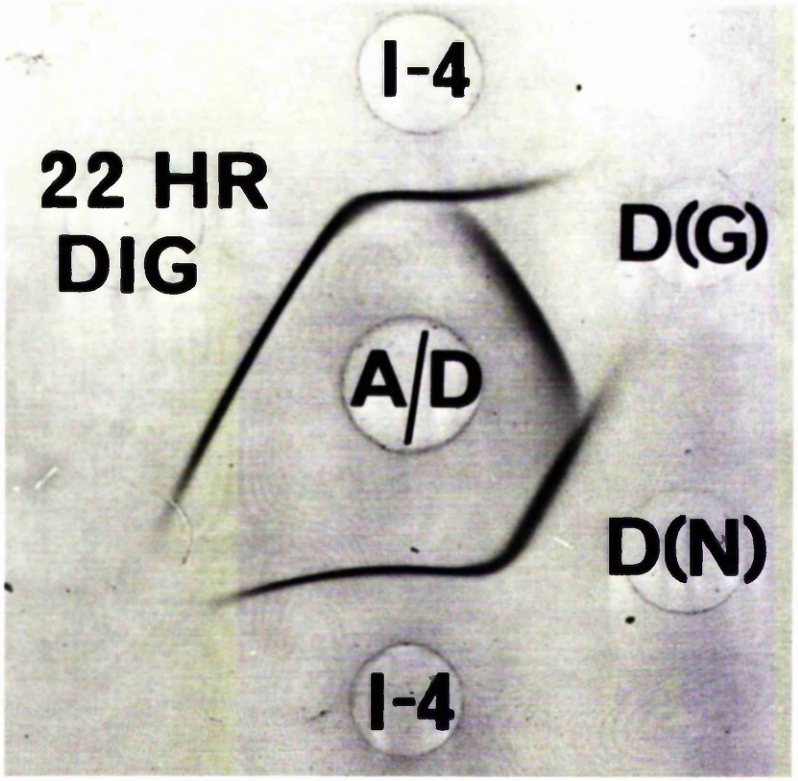


Fig. 14 Immunodiffusion of fibrinogen fraction I-4 (0.9 mg/ml) (I-4), fragment D prepared by the method of Gårdlund et al. (113) (0.5 mg/ml) (D(G)), fragment D prepared by the method of Nussenzweig et al. (265) (0.5 mg/ml) (D(N)), and a 22 hour plasmin digest of Kabi fibrinogen (0.5 mg/ml) (22 HR DIG) with anti-fragment D antiserum (A/D).

structure, either acetic acid treatment (pH 2.3) of fragment D in the method of Gårdlund et al. (113) or the subsequent dissolution of fragment D in 2-4M urea solutions, necessitated by its insolubility, resulted in a conformational change and loss of antigenicity in fragment D. The anomalous behaviour (228) and insolubility at neutral pH (59) of fragment D prepared by this method have been reported by other workers. Consequently, further studies were performed only on fragment D obtained by the method of Nussenzweig et al. (265).

4.2.6.4 Fragment E

The method of Gårdlund et al. (113) was slightly modified, in that a 4 hour plasmin digest of fibrinogen was heat-treated at 56°C for 1 hour to precipitate most of fragment D. The supernatant was applied to a column (84 x 2.5 cm) of Sephadex G-100 and eluted with 10% v/v acetic acid. The fragment E peak was dialysed against distilled water and freeze-dried. Immunodiffusion revealed that this preparation was contaminated with a small amount of fragment D, although SDS-polyacrylamide gel electrophoresis did not show any bands which corresponded to fragment D. However, a faint band of approximate molecular weight 41,000 was observed, which may correspond to fragment d of Kemp et al. (175), since the digest mixture had been exposed to denaturing conditions. This band was not removed by rechromatography on Sephadex G-100, but was easily removed by ion-exchange chromatography on a column (27 x 1.5 cm) of Whatman CM 52 resin, according to Furlan and Beck (98). A linear pH and ionic strength gradient was formed with 0.05M ammonium formate (pH 4.0) as starting buffer and 0.3M ammonium formate (pH 7.5) as final buffer; pure fragment E was obtained after freeze-drying.

4.2.7 Preparation of low molecular weight polypeptides from plasmin digests of fibrinogen

4.2.7.1 1½ min digest

Kabi fibrinogen was digested by plasmin and, after 1½ min, the reaction was stopped, as described above. Fibrinogen and the large molecular weight derivatives were heat-precipitated at 56°C for 30 min. The supernatant solution was fractionated on a column (92 x 2.5 cm) of Sephadex G-100, using the buffer system described by Marder et al. (222) (Fig. 20A). After dialysis against distilled water, the fractions were freeze-dried and examined by immunodiffusion and SDS-polyacrylamide gel electrophoresis.

4.2.7.2 3 min digest

This digest of fibrinogen was treated exactly as for the 1½ min digest. The elution profile from the gel filtration column is shown in Fig. 20B.

4.2.7.3 4 hour digest

Kabi fibrinogen was digested for 4 hours and heat-precipitated as described above. However, the supernatant solution was fractionated on a column (84 x 2.5 cm) of Sephadex G-100, using 10% v/v acetic acid as eluent, according to the method of Hessel (155) (Fig. 20C). The freeze-dried fractions were examined by immunodiffusion and SDS-polyacrylamide gel electrophoresis.

4.2.8 Reagents

Freund's Complete Adjuvant was obtained from Difco Laboratories, Streptokinase-Streptodornase-Varidase from Lederle Laboratories Division, aprotinin (Trasylol) from Bayer, soybean trypsin inhibitor (Type I-S) from Sigma London Chemical Company, and Whatman DE 52 and CM 52 celluloses from W. and R. Balston, Ltd.

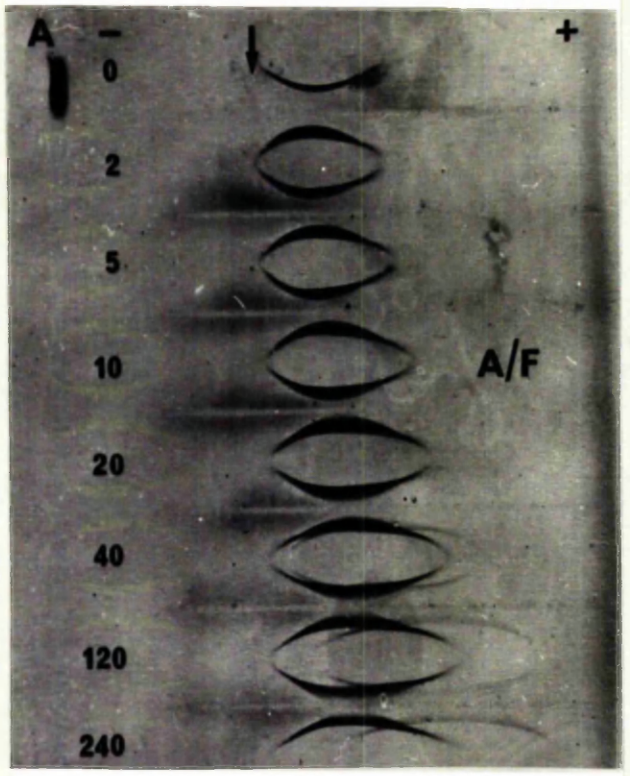
4.3 Results

4.3.1 Analysis of plasmin digests by electrophoresis

The plasmin digestion of fibrinogen was stopped at the times 2, 5, 10, 20, 40 min, 2 hours and 4 hours, by the addition of fibrinolytic inhibitors, and the progress of the reaction was examined by immuno-electrophoresis. Using commercial anti-fibrinogen antiserum in the troughs, the degradation of intact fibrinogen may be followed through to two antigenic species at 4 hours (Fig. 15A). These two final products were indicated by two intersecting precipitin arcs, the cathodal arc resulting from fragment D and the fainter anodal arc resulting from fragment E; these results were confirmed by the use of specific antisera for the fragments. The appearance of the E precipitin arc at 40 min corresponded to the appearance of the fragment E band in SDS-polyacrylamide gel electrophoresis (Fig. 16).

Using anti-Aα antiserum, immunoelectrophoresis of the same reaction mixtures revealed a slight cathodal shift of the precipitin arc at 2 min, which was thereafter transformed to two intersecting arcs of relatively low mobility (Fig. 15B). These two precipitin arcs persisted at 4 hours, although the more anodal arc became fainter in intensity. Examination of the polyacrylamide gels of the reaction mixtures showed the progressive degradation of fibrinogen to fragments X and Y and then to the final products, fragments D and E. Moreover, high sample loading (120 μg of protein from each digest mixture was applied to each SDS gel rod) permitted visualisation of a series of low molecular weight bands which appeared during the digestion process. Fig. 16 shows the early release of a polypeptide (band 1) at 2 min, which was itself degraded with time, while other polypeptides appeared on the gels (bands 2-6). The molecular weights, estimated from the relative mobilities of bands 1-6, were 44,000, 33,000, 26,000 23,000, 17,000 and $<14,000$, respectively.

Densitometric scanning of the lower half of the gel rods



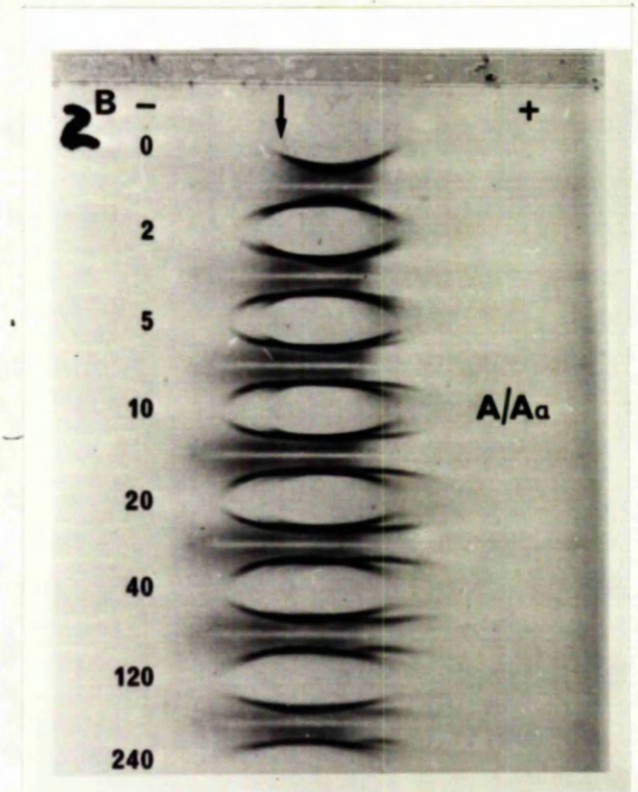


Fig. 15 Immunoelectrophoresis of plasmin digests of fibrinogen using A: anti-fibrinogen antiserum, and B: anti-Aα antiserum. Plasmin digestion of fibrinogen was stopped at the times shown (in minutes). 20 μg of the digests were applied to the agarose gels at the position indicated by the arrow.

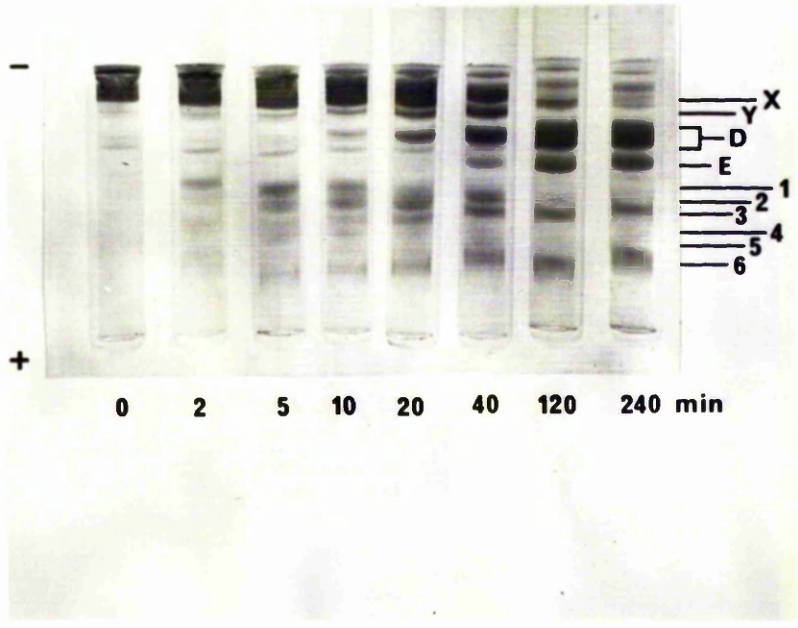


Fig. 16 SDS-polyacrylamide electrophoresis (5% w/v gels) of plasmin digests of fibrinogen. The degradation of fibrinogen was stopped at the times shown and 120 μg of the reaction mixture were applied to the gel rods. The position of fragments X, Y, D and E and bands 1-6 are indicated.

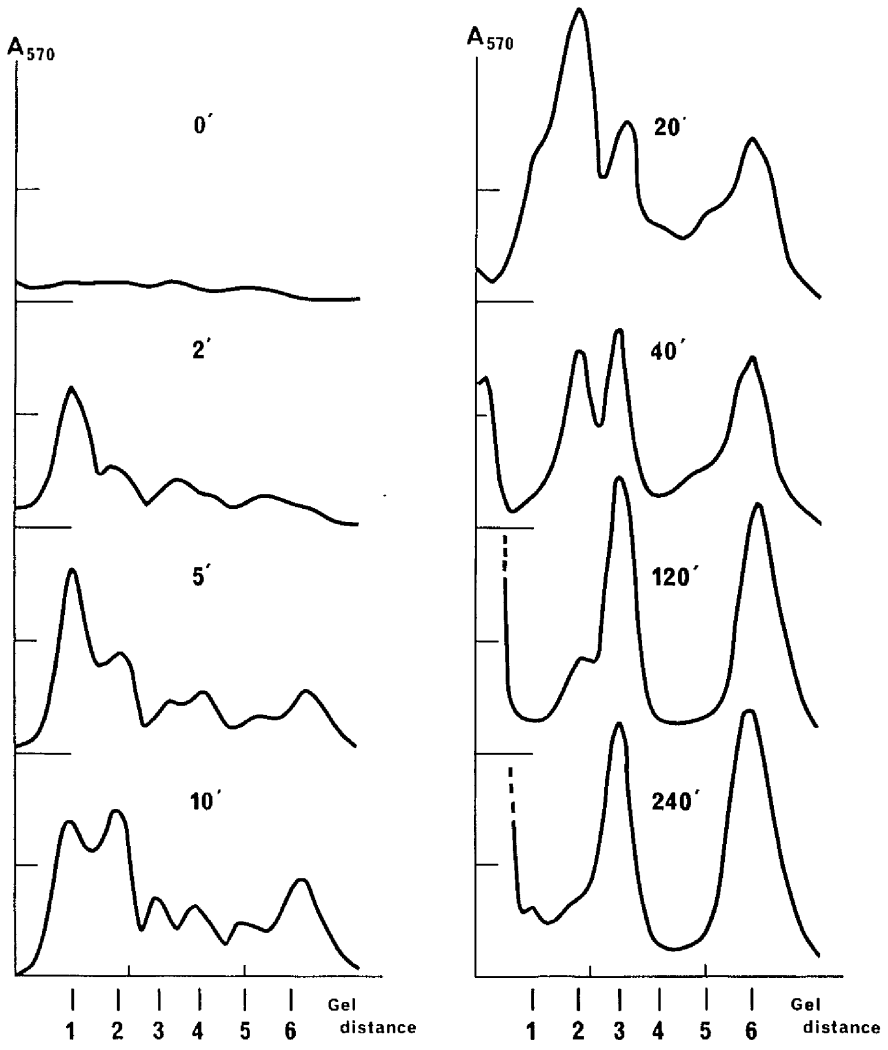


Fig. 17 Densitometric scans of the gels shown in Fig. 16 with the times of digestion shown in minutes. The gels were scanned from top to bottom and appear as a trace from left to right. Only partial scans are shown, corresponding to the lower part of each gel. Optical densities (570 nm) are indicated on the ordinates. Distances of migration are shown on the abscissa, along with vertical bars to indicate the position of bands 1-6.

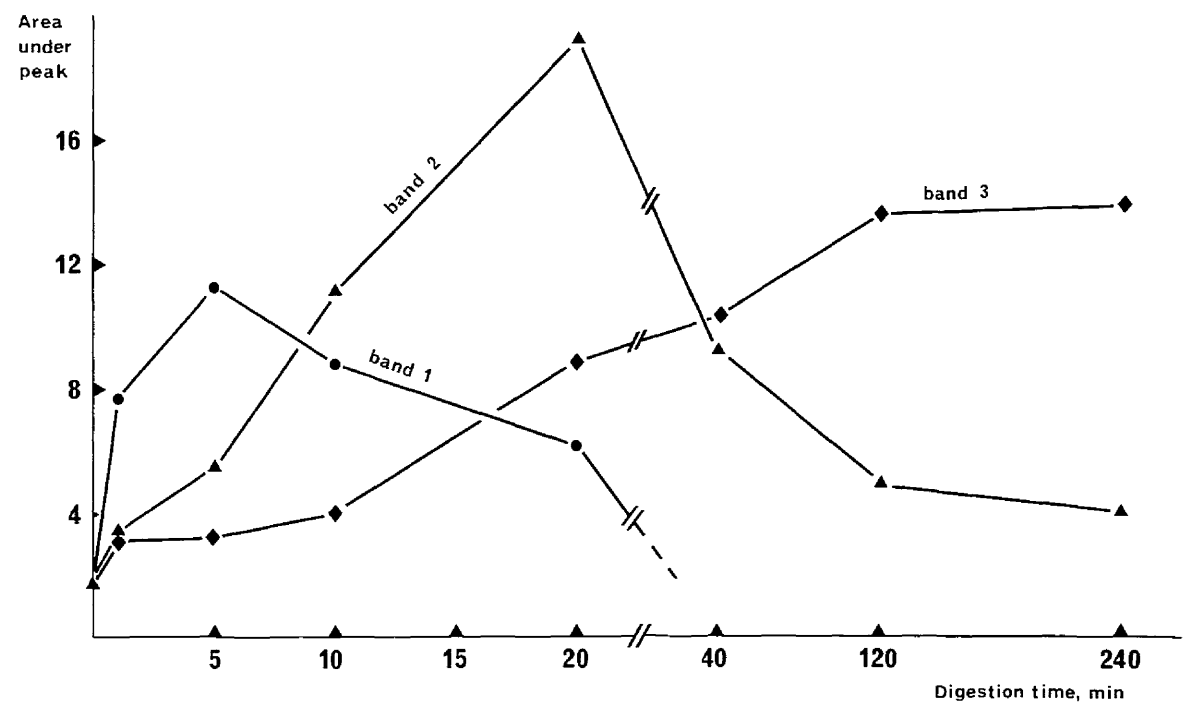


Fig. 18 The formation and degradation of bands 1, 2 and 3 during plasmin digestion of fibrinogen. The graphs indicate the intensities of the stained bands 1, 2 and 3 (estimated from areas under the peaks in Fig. 17 and expressed in arbitrary units) as the digestion of fibrinogen proceeds from 0 to 240 minutes.

(Fig. 17) confirmed the visual observations and permitted semi-quantitative assessment of the rates of appearance and disappearance of bands 1-6 by estimating the areas under each densitometric peak. Fig. 18 shows that bands 1 and 2 consisted of transient species, while the polypeptide in band 3 was plasmin-resistant to 4 hours of digestion. Bands 4 and 5 were also transient, while band 6 remained after 4 hours digestion (results shown in Fig. 17, only).

4.3.2 Identification of Aα chain antigens

In order to identify which of the fibrinogen fragments were responsible for the precipitin arcs observed on immunoelectrophoresis with anti-Aα antiserum (Fig. 15B), fibrinogen, fragments X, Y D and E, and several low molecular weight polypeptides were prepared as described and examined by SDS-polyacrylamide gel electrophoresis, immunodiffusion and immunoelectrophoresis.

4.3.2.1 Electrophoretic purity of fibrinogen degradation products X, Y, D and E

Fibrinogen and fragments X, Y, D and E were examined by SDS-polyacrylamide gel electrophoresis, using a 40 min digest of fibrinogen as reference (Fig. 19A). The faint low molecular weight band in the fragment X and Y gels was due to soybean trypsin inhibitor; minor bands also appeared on the fragment D and E gels above the major bands, but were unrelated to other fibrinogen fragments, as assessed by immunodiffusion using antisera to fragments D and E.

In order to confirm that the subunit structures of these preparations were as described in the literature, electrophoresis was also performed after reduction of their disulphide bonds (Fig. 19B). The observed polypeptides were assigned to their chains of origin by comparison of their molecular weights (Table III) and orders of appearance

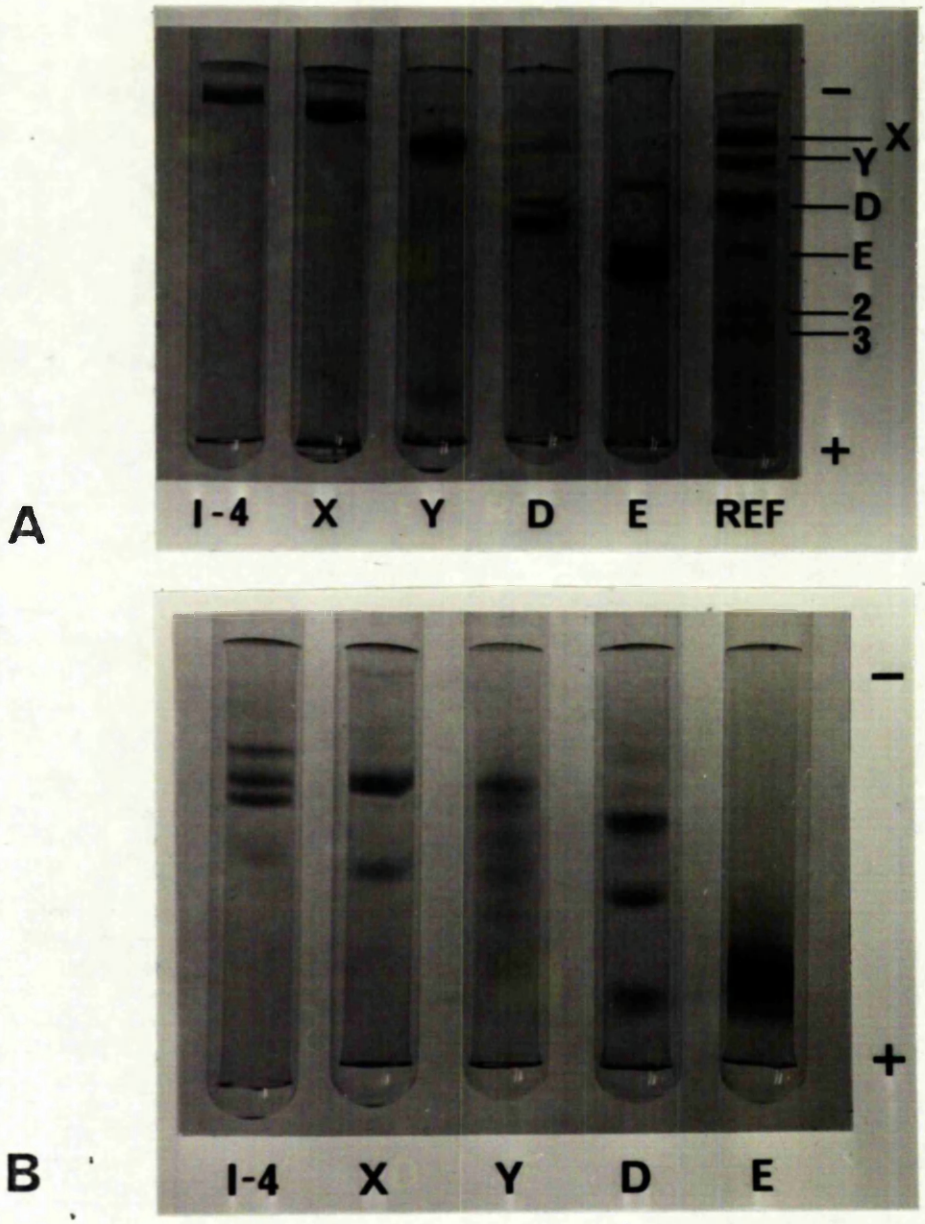


Fig. 19 A. SDS-polyacrylamide electrophoresis (5% w/v gels) of fibrinogen and purified degradation products. Each of the following quantities were applied to a gel rod: fibrinogen fraction I-4, 6 μg; fragment X, 12 μg; fragment Y, ca. 15 μg; fragment D, 6 μg; fragment E, 24 μg; the 40 minute plasmin digest of fibrinogen (REF.), 30 μg.

B. SDS-polyacrylamide electrophoresis (7% w/v gels) of fibrinogen and purified degradation products after reduction with dithiothreitol. Each of the following quantities were applied to a gel rod: fibrinogen fraction I-4, 10 μg; fragment X, 12 μg; fragment Y, ca. 30 μg; fragment D, 10 μg; fragment E, 60 μg.

TABLE III

Molecular weights of the major bands appearing on gels (Fig. 19B) after reduction of the sample

Reduced Sample	Molecular Weight	Polypeptide Chain of Origin[1]
Fibrinogen I-4	67,000 56,000 47,000	Aα Bβ γ
Fg. X	48,000 26,000	β', γ α'
Fg. Y	46,000 38,000 31,000 26,000 20,000 < 13,000	These bands were not assigned to their chains of origin
Fg. D	40,000 27,000 14,000	β'' γ''' α''
Fg. E	< 13,000	α^E, β^E, γ^E

[1] The symbols for the subunit chain fragments are those used by Pizzo et al. (272).

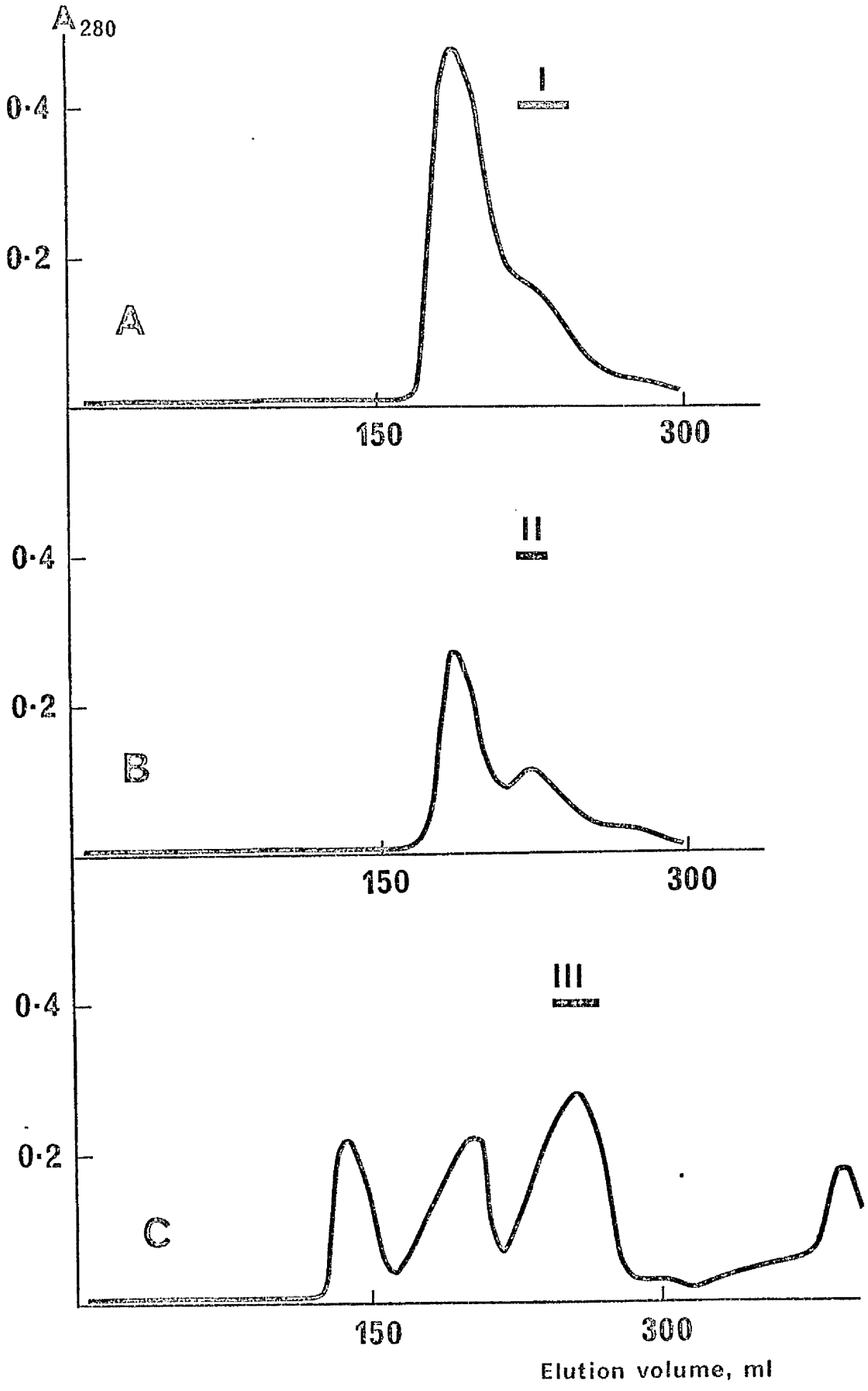

A280
0·4
0·2
A
I
150
300
0·4
0·2
B
II
150
300
0·4
0·2
C
III
150
300
Elution volume, ml

Fig. 20 Protein elution profiles on Sephadex G-100 (monitored at 280 nm) of the heat-stable polypeptides from: A. 1½ min digest, B. 3 min digest, and C. 4 hour digest of Kabi fibrinogen. The polypeptide fractions of interest (I, II and III) are indicated by horizontal bars. The same filtration column was used for A and B, but not for C.

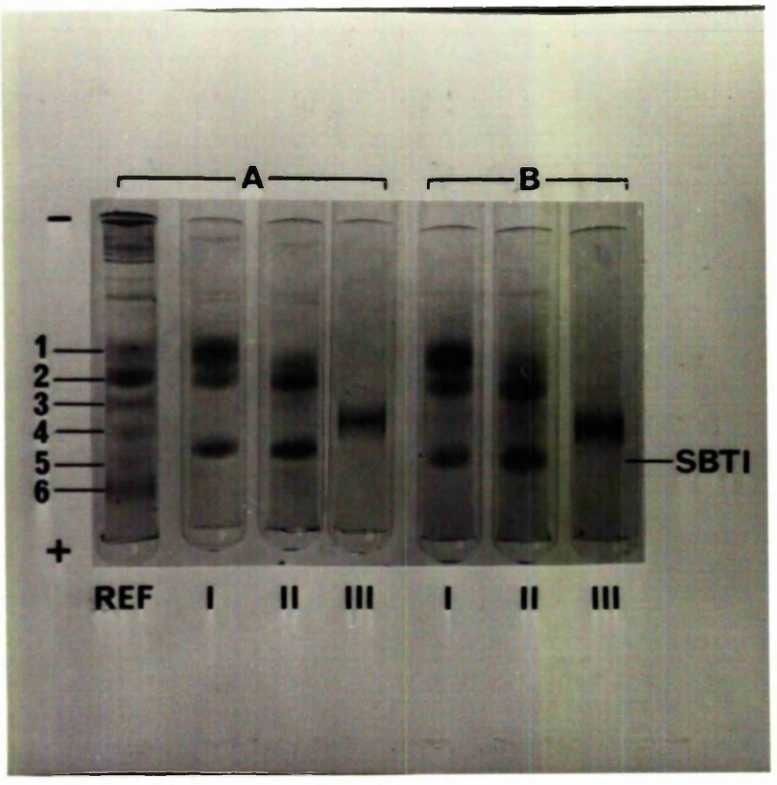


Fig. 21 SDS-polyacrylamide electrophoresis (7% w/v gels) of polypeptides released from fibrinogen after 1½ min, 3 min and 4 hours plasmin digestion, and isolated by gel filtration. Fractions I (60 µg), II (60 µg) and III (15 µg) refer to the corresponding fractions of Fig. 20, and are compared to the supernatant of a heat-precipitated, 10 min plasmin digest of fibrinogen (REF) with bands 1-6 indicated. Fractions I and II contain soybean trypsin inhibitor (SBTI). Samples A were non-reduced, while samples B were reduced with dithiothreitol prior to electrophoresis.

with those described by Pizzo et al. (272). The results showed that the polypeptide chain composition of these preparations were similar to other preparations described in the literature, except for the chains of fragment Y which could not be identified, although the bands of molecular weights 26,000 and 13,000 may be similar to the α' and (α^E, β^E, γ^E) bands, respectively, of Pizzo et al. (272).

4.3.2.2 Preparation and electrophoretic properties of the low molecular weight polypeptides

The supernatants after heat-precipitation of the 1½ min, 3 min and 4 hour plasmin digests of fibrinogen were applied to Sephadex G-100 columns and the elution profiles are shown in Fig. 20A-C. In all three cases, SDS-polyacrylamide gel electrophoresis indicated that the first peak consisted of several high molecular weight species, as previously reported (155), which had not been precipitated by the heat-treatment. These species might have been highly associated or crosslinked fibrinogen fragments, although only a weak reaction with anti-fibrinogen antiserum was obtained on immunodiffusion. In the case of the 4 hour digest (Fig. 20C), the second peak on the protein elution profile was due to fragment E, which is heat-stable (155).

The polypeptide fractions of interest were pooled as indicated and the electrophoretic patterns of each of the preparations, before and after disulphide reduction, are shown in Fig. 21. The unfractionated supernatant from a heat-precipitated, 10 min plasmin digest of fibrinogen was used as a reference, as the polypeptide bands 1-6 were clearly visible in this sample. Besides some incompletely separated high molecular weight material, the 1½ min digest preparation contained two major polypeptides, corresponding in size to bands 1 and 2 (molecular weights 44,000 and 33,000, respectively), while the 3 min digest preparation consisted of band 2 (molecular weight 33,000), along with a

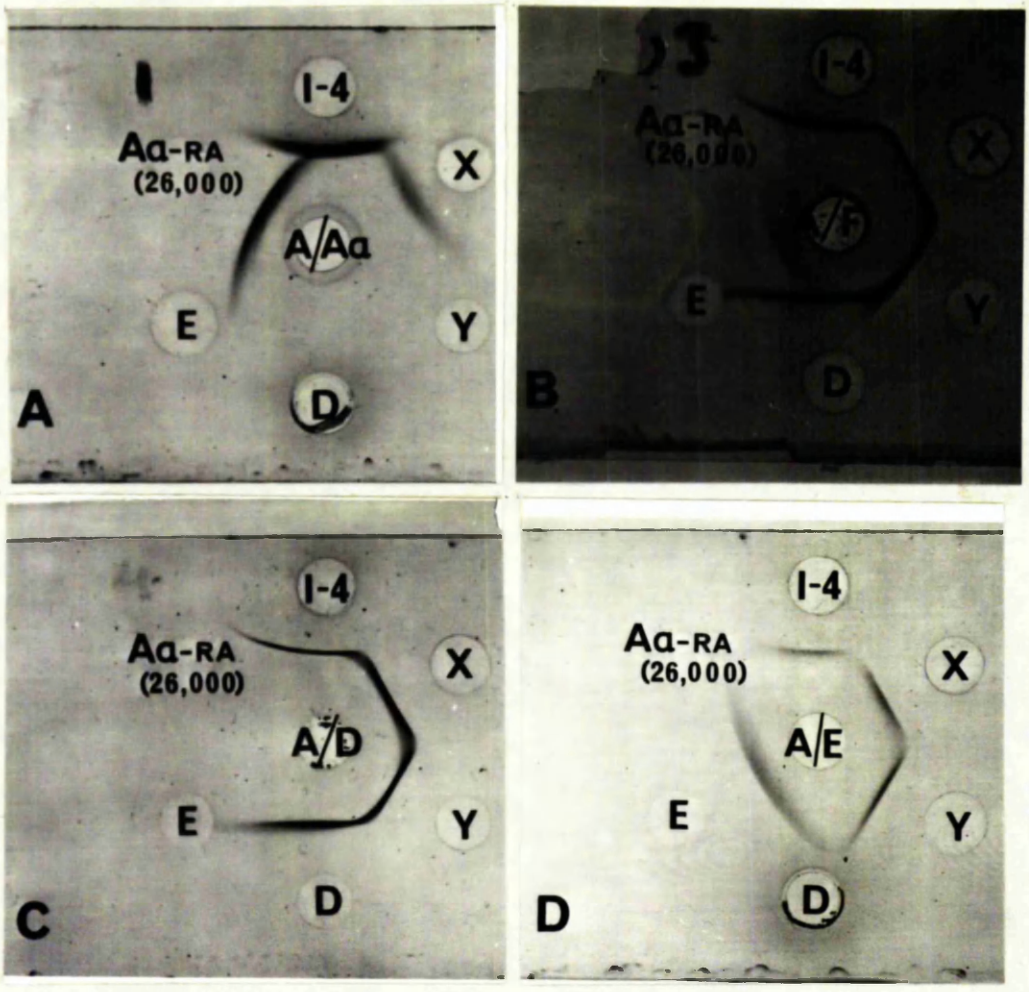


Fig. 22 Immunodiffusion with fibrinogen and purified degradation products. The antigens were applied at optimum concentrations for precipitin formation or at higher concentrations in attempts to produce precipitin formation. The agarose wells were filled with 8 µl samples as follows:

A. A/Aα, anti-Aα antiserum; I-4, fibrinogen fraction I-4, 1.3 mg/ml; X, fragment X, 4 mg/ml; Y, fragment Y, ca. 4 mg/ml; D, fragment D, 5 mg/ml; E, fragment E, 4 mg/ml; Aα-RA(26,000), the 26,000 molecular weight polypeptide, 0.06 mg/ml.

B. A/F, anti-fibrinogen antiserum; I-4, 0.9 mg/ml; X, 0.5 mg/ml; Y, ca. 1 mg/ml; D, 0.5 mg/ml; E, 0.3 mg/ml; Aα-RA(26,000), 2.2 mg/ml.

C. A/D, anti-fragment D antiserum; I-4, 0.9 mg/ml; X, 0.5 mg/ml; Y, ca. 2 mg/ml; D, 0.5 mg/ml; E, 4 mg/ml; Aα-RA(26,000), 2.2 mg/ml.

D. A/E, anti-fragment E antiserum; I-4, 0.9 mg/ml; X, 0.5 mg/ml; Y, ca. 1 mg/ml; D, 5 mg/ml; E, 0.3 mg/ml; Aα-RA(26,000), 2.2 mg/ml.

small amount of band 1. In both these preparations, the low molecular band was due to the presence of soybean trypsin inhibitor. Densitometric scanning of the gels revealed a band 1: band 2 ratio of 4:1 for the 1½ min digest preparation and 1:3 for the 3 min digest preparation. The 4 hour digest preparation revealed only a single band of molecular weight 26,000 (corresponding to band 3). Each of these polypeptides appeared to consist of a single chain, since there were no significant increases in electrophoretic mobility after disulphide bond reduction with dithiothreitol.

4.3.2.3 Immunological results

The immunodiffusion results on the purified fibrinogen fragments, shown in Fig. 22, indicated that the 26,000 molecular weight fragment reacted with anti-Aα antiserum only, and not with commercial anti-fibrinogen, anti-D or anti-E antisera, even at relatively high antigen concentrations; no reaction was observed with anti-fibrinogen antiserum in the presence of calcium ions. However, a faint precipitation was observed with anti-fibrinogen antiserum produced in this laboratory and the 26,000 molecular weight fragment. Similar results were obtained for the polypeptide fractions of the 1½ and 3 min digest preparations.

On account of the high purity (as judged by SDS-polyacrylamide gel electrophoresis) of the 26,000 molecular weight polypeptide, the most meaningful observations have come from studies on this fragment. Since it appeared to be derived from the Aα chain of fibrinogen, the author has named it Aα-related antigen (molecular weight 26,000), (Aα-RA(26,000)). In view of the similar immunological reactivity of the 44,000 and 33,000 molecular weight polypeptide fractions (although impure), and in accordance with the nomenclature adopted for the purified band 3 polypeptide, the band 1 and band 2 polypeptides have been tentatively named Aα-RA(44,000) and Aα-RA(33,000), respectively.

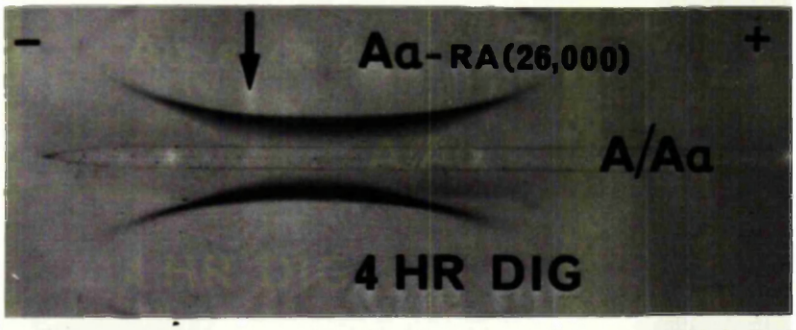
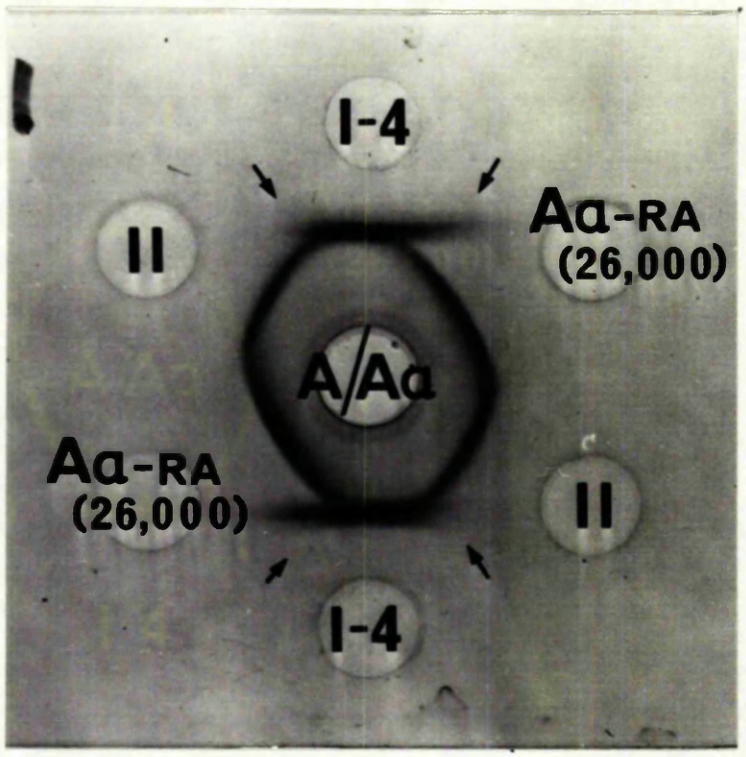


Fig. 23 Immunoelectrophoresis of Aα-RA(26,000) (2 μg) and a 4 hour plasmin digest of fibrinogen (4 HR DIG) (20 μg), using anti-Aα antiserum (A/Aα). The samples were applied on the agarose gel at the position shown by the arrow.

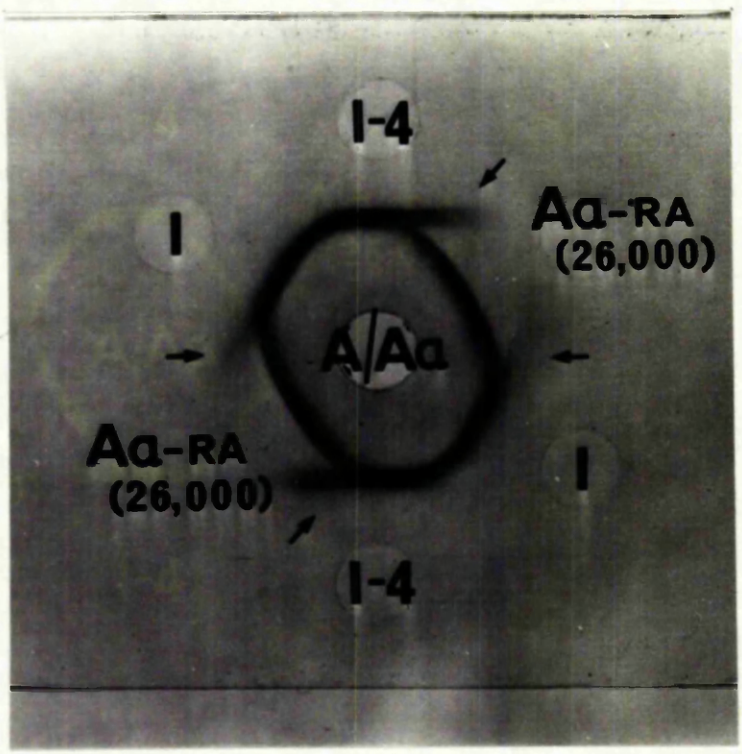
In Fig. 22A, anti-Aα antiserum reacted with fibrinogen fraction I-4, Aα-RA(26,000), and also with fragment X, which, however, yielded only a weak precipitin line at high antigen concentration. In addition, the precipitin line formed by fibrinogen spurred over those of Aα-RA(26,000) and fragment X, indicating that these fragments possessed fewer Aα antigenic determinants than the intact Aα chain in fibrinogen. This result was confirmed in immunodiffusion by the spurring of isolated carboxymethylated Aα chain over both fragment X and Aα-RA(26,000) (not shown). Fragments Y, D and E did not precipitate in agarose with anti-Aα antiserum, although one preparation of fragment Y produced a faint reaction.

The immunodiffusion results were confirmed by immunoelectrophoretic analysis of pure fibrinogen degradation products. When the troughs contained anti-Aα antiserum, precipitin arcs were obtained from fibrinogen fraction I-4, fragment X (very faint reaction, with electrophoretic mobility characteristic of fragment X) and Aα-RA (26,000), but not with fragments Y, D or E. Furthermore, as shown in Fig. 23, Aα-RA(26,000) produced a precipitin arc with an electrophoretic mobility identical to that of the major (cathodal) arc of a 4 hour plasmin digest of fibrinogen.

The Aα antigenicity of the 44,000 and 33,000 molecular weight polypeptide preparations was also investigated by immunodiffusion. Fig. 24A shows that fibrinogen fraction I-4 spurred over both Aα-RA (26,000) and the polypeptide preparation from the 3 min digest, which largely consisted of Aα-RA(33,000); furthermore, these latter two species appeared to show a reaction of identity. However, in Fig. 24B, a reaction of identity was observed between fibrinogen and the polypeptide preparation from the 1½ min plasmin digest, which was largely Aα-RA(44,000). Moreover, this preparation now spurred over Aα-RA(26,000).


I-4
II
Aα-RA
(26,000)
A/Aα
Aα-RA
(26,000)
II
I-4

A


I-4
I
Aα-RA
(26,000)
A/Aα
Aα-RA
(26,000)
I
I-4

B

Fig. 24 Immunodiffusion experiments with fibrinogen and Aα chain polypeptides. The centre wells were filled with anti-Aα antiserum (A/Aα) and the peripheral wells were filled with 8 µl samples as follows: fibrinogen fraction I-4, 1.3 mg/ml; Aα-RA(26,000), 0.1 mg/ml; polypeptides from the 1½ min plasmin digest of fibrinogen (I), 0.3 mg/ml; polypeptides from the 3 min plasmin digest of fibrinogen (II), 0.3 mg/ml. (Samples I and II refer to the pooled fractions of Fig. 20.) The arrows indicate spur formation between the precipitin lines.

4.4 Discussion

4.4.1 Electrophoretic analysis of plasmin digestion of fibrinogen

As shown in Chapter 3, anti-Aα antiserum precipitates in agarose gel with the isolated Aα chain and the intact Aα chain in human fibrinogen, in which most or all of the Aα epitopes appeared to be exposed. Therefore, with the use of this antiserum, it was considered possible to study the fate of the Aα antigenic determinant sites by immunoelectrophoretic analysis during plasmin degradation of human fibrinogen.

In order to relate the observations on the Aα chain to a known sequence of events, the progress of the degradation was also studied by SDS-polyacrylamide gel electrophoresis and immunoelectrophoresis using anti-fibrinogen antiserum. SDS-polyacrylamide gel electrophoresis demonstrated clearly the degradation of intact fibrinogen to the transient intermediate fragments X and Y and then to the final products, fragments D and E. The appearance of fragment E coincided with the formation (after 40 min digestion) of a second, non-identical precipitin arc on immunoelectrophoresis. However, the small fork on the main arc which distinguishes fragments X and Y from fragment D (222) was absent, possibly because the anti-fibrinogen antiserum used appeared to have a low content of anti-fragment E antibodies, as shown by the relatively weak fragment E immunoprecipitin arc (Fig. 15A). The use of anti-Aα antiserum confirmed the early plasmin cleavage of the Aα chain of fibrinogen, as established by others using a different technique (106, 234, 272) and, since two intersecting precipitin arcs were obtained, demonstrated that separate antigenic fragments of the Aα chain, with no common determinant sites, were formed (Fig. 15B).

Coincident with the cathodal shift of the initial precipitin arc

on immunoelectrophoresis against anti-Aα antiserum was the concomitant appearance of fragment X and a small polypeptide (band 1, molecular weight 44,000) on the polyacrylamide gel of the 2 min reaction mixture (Fig. 16). However, this band was degraded with time so that, after 5 min digestion, a series of low molecular weight bands (bands 2-6) appeared on the gels while two precipitin arcs formed on immunoelectrophoresis (Fig. 15B). Densitometry of the polyacrylamide gels revealed the progressive nature of the appearance of bands 1-6 (molecular weight 44,000- <14,000), followed by their disappearance, excepting bands 3 and 6 which appeared to be plasmin-resistant. Purification of the band 3 material, followed by immunoelectrophoresis with anti-Aα antiserum, revealed that it corresponded in electrophoretic mobility and in immunological reactivity to the more cathodal arc of a late fibrinogen digest. The band 3 polypeptide reacted only with anti-Aα antiserum on immunodiffusion and consisted of a single chain polypeptide. On this basis, therefore, an immunological nomenclature has been employed and the author has named the purified band 3 polypeptide, Aα-related antigen (molecular weight 26,000), (Aα-RA(26,000)); this fragment is undoubtedly very similar to one of the plasmin-derived fragments Hi2-Ala (molecular weight 23,000) (155) or Hi2-Met (molecular weight 21,000) (38), which have, however, been characterised by their amino acid compositions and NH_2-terminal amino acid sequence analyses. Other Aα chain fragments have been described in recent years, viz. the plasmin-derived fragments A (265, 317, 320) and H (137) (both of which appear to be similar or identical to Hi2-Met) and the cyanogen bromide-derived fragments Hi2-DSK (155) and F-CB3 (122) (both of which are immunologically related to the plasmin-produced derivatives).

While these fragments have molecular weights in the range 20,000-26,000, there have been several reports of plasmin-cleaved fragments of

the Aα chain with higher molecular weights and which appear earlier in the digestion process than the former fragments (55, 85, 98, 137, 155, 170, 235, 244, 306). In particular, Hessel (155) has established that a precursor-product relationship existed between early plasmin digest fragments (average molecular weight 50,000) and Hi2-Ala, and Ferguson et al. (85) have demonstrated a series of five α-derived fragments with molecular weights 21,000-40,000, resulting from initial plasmin cleavage of fibrin. Shen et al. (306) have described the first event in the plasmin degradation of fibrinogen as the release of a 44,000 molecular weight polypeptide from the COOH-terminal end of the Aα chain, while examination of the electropherograms of Kang and Triantaphyllopoulos (170) revealed the progressive release of polypeptides of molecular weights >33,000, 33,000 and 25,000 from plasmin digests of fibrinogen, thus providing adequate support for the results reported here. These electrophoretic and immunodiffusion results revealed the likely Aα chain origin of the polypeptide bands 1 and 2. Attempts to separate pure band 1 and band 2 material have not been entirely successful, probably because of their lability (137) and because of the similarity of their molecular weights. However, since these preparations from the 1½ min and 3 min digests consisted largely of band 1 and band 2, respectively, they were used in comparative studies of the Aα antigenic sites which are located on these polypeptides; they were tentatively named Aα-RA(44,000) and Aα-RA(33,000), respectively, in accordance with their antigenic characteristics.

4.4.2 Reactivity of fragments X, Y, D and E with anti-Aα antiserum

To locate other exposed antigenic sites of the Aα chain, the reactions of the purified degradation products of fibrinogen with anti-Aα antiserum were investigated by immunodiffusion. Fragment X at high

antigen concentration produced a faint reaction with this antiserum, and the subunit structure on electrophoresis was shown to consist of an unresolved β chain fragment and a γ chain of molecular weight ca. 48,000 along with a NH_2-terminal Aα chain remnant of molecular weight 26,000. It has been suggested that the Aα remnant of fragment X preparations may exhibit considerable size heterogeneity (98, 272), especially since Mosesson et al. (246) have recognised at least ten proteolytic cleavage sites on the Aα chain of fibrinogen, and the observed weak reaction of this fragment with anti-Aα antiserum may, in fact, derive from a species of fragment X with a NH_2-terminal Aα remnant of molecular weight $>26,000$. This result will be discussed more fully in Chapter 6.

The fragment Y preparation used in these studies did not react with anti-Aα antiserum, although a fragment Y preparation, isolated from an earlier digest, did show a faint precipitin line, identical with that of fragment X. These results remain unexplained at present, especially since the polypeptide bands of reduced fragment Y on SDS-polyacrylamide gel electrophoresis could not be assigned with any certainty to their chains of origin. Indeed, some discrepancies in the proposed sizes of the polypeptide components of fragment Y are evident in other studies (100, 218, 244).

Neither fragments D nor E produced a reaction in agarose with anti-Aα antiserum, although fragment D possessed an internal segment of the Aα chain (molecular weight 14,000) and fragment E contained the NH_2-terminal Aα remnant (molecular weight $<13,000$). By comparison, the NH_2-terminal disulphide knot of fibrinogen, N-DSK, which closely corresponds in its location in the fibrinogen molecule to fragment E (186), has been shown to react with anti-Aα antiserum in a radioimmunoassay (199, 289). However, it has been shown by Kudryk et al. (188) that

there are considerable differences in immunological behaviour between N-DSK and fragment E.

4.4.3 Antigenicity of Aα-related antigens with respect to:

4.4.3.1 anti-Aα antiserum

Since fragments D and E, and possibly fragment Y, do not react with anti-Aα antiserum, it appears that few antigenic determinants are located or exposed in the NH_2-terminal section of the Aα chain in these fragments. It might be that many of the determinants reside on the remaining 40,000-50,000 molecular weight section of the COOH-terminal of the Aα chain. Certainly, the strong precipitin lines obtained with Aα-RA(26,000) and the Aα-RA(33,000) and Aα-RA(44,000) preparations, at low antigen concentrations on immunodiffusion, support this view. Indeed, Gollwitzer et al. (120) have suggested that F-CB3, a cyanogen bromide-derived Aα fragment from bovine fibrinogen, contained most of the antigenic determinants of the Aα chain. On the other hand, the spur, formed by intact fibrinogen over the precipitin line of the Aα-RA(26,000) (Fig. 22A), and the formation of a second precipitin arc on immunoelectrophoresis, using anti-Aα antiserum (Fig. 15B), indicated the existence of Aα antigenic sites other than those present on Aα-RA(26,000). Immunodiffusion studies also showed that the Aα-RA(33,000) preparation, largely band 2 material, was similarly deficient in Aα antigenic determinants, and might be immunologically identical to Aα-RA(26,000) (Fig. 24A). However, the Aα-RA(44,000) preparation, largely band 1 material, possessed more Aα antigenic determinants than Aα-RA(26,000) and was immunologically identical to fibrinogen (Fig. 24B), suggesting that most or all of the antigenic determinants of the Aα chain might be located on the fragment Aα-RA(44,000). (N.B. The species responsible for the anodal precipitin arc on immunoelectrophoresis has

not yet been identified, but it was not due to the reaction of fragment X, on account of its differing electrophoretic mobility and because the arc persisted throughout the digestion of fibrinogen, even at a time when fragment X had been fully degraded. It is possible that this electrophoretically distinct Aα-related antigen is formed during proteolytic cleavage of the 44,000 molecular weight fragment and will be found among the fragments constituting the electrophoretic bands 4-6.)

4.4.3.2 anti-fibrinogen antiserum

The partial physical and immunological characterisation of Aα-RA(26,000) revealed the expected similarities of this fragment to the Hi2 fragments of Hessel (155) in that they reacted with anti-Aα antiserum, they had similar molecular weights, and they were single chain polypeptides. However, both Hi2-Ala and a precursor 50,000 molecular weight fragment reacted with anti-fibrinogen antiserum (155), whereas the Aα-related antigens of molecular weights 44,000, 33,000 and 26,000 did not precipitate on immunodiffusion, even over wide concentration ranges (0.03-2.2 mg/ml) and using anti-fibrinogen antiserum obtained from Hoechst Pharmaceuticals, Nordic Diagnostics, Dakopatts A/S and Calbiochem. This difference in the properties of Aα chain fragments may also be found by comparing the results of previous studies. Mosesson et al. (244) have shown that COOH-terminal fragments of the Aα chain reacted with anti-fibrinogen antiserum on immunoelectrophoresis, while Timpl et al. (329) reported that 20% of antibodies in anti-bovine fibrinogen antiserum react with the Aα fragment, bovine F-CB3, (but the species difference must also be considered here, especially in view of the finding that human F-CB3 and bovine F-CB3 were not immunologically related (203)). On the other hand, the present observations on Aα-RA(26,000) are supported by the findings of Nussenzweig et al. (264), Ekert and Muntz (82) and Takagi and Kawai (317) who failed to find

reactions between anti-fibrinogen antiserum and the small fibrinogen degradation products, A, B and/or C, which are derived from the Aα chain of fibrinogen (272). The discrepancy in these observations may be due simply to the differing sensitivities of immunological techniques, or it may be due to differences in the anti-fibrinogen antisera. In many laboratories, it is common practice to absorb anti-fibrinogen antiserum with normal human serum, which, however, has recently been shown to contain small amounts of antigenic Aα fragments (38, 119, 138, 318). Therefore, it is possible that, in the absorption process, some anti-fibrinogen antibodies may be unwittingly removed along with other undesired antibodies. Studies, in this laboratory, using unabsorbed anti-fibrinogen antiserum, have produced weak reactions with Aα-RA (26,000) and the Aα-RA(33,000) and Aα-RA(44,000) preparations.

Attempts to distinguish fibrinogen, fragment X and fragment Y immunologically by gel diffusion using various anti-fibrinogen antisera were unsuccessful, since antigenic identity was always observed (Fig. 22B). Similar results were found by Marder (216), but a later study revealed partial identity between fibrinogen on the one hand and fragments X and Y on the other (222). At present, however, there appears to be little doubt, from other immunological procedures (108, 277), that the formation of fragment X from fibrinogen is accompanied by a considerable loss in the antigenic character of the species. Since the major loss of polypeptide material occurs from the Aα chain of fibrinogen during its degradation to fragment X, it seems likely that the loss in antigenic determinants is associated with this cleavage. However, in view of the absence or weakness of a reaction of anti-fibrinogen antiserum with Aα-related antigens (molecular weights 26,000-44,000) or with the intact carboxymethylated Aα chain (Results, Chapter 3), the immunogenic sites associated with the whole Aα chain in intact fibrino-

gen are probably largely conformational in nature, and may be associated with the Bβ and γ chains, as suggested previously (28). The results of immunodiffusion studies carried out by the author in Dr. P. J. Gaffney's laboratory in London support this hypothesis, since no reaction was apparent between the intact Aα chain and anti-COOH/Aα (antiserum directed to sites in the COOH-terminal aspect of the Aα chain and obtained by fragment D and E absorption of anti-fibrinogen antiserum) (104).

Nevertheless, antibodies have been raised against the isolated Aα chain in rabbits, but it is likely that the determinant sites were sequential in nature, since any native conformational structure would almost certainly have been disrupted by the extreme denaturing conditions encountered during purification of the immunogen. A similar suggestion has also been made for the Aα chain of bovine fibrinogen; the existence of intramolecular competition between conformational and sequential determinants in fibrinogen has been proposed to account for the apparent increase in immunogenicity of the Aα chain in the isolated form, compared with that of the Aα chain in intact bovine fibrinogen (121).

The calcium binding centres of fibrinogen, involving the COOH-terminal section of the Aα chain, may determine the conformation of this section (97, 223). However, as precipitation reactions of the COOH-terminal polypeptides of the Aα chain (molecular weights 26,000-44,000) were not observed in the presence of calcium ions, the reduced antigenic character of these species, with respect to (commercial) anti-fibrinogen antibodies, did not appear to be related to the loss of calcium ions from the calcium binding centres.

APPENDIX TO CHAPTER 4

Upon the recent submission of a paper concerning the fragment Aα-RA(26,000) by this author, the Editors of the European Journal of Biochemistry recommended the undertaking of an amino acid composition analysis of that fragment. Such an analysis was performed immediately prior to the binding of this thesis.

Methods

The amino acid composition analysis was performed on two samples (1.0 and 1.5 mg) of Aα-RA(26,000) (from different batches). After hydrolysis of each sample for 22 hours at 110°C in 1 ml 6M HCl and 0.1 ml of mercapto-acetic acid in evacuated, sealed tubes, the amino acid mixture was analysed in a Locarte Amino Acid Analyser (Scientific Instruments, London), which was based on the original design of Spackman, D. H., Stein, W. H. & Moore, S. (1958) (Analytical Chemistry, 30, 1190-1206).

Results and Discussion

Absolute concentrations of the amino acids were determined, but, for the purposes of comparison, the results are shown as molar percentages in the Table.

A small peak of unknown origin appeared in the ornithine position of elution. No methionine was found in the fragment, while tryptophan was not determined. The low value obtained for the half-cystine content indicated that the majority of the polypeptides did not contain this amino acid. Generally, the amino acid distribution indicated a low content of nonpolar amino acids, while the contents of glycine, serine, proline and threonine were high. These results are similar to the composition analyses obtained by others (137, 155, 317, 320) and provide further evidence for the identity of Aα-RA(26,000) to the fragments A and H.

Table

The amino acid composition of Aα-RA(26,000)[1]

Residue	Content
Aspartic acid	5.0
Threonine	10.5
Serine	20.5
Glutamic acid	7.6
Proline	12.7
Glycine	21.1
Alanine	2.7
Valine	2.4
Half-cystine	0.4
Isoleucine	0.6
Leucine	1.3
Tyrosine	1.1
Phenylalanine	1.9
"Ornithine"[2]	1.0
Lysine	2.0
Histidine	1.8
Arginine	7.3

[1] Values given are the means from two different batches of Aα-RA(26,000) and are presented as molar percentages.

[2] A small peak of unknown origin appeared in the orinthine position.

Acknowledgements

Grateful thanks are due to Dr. J. Shepherd and his staff, Department of Biochemistry, Glasgow Royal Infirmary, for assistance with the sample hydrolysis, and to Mr. B. Birrell of the same department for performing the amino acid composition analysis.

CHAPTER 5: GENERATION OF Aα FRAGMENTS IN HUMAN PLASMA

5.1 Introduction

In Chapter 4, studies in a purified system revealed that plasmin degradation of fibrinogen caused the release of Aα chain fragments from the COOH-terminal aspect of the polypeptide. In order to discover if the release of such fragments from fibrinogen occurred in a plasma environment, immunodiffusion with anti-Aα antiserum was used to study the effects of *in vitro* activation of the plasma fibrinolytic system by urokinase. In addition, a number of human sera from individuals, after *in vivo* activation of the plasma fibrinolytic system, have been studied briefly and the results are given here.

5.1.1 In vitro studies

When urokinase is added to human plasma in vitro, the fibrinolytic system is activated, generating plasmin which may digest formed fibrin clots. However, it was disputed for several years whether plasma fibrinogen was similarly degraded by the fibrinolytic enzyme system or was protected from proteolysis by fibrinolytic inhibitors, although, in purified systems, fibrinogen was readily cleaved to smaller, non-clottable derivatives.

In 1960, Bergström *et al.* (16) added urokinase to plasma in a case of liver cirrhosis to produce intense fibrinolysis. However, almost no fibrinogenolysis was measurable using a clottable fibrinogen assay. On the other hand, Fisher *et al.* (93) showed that considerable degradation of human plasma fibrinogen occurred upon plasminogen activation by urokinase, the extent of degradation being observed by immunoelectrophoresis. Several years later, Gallimore *et al.* (109) added urokinase to plasma at concentrations sufficient to cause rapid lysis of plasma fibrin clots, but these workers were unable to detect

the breakdown of a significant amount of plasma fibrinogen, since no FDP were measurable in the serum from such urokinase-treated plasma.

Recently, these doubts have been resolved and several investigators have demonstrated conclusively the degradation of fibrinogen in plasma by estimations of whole fibrinogen (131, 258) and by examination of the subunit structure of fibrinogen (206, 301) after incubation of urokinase in plasma. In addition, the latter studies (206, 301) have shown that the initial sequence of fibrinogen chain degradation is similar to that observed in purified systems (272) in which the earliest plasmin cleavage sites are located on the Aα chain of fibrinogen.

In this study, the plasma fibrinolytic system was activated with urokinase and, after clotting, the serum was examined for the presence of Aα-related antigens by means of anti-Aα antiserum.

5.1.2 In vivo studies

It might be reasonably expected that the level of FDP in human serum would be a useful measure of the extent of *in vivo* fibrin(ogen)olysis. However, when an immunochemical method for detecting FDP (fragments X, Y, D and E) was used as a means of testing for primary or secondary fibrinolysis (180, 196), unsatisfactory results were obtained in a group of patients with thrombotic and other diseases. There was no apparent correlation between the fibrinolytic activity (FDP measurements) and the course of the disease, and, therefore, no useful diagnostic or prognostic information was obtained from the results (196).

Attempts to elucidate the mechanism of fibrinogen/fibrin catabolism *in vivo* have been without success. While Mosesson (240) favoured fibrinogenolytic degradation by plasmin or plasmin-like enzymes as a major catabolic pathway, Collen *et al.* (61) claimed that Mosesson's partially degraded fibrinogen species were artefactual in nature.

Fibrinolytic enhancement by *in vivo* infusion of chemical agents may (66) or may not (215) produce an increase in circulating FDP. In the case of liver cirrhosis, Bergström *et al.* (16) demonstrated intense fibrinolytic activity in the absence of fibrinogenolysis, and Gurewich *et al.* (131) found that a potent plasminogen activator did not degrade plasma fibrinogen, but caused rapid lysis of plasma fibrin clots. Induction of activated fibrinolysis by tourniquet has resulted in an increase of circulating FDP in one case (49), but no significant change was observed after venous occlusion in other cases (47, 201). In addition, normal levels of FDP have also been found after venous occlusion (134, 144, 180).

In this second part of the study, human sera, before and after venous occlusion of the arm, have been examined by means of anti-Aα antiserum in order to detect cleaved Aα-related antigens.

5.2 Materials and Methods

5.2.1 Preparation of fibrinogen and fibrinogen fragments

Fibrinogen fraction I-4 was prepared as described in Chapter 2. Aα-RA(26,000) and the preparation Aα-RA(44,000), which was contaminated with Aα-RA(33,000), were obtained from plasmin digests of fibrinogen, as described in Chapter 4.

5.2.2 Antisera to fibrinogen and the Aα chain

Antiserum to the carboxymethylated Aα chain was raised in rabbits as described in Chapter 3. Antiserum to human fibrinogen was obtained from Hoechst Pharmaceuticals.

5.2.3 Immunodiffusion, immunoelectrophoresis and SDS-polyacrylamide gel electrophoresis

These techniques were carried out as described in Chapters 2 and

3.

5.2.4 In vitro activation of plasma fibrinolytic system

1 ml normal citrated plasma was incubated at 37°C with 0.02 ml urokinase, yielding a final concentration of approximately 100 Ploug units/ml. 0.02 ml 0.15M NaCl was incubated with 1 ml of the same plasma as control. After 1 hour, the induced fibrinolytic activity in both samples was inhibited by aprotinin (final concentration 200 u/ml). Both plasmas were clotted with bovine thrombin (final concentration 10 NIHu/ml) and the sera were obtained by removal of the clots and centrifugation of the samples. Serum from the plasma incubated with urokinase was designated S-UK and serum from the control plasma was designated S-NaCl.

The physico-chemical properties of the Aα-related antigen(s) in human serum were examined by fractional precipitation by saturated ammonium sulphate, gel filtration on Sephadex G-100 and SDS-polyacrylamide gel electrophoresis of the serum immunoprecipitates, according to Gaffney et al. (105). The immunological behaviour of Aα-related antigen(s) in human serum was examined by immunodiffusion and immuno-electrophoresis.

5.2.5 In vivo activation of plasma fibrinolytic system

A local increase in fibrinolytic activity in one arm was induced by means of venous occlusion, according to Walker et al. (334), and a sample of blood was obtained after 15 min. A sample of blood was obtained from the non-occluded arm before application of the sphygmomanometer cuff. The sera, obtained from a group of 28 normal volunteers and patients in this hospital, were treated as described in Chapter 3. The fibrinolytic activities of the corresponding plasma samples were assessed by measuring areas of lysis produced by

resuspended euglobulin precipitates on plasminogen-rich bovine fibrin plates (334).

The immunological behaviour of Aα-related antigen(s) in the pre- and post-occlusion sera was examined by immunodiffusion, single radial immunodiffusion (214) and Laurell electrophoresis (194). In addition, the plasma from an afibrinogenaemic patient (no detectable fibrinogen by single radial immunodiffusion) was examined by immunodiffusion.

Carbamylation of the serum samples was performed with potassium cyanate, as described elsewhere (260).

5.2.6 FDP assay

Non-clottable fibrin(ogen) degradation products were estimated in serum by the tanned red cell haemagglutination inhibition assay (233), using the Wellcome FDP Kit.

5.2.7 Ethanol gelation test

The detection of soluble fibrin monomer was performed according to the modification of Gurewich *et al.* (134).

5.2.8 Reagents

Urokinase was obtained from Leo Pharmaceutical Products and bovine thrombin from Parke-Davis.

5.3 Results

5.3.1 In vitro studies

S-UK and S-NaCl (sera obtained from the incubation of plasma with urokinase and saline, respectively) were examined qualitatively for the presence of fibrinogen- and Aα-related antigens by means of immunodiffusion against the appropriate antisera. Fig. 25 shows that

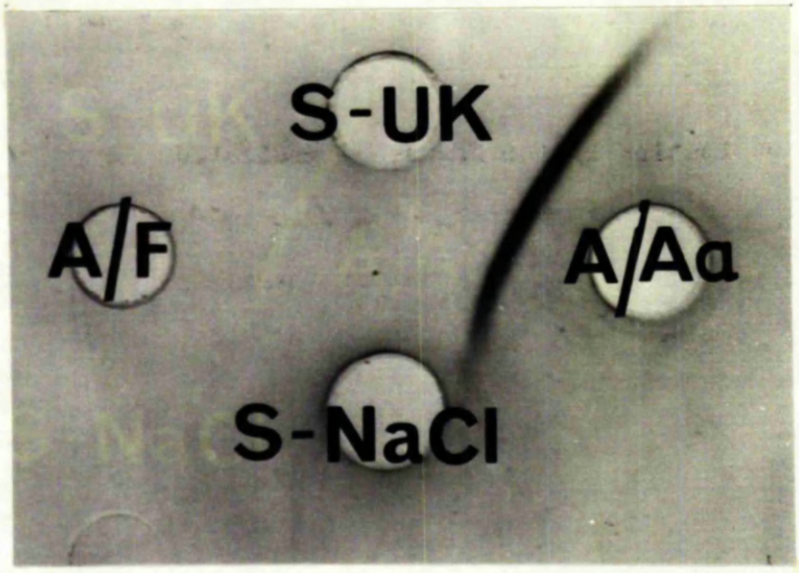


Fig. 25 Immunodiffusion of S-UK and S-NaCl with anti-fibrinogen (A/F) and anti-Aα (A/Aα) antisera.

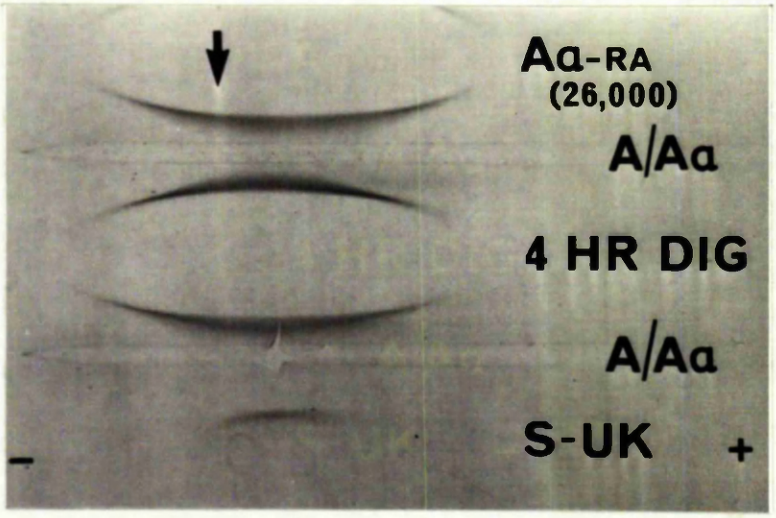


Fig. 26 Immunoelectrophoresis of Aα-RA(26,000) (2 mg/ml), a 4 hour plasmin digest of fibrinogen (4 HR DIG) (20 mg/ml) and S-UK, using anti-Aα antiserum (A/Aα). The 1 µl samples were applied on the agarose gels at the position shown by the arrow.

neither S-UK nor S-NaCl reacted with anti-fibrinogen antiserum, whereas quantitative estimation by the FDP assay indicated a small rise in fibrinogen degradation products in S-UK (Table IV). On the other hand, both S-UK and S-NaCl reacted with anti-Aα antiserum. However, the precipitin line of S-NaCl was very faint and cannot easily be seen in Fig. 25, while that of S-UK was quite distinct. It was not possible to ascertain if these two precipitin lines exhibited reactions of identity.

Table IV

FDP levels in serum, after incubation of the plasma samples in the presence or absence of urokinase (means of 4 samples)

Sample	FDP level (μg/ml)
S-NaCl	5.0
S-UK	10.0

Salt precipitation with saturated ammonium sulphate revealed that most of the Aα-related antigen(s) in S-UK precipitated at 40-50% saturation, along with a smaller amount at 30-40% saturation.

A small molecular size was indicated for the antigen(s) in S-UK by their elution volume on gel filtration; when the Aα-RA(44,000) preparation and S-UK were chromatographed on the same column of Sephadex G-100, similar elution volume/void volume ratios were obtained (V_e/V_o = 1.38 for S-UK and 1.31 for the Aα-RA(44,000) preparation).

A more accurate measurement of the molecular weight of the antigenic species by SDS-polyacrylamide gel electrophoresis of the immunoprecipitate obtained after incubating anti-Aα antiserum with S-UK was not possible, because of the large number of contaminant bands besides that of IgG, despite repeated careful washing of the precipitate.

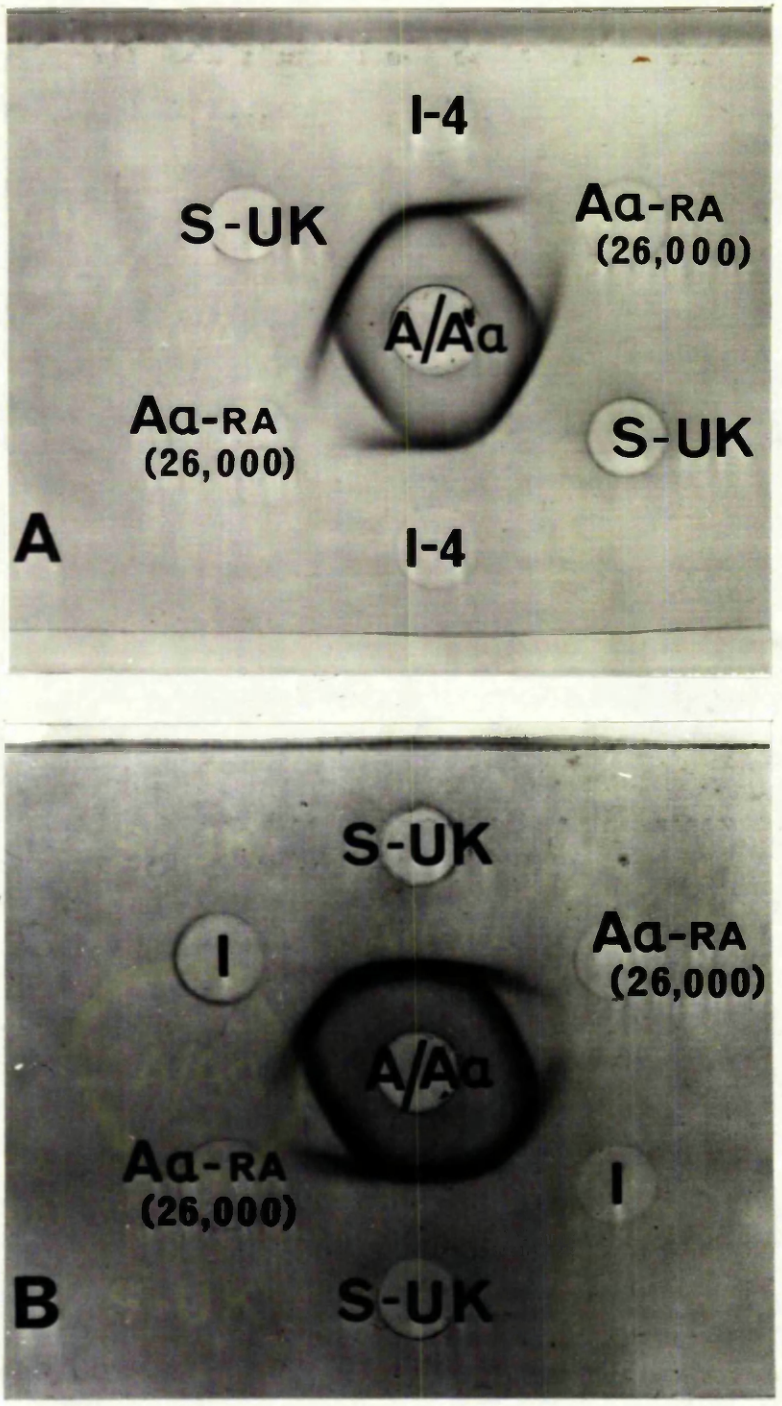


Fig. 27 Immunodiffusion of fibrinogen fraction I-4 (1.3 mg/ml), Aα-RA(26,000) (0.05 mg/ml), polypeptides from the 1½ min plasmin digest of fibrinogen (I), (0.3 mg/ml), and S-UK with anti-Aα antiserum (A/Aα).
(Sample I refers to the pooled fraction of Fig. 20A.)

Immunoelectrophoresis established that the Aα-related antigen(s) in S-UK had the same mobility as the fragment Aα-RA(26,000), which, in turn, corresponded to the major (cathodal) immunoprecipitin arc of a 4 hour plasmin digest of fibrinogen (Fig. 26). Similar electrophoretic experiments with anti-Aα antiserum indicated that the S-UK antigen(s) and the Aα-RA(44,000) preparation, as well as Aα-RA(26,000), possessed identical electrophoretic mobilities.

The immunological behaviour of the Aα-related antigen(s) in S-UK was further investigated by gel diffusion, using anti-Aα antiserum. Fig. 27A shows that both fibrinogen fraction I-4 and the Aα-related antigen(s) in S-UK spurred over Aα-RA(26,000), while a reaction of identity was observed between S-UK and fibrinogen fraction I-4. When the Aα-related antigens in S-UK were compared with the Aα-RA(44,000) preparation, a reaction of identity was obtained (Fig. 27B). In addition, the latter preparation showed spur formation over Aα-RA(26,000).

5.3.2 In vivo studies

Limited studies on a number of serum samples from normal individuals and patients have been performed, although no attempt was made to distinguish these two groups.

By double diffusion analysis, 77 serum samples have been examined. Faint precipitin lines were observed in each case when anti-Aα antiserum was employed, but none were seen with anti-fibrinogen antiserum. The intensity of these precipitin lines usually increased when the post-occlusion serum was compared with the pre-occlusion sample (28 pairs of samples). However, no significant increase in FDP levels were found by the tanned red cell haemagglutination inhibition assay. In the few pre- and post-occlusion samples which were examined by the ethanol gelation test, no demonstrable fibrin monomer was present. No

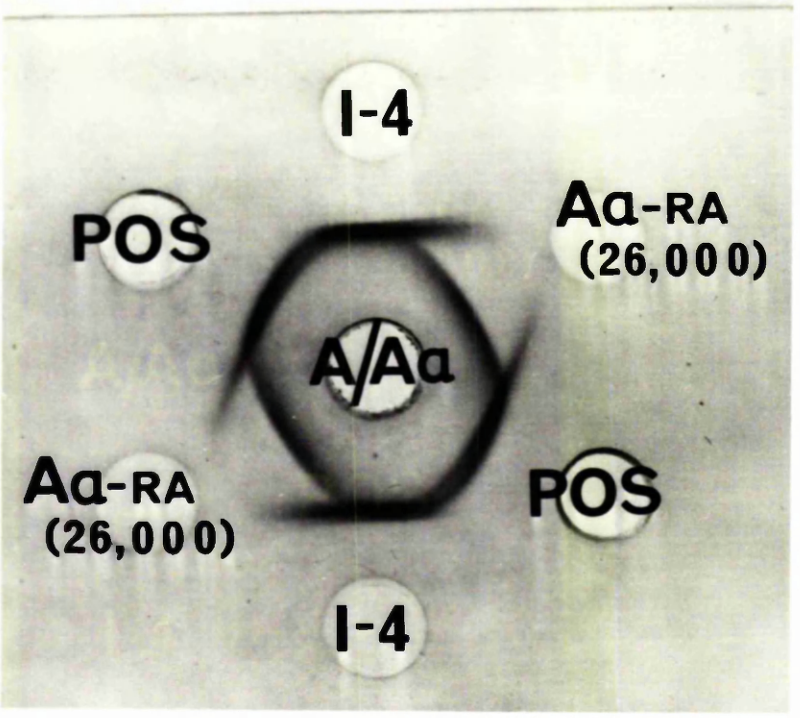


Fig. 28 Immunodiffusion of fibrinogen fraction I-4 (1.3 mg/ml), Aα-RA(26,000) (0.05 mg/ml) and the concentrated post-occlusion serum sample from D.McD. (POS) with anti-Aα antiserum (A/Aα).

precipitin line was observed when afibrinogenaemic plasma was investigated with either anti-fibrinogen or anti-Aα antisera.

Two post-occlusion serum samples were 2x concentrated by means of a B15 Amicon Concentrator and their immunological behaviour was compared with that of fibrinogen fraction I-4 and Aα-RA(26,000). As an example, Fig. 28 shows that the serum Aα-related antigen(s) contain more determinant sites than Aα-RA(26,000) and appear to be more closely related to fraction I-4, thus behaving in an analogous manner to the Aα-RA(44,000) preparation. In fact, there may be two Aα-related antigens in such serum samples, since closer examination of Fig. 28 reveals that part of the serum precipitin line does not meet that of fibrinogen in a line of complete identity. As yet, the nature of the Aα-related antigen(s) in pre-occlusion serum has not been investigated.

Single radial immunodiffusion clearly indicated an increase in the level of Aα-related antigen(s) in the post-occlusion serum samples (Fig. 29; the origin of the inner precipitin ring is not known at present). The illustrated example showed an increase in precipitin area in the post-occlusion sample of approximately 100%, while the fibrinolytic activity of the corresponding plasma euglobulin fraction increased by 480%. In two cases, venous occlusion produced no increase in the fibrinolytic activity of plasma euglobulin fractions and, correspondingly, no increase in the areas bounded by the precipitin rings of the post-occlusion serum samples.

Attempts have also been made to quantitate the levels of Aα-related antigen(s) in pre- and post-occlusion sera by Laurell electrophoresis. Although the electrophoretic conditions have not yet been perfected, Fig. 30 indicated the large increase in Aα-related antigen(s) when the post-occlusion sample was compared with the pre-occlusion sample. The imperfectly formed Laurell "rockets" were probably due to

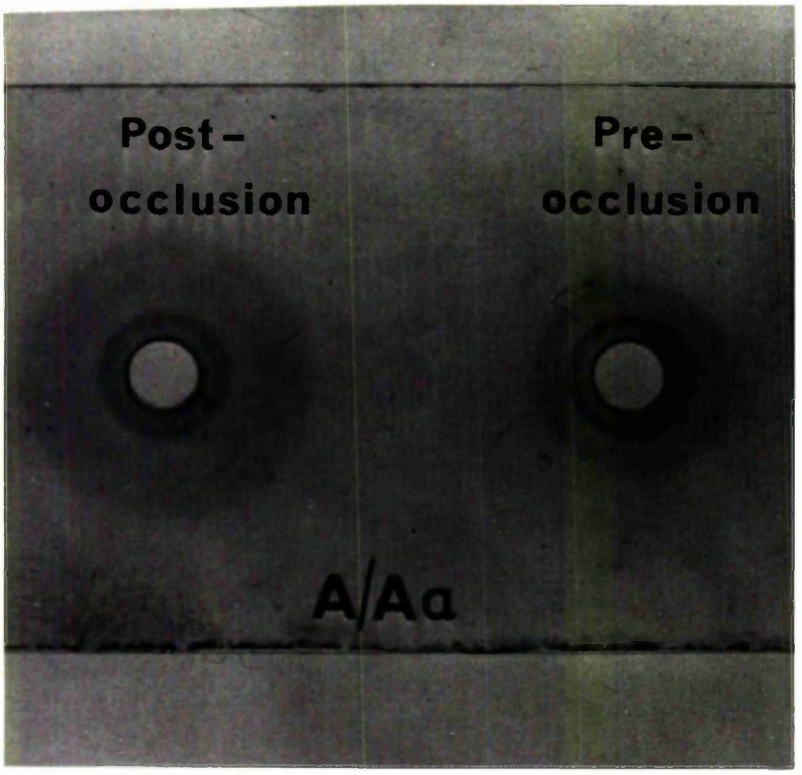


Fig. 29 Single radial immunodiffusion of serum samples from patient W.M. Pre-occlusion serum was obtained from a non-occluded arm and post-occlusion serum was obtained from the other arm after 15 min venous occlusion. 10 µl serum samples were applied to the agarose gel, which contained 2% v/v anti-Aα antiserum (A/Aα).

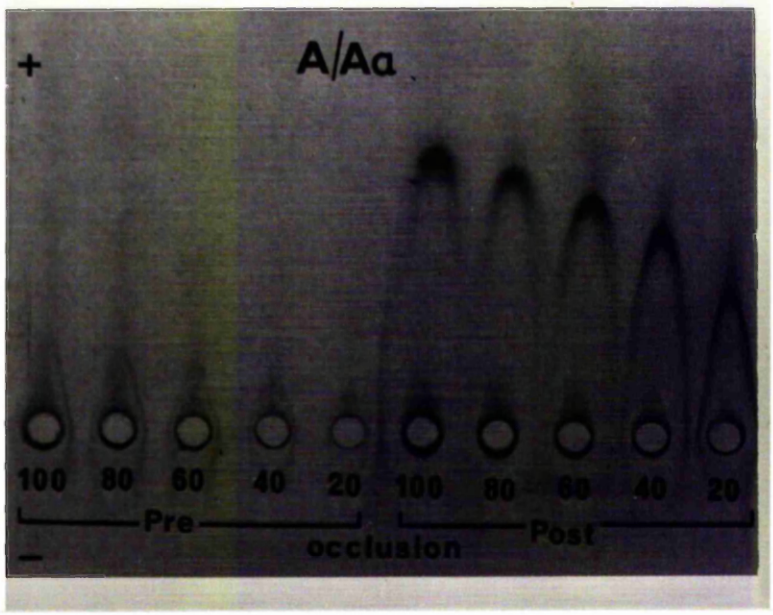


Fig. 30 Laurell electrophoresis of 100%, 80%, 60%, 40% and 20% dilutions of serum samples from patient H.C. Pre-occlusion serum was obtained from a non-occluded arm and post-occlusion serum was obtained from the other arm after 15 min venous occlusion. 10 µl serum samples were applied to the agarose gel, which contained 2% v/v anti-Aα antiserum (A/Aα). Electrophoresis was conducted overnight (17 hours) at 150V.

rapid diffusion of the antigen(s) because of their low molecular weight and/or the low electrophoretic mobility of the antigenic species (cf. the low electrophoretic mobility of Aα-RA(26,000) and S-UK antigen(s) in Fig. 26). Carbamylation of the serum samples, in order to increase the electrophoretic mobility, did not greatly alter the shape of the "rockets".

5.4 Discussion

5.4.1 In vitro studies

With a view to quantitating the amount and extent of fibrin(ogen)-olysis using anti-Aα antiserum, the effect on plasma fibrinogen of urokinase-induced activation of the plasma fibrinolytic system has been re-examined by an immunological technique. Instead of estimating the plasma fibrinogen or inspecting the molecular condition of plasma fibrinogen after incubation with urokinase, immunodiffusion has been used to detect qualitatively the presence or absence of fibrinogen- and Aα-related antigens in the resulting serum, after removal of the fibrin clot with thrombin.

The results showed that both S-UK and S-NaCl (sera obtained from plasma incubated with urokinase and saline, respectively) contained one or more species which demonstrated Aα antigenicity, but did not precipitate with anti-fibrinogen antiserum. While the precipitin line from the reaction of anti-Aα antiserum and S-NaCl was faint, that of S-UK was clearly visible (Fig. 25), indicating that activation of plasminogen by urokinase had resulted in considerable fibrinogenolysis with the release of Aα chain fragments from plasma fibrinogen. Over the period of observation, the extent of plasma fibrinogen degradation appeared to be limited, since only a low level of fibrinogen-related antigens were detected in the serum. (Although immunodiffusion with

anti-fibrinogen antiserum, as used here, is not capable of detecting fragment D or fragment E levels below ca. 30 µg/ml, the tanned red cell haemagglutionation inhibition assay is sensitive to 2 µg (fibrinogen equivalent) FDP/ml (233).

These results resembled those of Gallimore et al. (109) who found no evidence of FDP in human serum after incubation of plasma and urokinase (75 Ploug units/ml), and those of others (206, 301) who demonstrated extensive degradation of the Aα chain of clottable plasma fibrinogen after incubation with urokinase. Although Ly et al. (206) found low levels of serum FDP, they stated that failure to detect FDP in serum does not necessarily preclude fibrinogenolysis, while Konttinen et al. (181), in a study of streptokinase-induced fibrinolytic activity in plasma, have pointed out that, if the fragments liberated during fibrinogen degradation are related to the small A, B and C peptides (265, 272), they would probably not be measurable by the immunological methods currently used for the demonstration of fibrinogen-fibrin-related antigens.

Since S-UK contained polypeptides which exhibited Aα, but not fibrinogen, antigenicity, and since gel filtration studies indicated that the order of size of the antigen(s) was comparable to that of the purified Aα-related antigens, the immunological behaviour of these antigenic fragments in S-UK was compared with those of Aα-RA(26,000) and the Aα-RA(44,000) preparation. The electrophoretic mobility of the Aα-related antigen(s) in S-UK was the same as those of Aα-RA(26,000) (Fig. 26) and the Aα-RA(44,000) preparation. Immunodiffusion with anti-Aα antiserum revealed that the S-UK antigen(s) and Aα-RA(26,000) displayed only partial immunological identity, the S-UK antigen(s) possessing more Aα antigenic sites than the 26,000 molecular weight polypeptide (Fig. 27A). The reaction of identity with the Aα-RA(44,000)

preparation (Fig. 27B), which contained most or all of the antigenic sites of the Aα chain of fibrinogen, indicated that the S-UK antigen(s) were more closely related to the 44,000 molecular weight polypeptide than to the 26,000 molecular weight polypeptide.

5.4.2 In vivo studies

The existence of Aα chain fragments in every individual sera examined, except the afibrinogenaemic patient, was confirmed by immunodiffusion. In addition, the results of double diffusion analysis, single radial immunodiffusion and Laurell electrophoresis using anti-Aα antiserum revealed that venous occlusion generally produced an increase in the level of Aα-related antigen(s) in serum, and was accompanied by an increase in the fibrinolytic activity of the plasma, as assessed by fibrin plates. In two individuals, however, there was no increase in either plasma fibrinolytic activities or serum Aα-related antigens. Since detectable fibrin monomers were not generated by venous occlusion, these results indicated that activation of the plasma fibrinolytic system caused direct fibrinogenolysis, a result which is in direct contrast to those of others (16, 47, 109, 134, 201) and which would be overlooked by immunological techniques employing anti-fibrinogen antiserum. Although attempts at correlating the increases in Aα-related antigens in human sera with other haematological parameters (e.g. areas of lysis on fibrin plates) must await greater characterisation of the antigenic species, the magnitude of the increase of these antigenic species after venous occlusion was generally greater than can be accounted for by the effects of haemoconcentration.

Preliminary characterisation of Aα-related antigen(s) in human sera (Fig. 28) indicated at least one antigenic species which behaved immunologically in a manner similar to Aα-RA(44,000) and a possible

second species with fewer antigenic determinants. It is not yet known if greater plasma fibrinolytic activity and longer incubation times result in further degradation of the Aα fragments to species resembling Aα-RA(26,000).

5.4.3 Aα chain fragments in serum: recent investigations

The above study is relevant to some of the recent investigations of peptides which have been derived by enzymatic and chemical means from the Aα chain of fibrinogen (38, 119, 122, 137, 138, 155, 317, 318, 320). Some investigators have described the existence of Aα chain fragments in human serum and have indicated that their quantitation may have physiological and pathological significance (38, 119, 138, 318). While Gollwitzer *et al.* (119) have found a serum concentration range of 1-10 pmoles/ml for normal healthy subjects, Blombäck *et al.* (38), Harfenist *et al.* (138) and Takagi and Kawai (318) have described a range of 50-200 pmoles/ml. From a comparison of the precipitin ring sizes obtained for Aα-RA(26,000) with the ring sizes for normal serum in single radial immunodiffusion tests, approximate calculations for normal serum concentrations in this study agree more closely with the latter range.

Although previous authors have described an increase of serum Aα fragment concentrations in various coagulation disorders (119, 138, 318) and in one Arvin-treated patient (38), no one has yet described such an increase after venous occlusion of the limbs, as was found in this study. Moreover, since some workers found that exercise-induced activation of the plasma fibrinolytic system leads to an increase in serum FDP levels (66, 86), while others have detected no change in serum FDP levels (5, 132, 185, 288), it will be interesting to re-examine this issue of exercise-induced fibrinogenolysis by means of an immunological assay

employing anti-Aα antiserum, especially in view of the recent finding of an increase in degraded Aα chains in plasma fibrinogen after exercise (60).

5.4.4 Speculation on a fibrinogenolytic mechanism

The mechanism of plasmin action, assuming that enzyme to be responsible for the observed degradation of fibrinogen during the in vivo and in vitro studies, is difficult to explain. During activation of the plasma fibrinolytic system in other studies (131, 132, 134, 201, 288), the special protection from degradation afforded to soluble fibrinogen was attributed to the presence of circulating antiplasmins and/or anti-activators. In addition, it has been pointed out that antiplasmins exist in plasma in considerable molar excess over potential plasmin (134, 258) and that some of these antiplasmins are fact-acting (3, 78). The observed Aα chain degradation in plasma fibrinogen may have occurred in the short time between plasmin generation and inhibition of the proteolytic activity by antiplasmins or it may be a direct result of the small amount of free plasmin postulated by Nanninga (258) to be present in plasma. On the other hand, Harpel and Mosesson (140) demonstrated that plasmin activity was inhibited incompletely by α_2-macroglobulin and that the enzyme-inhibitor complex retained a small amount of fibrinogenolytic activity, which was protected from inactivation by other enzyme inhibitors. An attractive hypothesis that α_2-macroglobulin "traps" by conformational change, rather than inhibits, proteolytic enzymes has been proposed (13). By this means, the bound enzymes may demonstrate a part of its normal activity with low molecular weight substrates, but little or none with large proteins because of steric hindrance. If the Aα chain were an integral unit of a compact fibrinogen molecule, one might expect complete protection from the residual proteolytic activity of such a trapped enzyme. However, present evidence indicates that the

Aα chain of fibrinogen is located on the surface of the molecule (Discussion, Chapter 3) and that the Aα fragments which are cleaved by plasmin have a hydrophilic nature and are surface-oriented, since they have a high content of polar amino acids and are highly deficient in hydrophobic amino acids (30, 122, 137, 155, 317, 320). The COOH-terminal aspect of the Aα chain appears to have a random coil arrangement on the basis of circular dichroism studies of fragment A (320) and a less ordered structure than the rest of the molecule since the helical content of fibrinogen was increased after removal of the COOH-terminal half of the Aα chain (243). Indeed, a large part of the Aα chain may protrude from the bulk of the fibrinogen molecule, since its removal caused an increase in the diffusion coefficient and a decrease in the frictional ratio of fibrinogen (239), and crystallisation of fibrinogen occurs only after cleavage by proteolysis of a similar part of the Aα chain (330). Therefore, as depicted in the molecular models of Doolittle (68) and Mosesson and Finlayson (243), the accessibility to the external environment of the COOH-terminal aspect of the Aα chain may explain, in part, its ease of removal during activation of the plasma fibrinolytic system, even by such complexes as plasmin-α_2-macroglobulin (140).

From a physiological view, the effect of enzymic cleavage of a 44,000 molecular weight polypeptide from the Aα chain of fibrinogen as a result of activation of the plasma fibrinolytic system would prevent crosslinking of the α chains of the damaged species (105), should subsequent fibrin formation occur, since the two crosslinking acceptor sites are located on the cleaved Aα polypeptide (85, 91, 320). Furthermore, damage to fibrinogen by plasmin proteolysis of the Aα chain, and subsequent fibrin formation, was shown to produce fibrin gels possessing only 1% of the mechanical strength of gels formed from intact fibrinogen (306). Therefore, it is conceivable that, after <u>in vivo</u> vascular injury,

fibrin formation from such damaged fibrinogen may lead to defective stabilisation of the haemostatic plug.

CHAPTER 6: THE LOCATION OF Aα-RA(26,000) IN THE Aα CHAIN OF HUMAN FIBRINOGEN

6.1 Introduction

In recent years, several groups of workers have described a number of plasmin-derived fragments from the Aα chain of human fibrinogen (96, 137, 155, 317, 320). The fragments consisted of single chain polypeptides of molecular weight 20,000-25,000 and appeared to be similar to each other in amino acid composition. In some cases (96, 137, 155, 320), NH_2-terminal amino acid sequence analyses were performed and the results were in good agreement with one another and could be related to the sequences in cyanogen bromide-derived Aα chain fragments of similar properties (70, 96, 155, 320). These fragments were usually described as COOH-terminal Aα chain fragments, since they were not derived from sections of the Aα chain which participated in interchain disulphide bonding (137, 155, 317). However, the extensive amino acid sequencing data of Doolittle and coworkers (70, 320) have provided evidence for a more accurate location of one of these polypeptides, fragment A, in a central region of the Aα chain.

Since the fragment Aα-RA(26,000) is likely to be similar to those mentioned above, the location of this fragment in the Aα chain of fibrinogen was studied by immunological means in order to provide additional information to that given by Doolittle and coworkers (70, 320). The investigation was conducted by comparing the immunological behaviour of the intact Aα chain of fibrinogen and the fragment Aα-RA(26,000) with that of other fragments which contain or consist of various sections of the Aα chain, viz. fibrinogen fraction I-9, fragment X and the COOH-terminal Aα polypeptides cleaved by ancrod (75, 96, 230, 273).

6.2 Materials and Methods

6.2.1 Human fibrinogen fractions

Kabi fibrinogen was used directly for digestion by ancrod.

Fibrinogen fraction I-4 was prepared as described in Chapter 2.

High solubility fibrinogens, fractions I-8 and I-9, were prepared from outdated ACD plasma, according to Mosesson and Sherry (248). On electrophoresis on 7.5% w/v acrylamide gel in 2M urea/5% v/v acetic acid, both fractions I-8 and I-9 gave rise to a major band near the top of the gel along with a minor impurity of higher mobility. When assessing the purity of these fractions by immunodiffusion against anti-human serum, there was no reaction with fraction I-8, but fraction I-9 showed a very weak reaction which, however, could not be identified by using antisera to albumin, antithrombin III, factor VIII or plasminogen.

6.2.2 Fragment X

Fragment X was prepared as described in Chapter 4.

6.2.3 COOH-terminal polypeptides from the $A\alpha$ chain of fibrinogen

Using a modification of the method of Edgar and Prentice (75), Kabi fibrinogen was digested by ancrod. The fibrinogen and salts were dissolved in distilled water to a fibrinogen concentration of 20 mg/ml, and tetrasodium ethylenediamine tetra-acetate (Na_4EDTA) was added to a concentration of 0.03M in an attempt to inhibit the crosslinking contaminants present in some ancrod preparations (273). The fibrinogen solution was incubated for 1 hour at 37°C with ancrod at a concentration of 10 international units/ml. Since EDTA appeared to inhibit fibrin clot formation by ancrod, the supernatant containing non-clottable polypeptides was obtained by adding bovine thrombin to a final concentration of 20 NIHu/ml, or by heating the solution to 56°C, and

removing the fibrin clot on a glass rod. The proteolytic activities of both ancrod and thrombin were inhibited by adding phenylmethyl sulphonyl fluoride (PMSF) in isopropanol solution to a concentration of 4mM. Besides several low molecular weight polypeptides, SDS-polyacrylamide gel electrophoresis of the supernatant solution indicated the presence of high molecular weight species; partial separation was achieved by applying the supernatant solution (5 ml) to a column (91 x 2.5 cm) of Sephadex G-100 and eluting with 10% v/v acetic acid. The optical density profile of the protein elution is shown in Fig. 31, along with the electrophoretic gel patterns of freeze-dried fractions 1 and 2.

6.2.4 Immunological and electrophoretic techniques

Immunodiffusion and immunoelectrophoresis were performed as described in Chapter 3.

Polyacrylamide gel electrophoresis in the presence of SDS or 2M urea/5% v/v acetic acid was performed as described in Chapter 2. Densitometry of the gels was described in Chapter 4.

6.2.5 Antisera

Antiserum to the carboxymethylated Aα chain of fibrinogen was raised in rabbits as described in Chapter 3.

Antisera to human serum, albumin, antithrombin III, factor VIII, plasminogen and fibrinogen was obtained from Hoechst Pharmaceuticals.

6.2.6 Reagents

Ancrod (Arvin) was obtained from Berk Pharmaceuticals Ltd. and bovine thrombin from Leo Pharmaceutical Products.

6.3 Results

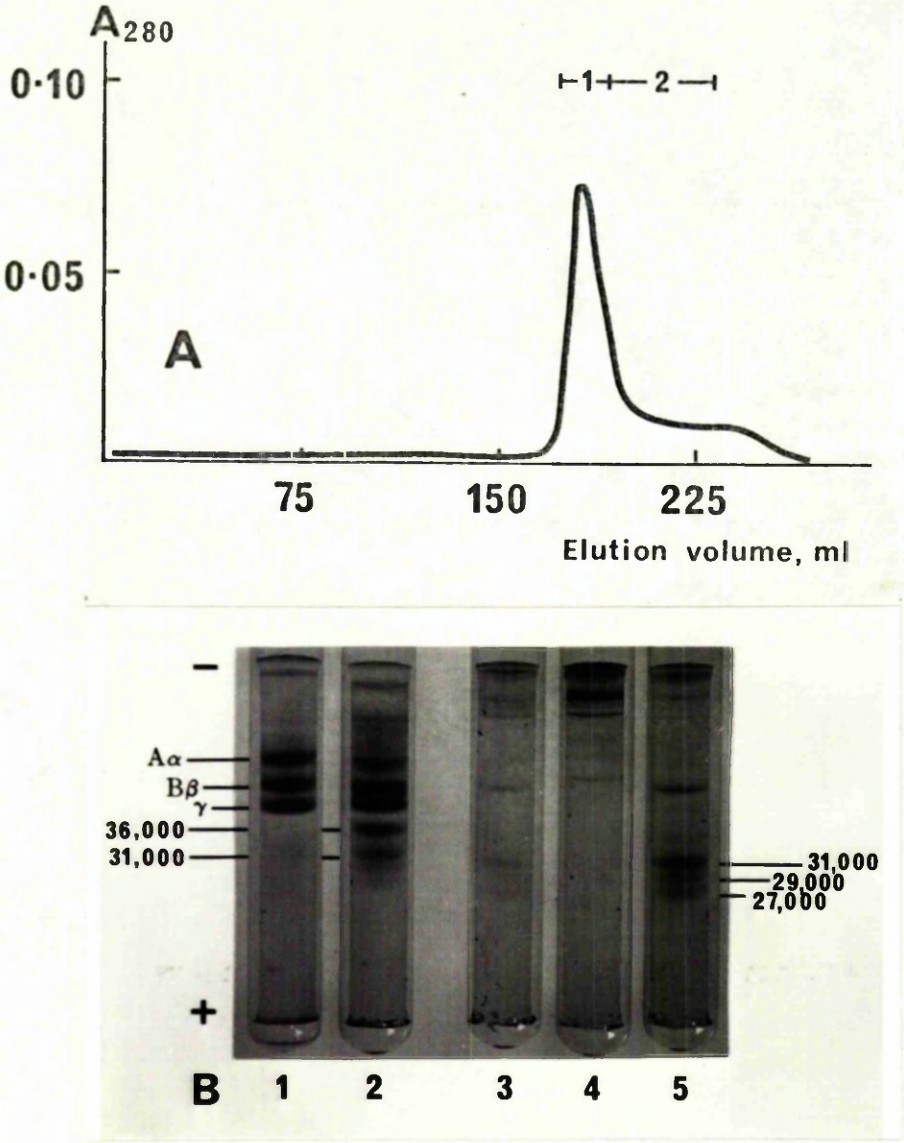


Fig. 31 A. Protein elution profile (monitored at 280 nm) of the supernatant solution remaining after removal of the clottable protein from ancrod-digested fibrinogen. The gel filtration was performed on Sephadex G-100 and the fractions were pooled as shown.

B. SDS-polyacrylamide electrophoresis (7% w/v gels) of: 1. Kabi fibrinogen (11 μg); 2. ancrod-digested fibrinogen (12 μg); 3. supernatant solution after removal of the clottable protein from ancrod-digested fibrinogen (3 μl); 4. fraction 1 (9 μg); 5. fraction 2 (14 μg). The samples applied to gels 1 and 2 were reduced with dithiothreitol prior to electrophoresis. Fractions 1 and 2 refer to the pooled fractions shown in A. The positions of the Aα, Bβ and γ chains, and low molecular weight polypeptides are indicated.

6.3.1 Polypeptide chain structure of the fibrinogen derivatives

Although the subunit structures of fibrinogen and the fibrinogen derivatives, fractions I-8 and I-9, fragment X, the Aα chain, Aα-RA(26,000), and the COOH-terminal fragments, have been well-defined by others, their chain compositions were confirmed in this study by SDS-polyacrylamide gel electrophoresis.

Of the single chain polypeptides, the intact Aα chain and Aα-RA(26,000) have molecular weights of 67,000 and 26,000 and have been described in Chapters 2 and 4, respectively. COOH-terminal fragments were derived from the Aα chain of fibrinogen by ancrod digestion, as described in Materials and Methods and as shown in Fig. 31B. Gel 2 indicates that most of the Aα chain has been cleaved to form several polypeptides of lower molecular weight, the two major fragments having molecular weights of 36,000 and 31,000. After removal of the clottable material or the heat-precipitable species, the supernatant (Fig. 31B, gel 3) was fractionated by gel filtration (Fig. 31A), into a leading peak (fraction 1), containing high molecular weight species, and a trailing shoulder (fraction 2), consisting largely of several low molecular weight polypeptides (Fig. 31B, gels 4 and 5, respectively). The series of 3 polypeptides in fraction 2 had molecular weights of 31,000 (major band), 29,000 and 27,000, and were not altered by disulphide bond reduction. Similar series of Aα chain fragments from ancrod digestion of fibrinogen have already been reported (75, 96, 273). The single band in the middle of the gel had a molecular weight 54,000 and may have been a dimer of one of the lower molecular weight bands. Since immunodiffusion tests revealed that the high molecular weight material of fraction 1 did not react with anti-fibrinogen or anti-Aα antiserum, the fraction was discarded. Fraction 2 was retained for investigation of the immunological properties of COOH-terminal Aα chain polypeptides.

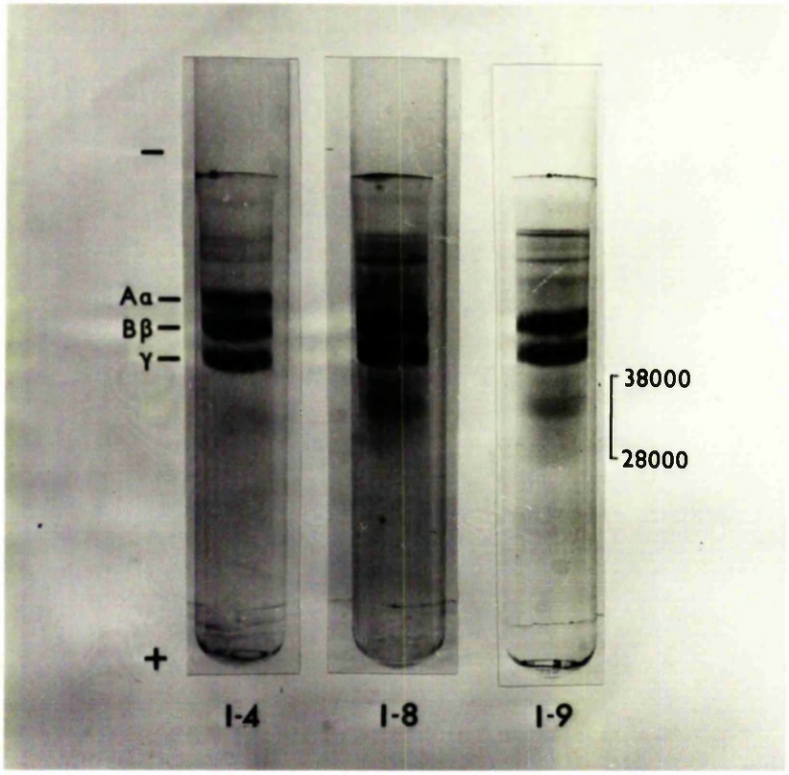


Fig. 32 SDS-polyacrylamide electrophoresis (7% w/v gels) of reduced fibrinogen fractions I-4, I-8 and I-9. The positions of the intact subunit chains are shown, along with two molecular weight graduations.

Of the multiple chain fibrinogen derivatives, the structure of fragment X has already been described (Fig. 19B and Table III, Chapter 4) and consists of intact γ chains, degraded Bβ chains and a NH_2-terminal Aα remnant of molecular weight 26,000. In addition, a faint band of molecular weight 31,000 was also seen. Examination of the subunit structures of fibrinogen fractions I-4, I-8 and I-9 by SDS-polyacrylamide gel electrophoresis, after sample reduction by dithiothreitol, indicated that the Aα chain had been largely degraded in fractions I-8 and I-9 (Fig. 32). (The origin of the high molecular weight bands seen in these gels is not known, but may be due to impurities or to incompletely reduced fibrinogen.) Estimation of the molecular weights yielded values of 56,000 and 46,000 for the Bβ and γ chains, respectively. The Aα chain in fraction I-4 had a molecular weight of 67,000, but there was no corresponding band in fraction I-9, while there was a very faint band in fraction I-8. Densitometry of the gels revealed that the staining pattern below the γ chains of fractions I-8 and I-9 consisted of 4 bands of molecular weights 38,000, 34,000, 31,000 and 29,000, of which the second band was the most abundant. A small amount of the 34,000 molecular weight material was seen in the fraction I-4 preparation. These bands represent the Aα remnants which remain bound to the core of the fibrinogen molecule at the NH_2-terminal end in these high solubility species (246).

6.3.2 Immunological properties of the fibrinogen derivatives

On immunodiffusion, all of the fibrinogen derivatives reacted with anti-Aα antiserum, but only fractions I-4, I-8, I-9 and fragment X reacted with anti-fibrinogen antiserum, showing reactions of identity, while the intact Aα chain, Aα-RA(26,000) and the COOH-terminal polypeptides did not react.

Comparison of the precipitin lines of some of these fragments by immunodiffusion with anti-Aα antiserum revealed that the intact Aα chain spurred over both Aα-RA(26,000) and fraction I-9 (Fig. 33). A similar, but smaller, spur was usually obtained with fraction I-8. Furthermore, a reaction of identity was indicated with fraction I-9 and Aα-RA(26,000). As expected, a similar precipitation pattern to that in Fig. 33 was obtained when the Aα chain was replaced by fibrinogen fraction I-4.

Further immunological investigation with fragment X, a species with a shorter NH_2-terminal Aα remnant than that of fraction I-9, revealed that anti-Aα antiserum also produced a reaction of identity with Aα-RA(26,000) and fragment X (Fig. 34). However, a high concentration of fragment X was required (4 mg/ml) and even then, the precipitin line was rather weak. Re-examination of the gel electropherogram of intact fragment X (Fig. 19A, Chapter 4) failed to show any contamination of the sample with Aα-RA(26,000), although a very faint band of molecular weight 31,000 was seen when fragment X was reduced by dithiothreitol (Fig. 19B, Chapter 4). Further evidence that the reactivity with anti-Aα antiserum was indeed due to fragment X was obtained from immunoelectrophoresis (Fig. 35) which showed that both fragment X precipitin arcs, with anti-Aα and anti-fibrinogen antisera, corresponded to each other in mobility, but differed from that of Aα-RA(26,000).

When fragments from the other end of the Aα chain, the COOH-terminal polypeptides, were subjected to gel diffusion analysis, the precipitin line from fibrinogen fraction I-4 spurred over those of Aα-RA(26,000) and the COOH-terminal polypeptide preparation (Fig. 36). However, the precipitin lines of Aα-RA(26,000) and the COOH-terminal Aα polypeptides revealed a pattern of complete intersection.

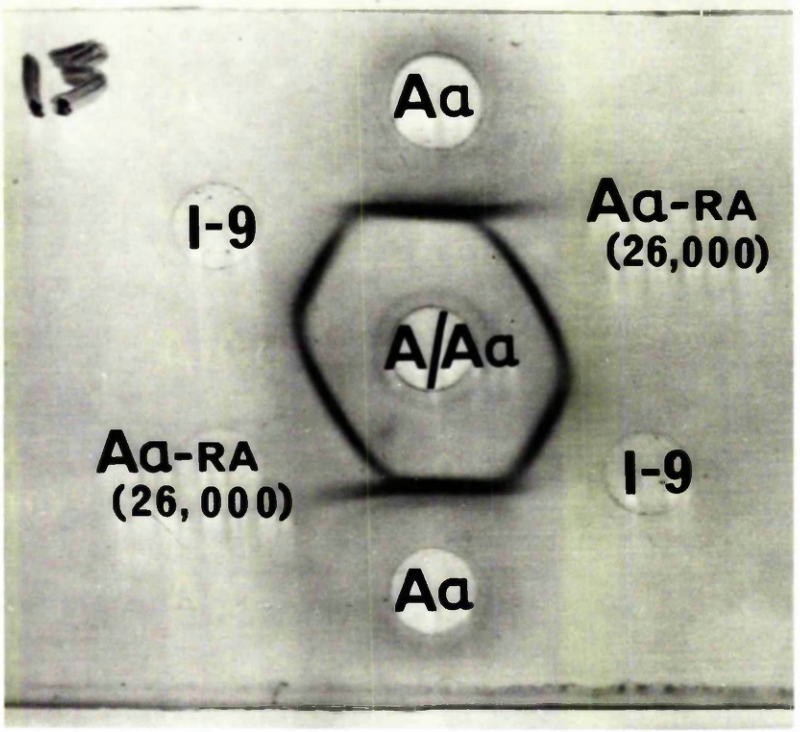


Fig. 33 Immunodiffusion of Aα chains (1.0 mg/ml), fibrinogen fraction I-9 (1.5 mg/ml) and Aα-RA(26,000) (0.05 mg/ml) with anti-Aα antiserum (A/Aα).

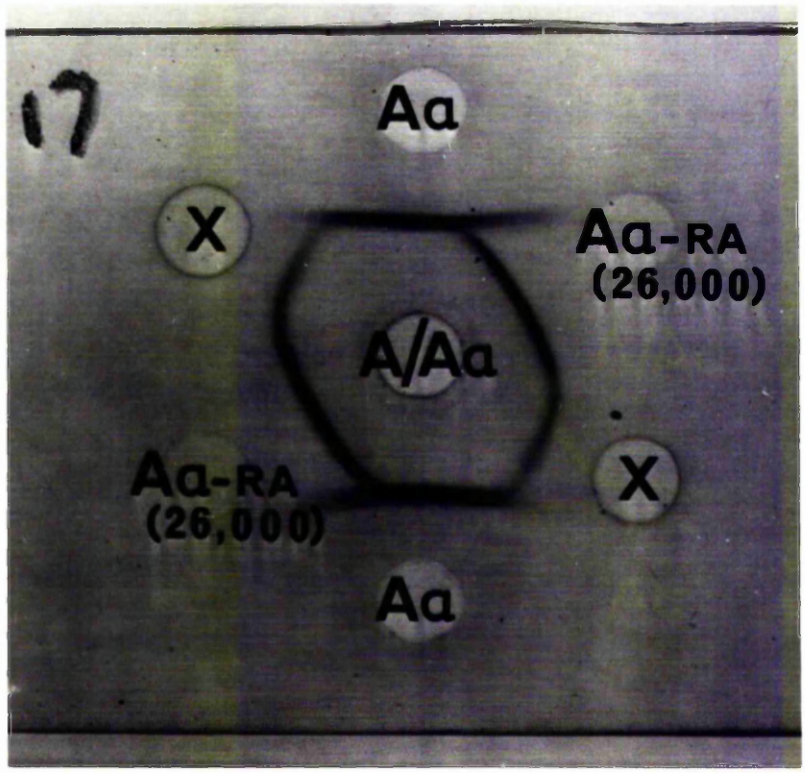


Fig. 34 Immunodiffusion of Aα chains (1.0 mg/ml), fragment X (4.0 mg/ml) and Aα-RA(26,000) (0.05 mg/ml) with anti-Aα antiserum (A/Aα).

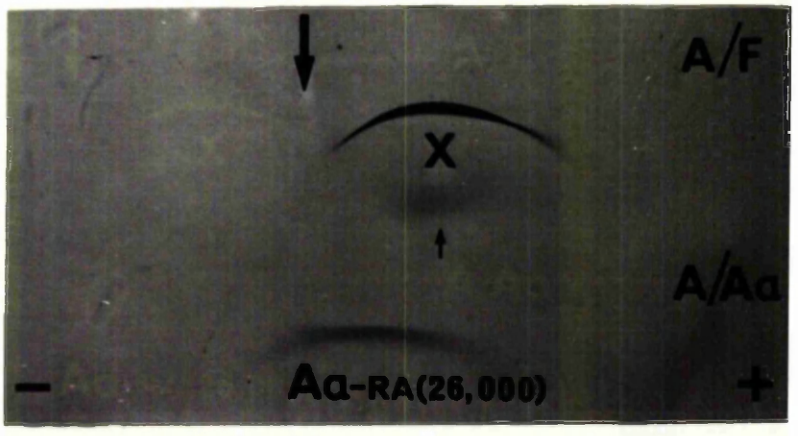


Fig. 35 Immunoelectrophoresis of fragment X (12 μg) and Aα-RA(26,000) (0.2 μg), using anti-fibrinogen (A/F) and anti-Aα (A/Aα) antisera. The samples were applied on the agarose gel at the position indicated by the large arrow. The small arrow indicates the faint immunoprecipitin arc formed by fragment X and anti-Aα antiserum.

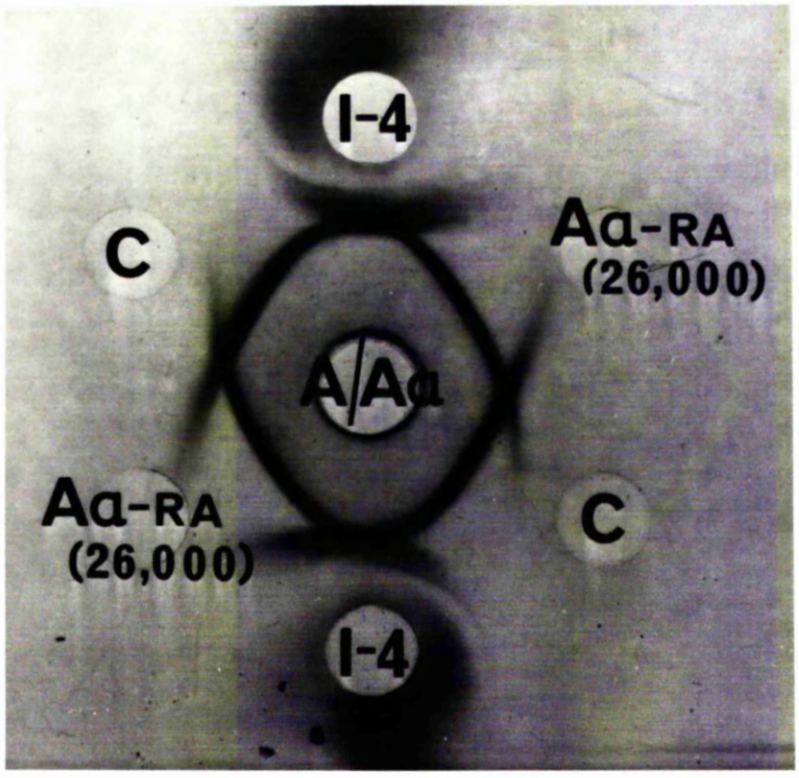


Fig. 36 Immunodiffusion of fibrinogen fraction I-4 (1.0 mg/ml), Aα-RA(26,000) (0.05 mg/ml) and the COOH-terminal Aα polypeptides (C) (0.6 mg/ml) with anti-Aα antiserum (A/Aα). Sample C was obtained from fraction 2 of Fig. 31A.

6.4 Discussion

6.4.1 Comments on the chain structures of the fibrinogen derivatives

The intact Aα chain and Aα-RA(26,000) species have been described in Chapters 2 and 4, respectively. The free, single-chain, COOH-terminal Aα polypeptides were obtained by ancrod digestion of human fibrinogen and isolated by chromatographic separation of the low molecular weight fragments. Polyacrylamide gel electrophoresis revealed the presence of 3 polypeptides of molecular weights 31,000, 29,000 and 27,000, besides some other contaminants. Although the progress of ancrod digestion with time was not studied, others (75, 96, 273) have found that continued ancrod digestion of the largest cleaved polypeptide (molecular weight 31,000) accounted for the progressive appearance of the smaller molecular size species. However, it has been suggested (96) that at least part of the observed heterogeneity in the molecular size of these polypeptides may be related to the absence of small peptides from the extreme COOH-terminal end of the Aα chain in native fibrinogen (63, 255). No evidence was found here or elsewhere (75, 96, 273) for ancrod-induced proteolysis of Bβ or γ chains.

SDS-polyacrylamide gel electrophoresis of the reduced fibrinogen fractions I-4, I-8 and I-9 revealed the presence of intact Bβ and γ chains in all three fractions, but extensively degraded Aα chains were observed in the high solubility fibrinogens, fractions I-8 and I-9. Four Aα remnant bands of molecular weight 29,000-38,000 (major band: 34,000) probably correspond to the $A\alpha/_{7}$-$A\alpha/_{10}$ bands of Mosesson et al. (246). While fraction I-4 possessed an intact Aα chain, the presence of a small amount of material with molecular weight 34,000 probably represents a proportion of degraded (from the COOH-terminal end) Aα chains (246), and confirms the observation of Mosesson et al. (246) that all plasma fibrino-

gen fractions contain at least some degraded Aα chains.

As described in Chapter 4, the fragment X preparation had intact γ chains, slightly degraded Bβ chains and extensively degraded NH_2-terminal Aα remnants (molecular weight 26,000). A faint band (molecular weight 31,000) of unknown origin was also seen, but, in view of the findings of others (98, 272) that the Aα remnants of fragment X may exhibit considerable size heterogeneity, this band may be derived from the Aα chain of fibrinogen. Indeed, some degree of heterogeneity of the Aα remnant of fragment X might be expected, since Mosesson et al. (246), in a study of circulating fibrinogen catabolites, have identified nine plasmin-like cleavage sites on the COOH-terminal aspect of the Aα chain.

Although Marder et al. (222) suggested that fragment X and fraction I-8 represented the same intermediate degradation product, and Gaffney (103) and Pizzo et al. (272) have attempted to relate their fragment X preparations to the high solubility fibrinogen, fraction I-8, on the basis of the intactness of the Bβ chain, Mosesson et al. (241, 246, 247) have convincingly demonstrated that the major structural differences between circulating high solubility fibrinogen fraction I-8 or I-9 and the in vitro plasmin-derived counterparts, I-8D or I-9D, lay in the relative abundance of different NH_2-terminal remnants of the Aα chain. Thus, these authors showed that $Aα/_8$ and $Aα/_9$ (molecular weights 34,000 and 32,000, respectively) were most abundant in fractions I-8 and I-9, whereas $Aα/_{11}$ (molecular weight 25,000) predominated in the I-9D counterpart which was derived from the plasmin degradation of pure fibrinogen. Such a difference in molecular size of the NH_2-terminal Aα remnants between the high solubility fibrinogens and the early derivatives of fibrinogen, produced by plasmin degradation in vitro, were reproduced in this study; this difference underlined the uncertainty as to whether plasmin alone was responsible for the formation of circulating

fibrinogen catabolites *in vivo* (247).

6.4.2 Immunological behaviour of the fibrinogen derivatives

All of the derivatives examined reacted with anti-Aα antiserum on immunodiffusion, but differences in their immunological behaviour gave insights into the probable location of the epitopes of the Aα chain.

The appearance of a reaction spur of fibrinogen fraction I-4 or intact Aα chain over fractions I-8 and I-9 indicated that one or more antigenic determinants of the Aα chain had been lost during removal of the COOH-terminal end of the Aα chain in the high solubility fibrinogens. Since the intact Aα chain also spurs over Aα-RA(26,000), the same conclusions are valid. However, immunological reactions of identity were obtained with fraction I-9 and Aα-RA(26,000), indicating that both of these fragments contained identical sets of antigenic determinants (Fig. 33). As there are no reports at present on the occurrence of internal antigenic homology of the Aα chain, in spite of the occurrence of small segments of internal sequence homology (64, 70), the identical immunological reactivity of these two fibrinogen derivatives with anti-Aα antiserum was consistent with considerable sequential overlap (with respect to their positions of origin on the intact Aα chain) of the Aα chain fragments of fraction I-9 and Aα-RA(26,000).

When fragment X replaced fraction I-9 in the gel diffusion experiment (Fig. 34), a similar precipitin pattern was observed, and a reaction of identity between fragment X and Aα-RA(26,000) was seen. Since the intensity of the fragment X reaction was weaker than that of fraction I-9 (confirmed by estimating the titre of each species) and since the molar concentration of fragment X applied in Fig. 34 was considerably greater than that of fraction I-9 in Fig. 33, the reaction

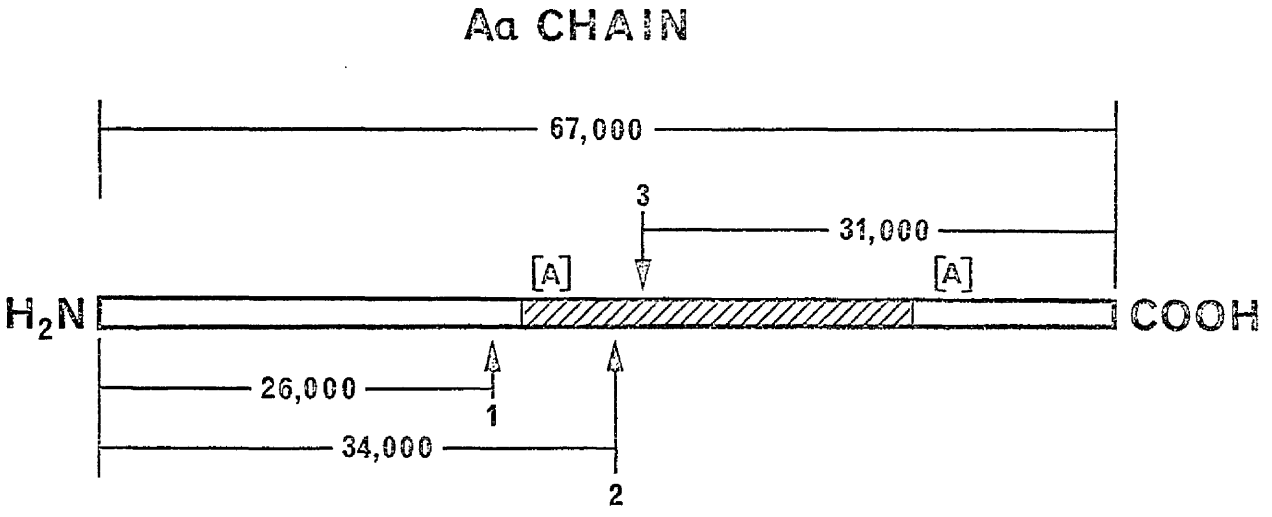


Fig. 37 Diagrammatic representation of the Aα chain of human fibrinogen. The hatched section indicates the approximate site of origin of Aα-RA(26,000) in the intact Aα chain. The numbered arrows indicate the following sites of enzymic cleavage of the Aα chain in intact fibrinogen:

1. plasmin cleavage to form fragment X with its major NH_2-terminal Aα remnant of molecular weight 26,000;
2. *in vivo* cleavage by an unknown enzyme to form fractions I-8 and I-9 with their major NH_2-terminal Aα remnant of molecular weight 34,000;
3. ancrod cleavage, removing the COOH-terminal polypeptides of molecular weight 31,000.

The proposed locations of two of the antigenic determinant sites are indicated by the symbol [A].

of identity between fragment X and Aα-RA(26,000) might be due to the existence of a small population of fragment X species with extended Aα remnant chains (molecular weight > 26,000) which overlapped in amino acid sequence with the fragment Aα-RA(26,000). Consistent with this hypothesis were the observations that

(i) a faint band of molecular weight 31,000 appeared on the acrylamide gels of reduced fragment X, and

(ii) fragments D and E, the Aα chain remnants of which constitute the NH_2-terminal Aα chain remnant of fragment X (218, 272), did not react with anti-Aα antiserum.

When the reactions of the COOH-terminal polypeptides of the Aα chain and Aα-RA(26,000) were compared, the precipitin lines intersected each other (Fig. 36). Close examination of the intersection of the precipitin lines and application of the criteria of Crowle (65) indicated that this was probably a true reaction of non-identity rather than double spur formation. These results indicated that the COOH-terminal polypeptides (molecular weights 31,000, 29,000 and 27,000)cleaved by ancrod from the Aα chain of fibrinogen, did not share antigenic determinant sites with Aα-RA(26,000), but included determinant sites not contained in Aα-RA(26,000). The above results have been summarised in Fig. 37, which shows that the antigenic determinant sites of Aα-RA(26,000) must lie towards the NH_2-terminal end of its structure. Such a constraint is required to accommodate the information that the antigenic sites of Aα-RA(26,000):

(i) are removed from the parent fibrinogen molecule by the early plasmin cleavage of a COOH-terminal polypeptide of molecular weight 44,000 (Chapter 4);

(ii) are included in the NH_2-terminal Aα remnant (molecular weight 34,000) of fraction I-9; and

(iii) are not contained in the ancrod-cleaved COOH-terminal polypeptides of the Aα chain.

While the immunological findings permitted the location of Aα-RA(26,000) in a central section of the Aα chain, the NH_2-terminal end of Aα-RA(26,000) was not contiguous with the COOH-terminal end of the Aα remnant of fragment X. Both Hessel (155) and Fretto et al. (96) have demonstrated additional peptide material at the NH_2-terminal end of the larger precursor Aα polypeptides compared with Hi2-Met, a fragment which is most likely the same as Aα-RA(26,000). In addition, Takagi and Doolittle (320) have described the loss of three small peptides from the NH_2-terminal end of an early plasmin cleavage fragment to yield fragment A. However, these authors (320) as well as others (96), have indicated that the NH_2-terminal end of fragment A lies near residue 250 in the Aα chain, which corresponds to an approximate molecular weight position of 28,500 (91). This finding is consistent with the proposed position of Aα-RA(26,000) in the Aα chain since fragment A and Aα-RA(26,000) appear to be similar species (317, 320).

If the position of fragment Aα-RA(26,000), as indicated above, is correct, it is difficult to reconcile this idea with the suggestion that the formation of the high solubility fibrinogens, fractions I-8 and I-9, represents a major catabolic pathway for fibrinogen, mediated by plasmin or plasmin-like enzymes (241, 246, 247). Studies on the degradation of purified fibrinogen have shown that plasmin largely removes a COOH-terminal Aα polypeptide which contains the antigenic determinant sites of Aα-RA(26,000) (Chapter 4), whereas fractions I-8 and I-9 appear to have retained these sites on their constituent chains. Furthermore, the plasma environment does not appear to have modified the initial cleavage pattern of plasma fibrinogen, such as to cause the progressive cleavage of COOH-terminal fragments of the Aα chain of fibrinogen up to

the stages represented by fractions I-8 and I-9 and then beyond that stage to release the antigenic determinant sites of Aα-RA(26,000); evidence was presented in Chapter 5 that activation of the plasma fibrinolytic system *in vitro* and *in vivo* caused the release from plasma fibrinogen of fragments which were immunologically identical to Aα-RA(44,000) and therefore, inconsistent with the formation of fractions I-8 and I-9. Therefore, the enzyme(s) responsible for the production of fractions I-8 and I-9 appear(s) to cleave the Aα chain at a position close to the initial peptide bond, or indeed the same peptide bond, cleaved by ancrod. At the present time, therefore, it is not known if fibrinogen fractions I-8 and I-9 are derived from intact fibrinogen by an alternative or minor catabolic pathway of plasmin or if the enzyme responsible for their formation is more closely related to ancrod than to plasmin in its specificity of action.

CHAPTER 7: ANTISERA TO THE CARBOXYMETHYLATED Bβ AND γ CHAINS OF FIBRINOGEN

7.1 Introduction

The major part of the present thesis consists of the characterisation of anti-Aα antiserum and its use in the investigation of fibrinogen degradation. The antiserum was adequate for this purpose, since it reacted with the Aα polypeptide chain, either in its isolated form or in intact fibrinogen.

In order to investigate the immunological behaviour of the Bβ and γ chains of fibrinogen by the technique of immunodiffusion, antisera to the isolated, carboxymethylated polypeptides were raised in rabbits (at the third attempt). From limited studies, some characteristics of anti-Bβ and anti-γ antisera are presented in this chapter.

7.2 Materials and Methods

7.2.1 Preparation of antisera to the carboxymethylated Aα, Bβ and γ chains of human fibrinogen

The carboxymethylated Aα, Bβ and γ chains of fibrinogen fraction I-4 were prepared as described in Chapter 2. Antisera to each of these chains were raised in 6, 3 and 3 rabbits, respectively, and were heat-inactivated, as described for the Aα chain in Chapter 3.

7.2.2 Commercial antisera

Antisera to fibrinogen and its fragments were obtained from a number of sources, as listed in Chapter 3.

7.2.3 Immunological techniques

Immunodiffusion and immunoelectrophoresis were performed as described for the Aα chain of fibrinogen in Chapter 3.

7.2.4 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence or absence of SDS, was performed as described in Chapter 2.

7.2.5 Preparation of polypeptide chains of fibrinogen from small samples of plasma

Using the sulphite precipitation method of Rampling and Gaffney (291), 0.9 ml of 10.5% w/v sodium sulphite solution was added to 0.1 ml of normal citrated plasma. After incubating for 15 min at 37°C, the precipitated fibrinogen was centrifuged at 1,000 g for 30 min and subsequently washed in 0.4 ml sodium sulphite solution. The suspension was centrifuged again to yield a deposit of fibrinogen chains.

For examination of the precipitated fibrinogen by SDS-polyacrylamide gel electrophoresis, the deposit was dissolved in 0.5 ml of 1% w/v SDS/phosphate buffer. For immunodiffusion studies, the deposit was dissolved in 0.1 ml 8M urea/barbitone buffer.

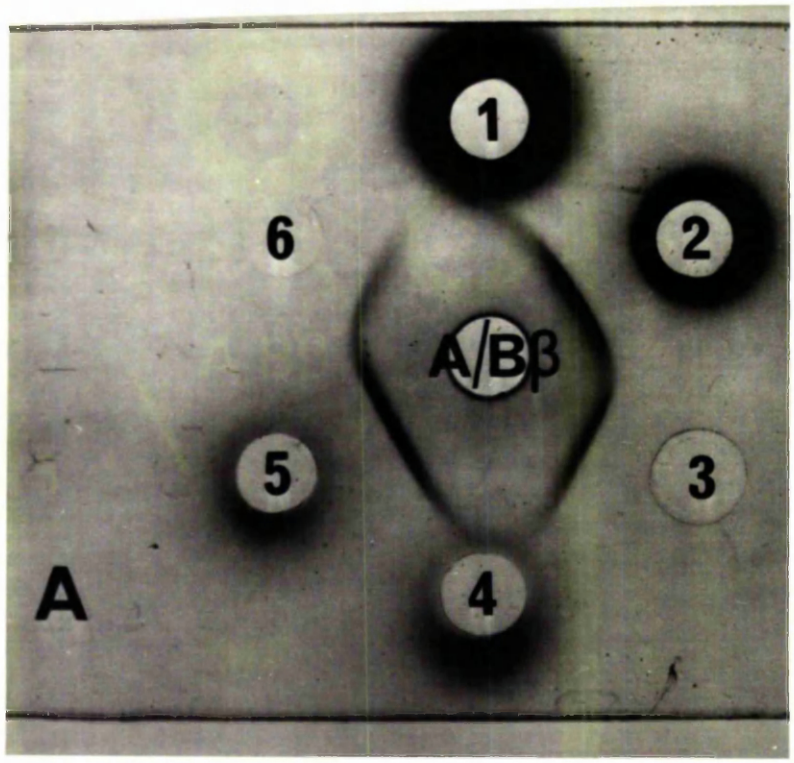
7.2.6 Reagents

Fibrinogen fraction I-4, normal human plasma and serum were obtained as indicated in Chapters 2 and 3. In addition, the series of plasmin digests of fibrinogen (Chapter 4) was investigated in this study.

7.3 Results

7.3.1 Purity of the Bβ and γ polypeptide chains

The purity of the Bβ and γ chains of fibrinogen was assessed by polyacrylamide gel electrophoresis, with or without the presence of SDS, and the results are shown in Figs. 5B, 6B and 7 (Chapter 2). Besides the major polypeptide band, a number of minor bands of lower



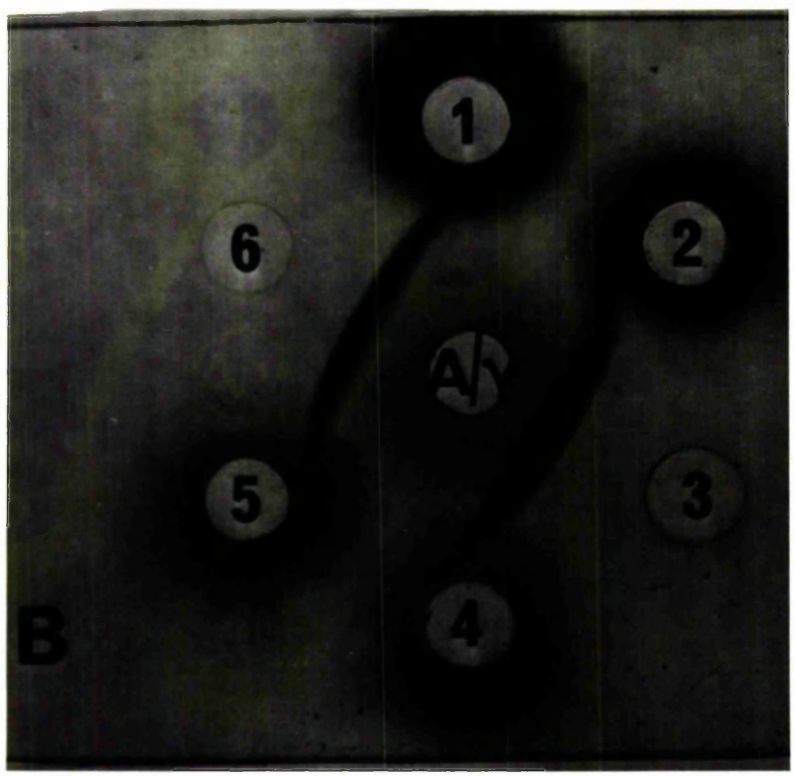


Fig. 38 Immunodiffusion of Aα chains (1) 5 mg/ml, (4) 1 mg/ml; Bβ chains (2) 5 mg/ml, (5) 1 mg/ml; and γ chains (3) 5 mg/ml, (6) 1 mg/ml; with antiserum in centre wells. A. Anti-Bβ antiserum (A/Bβ). B. Anti-γ antiserum (A/γ).

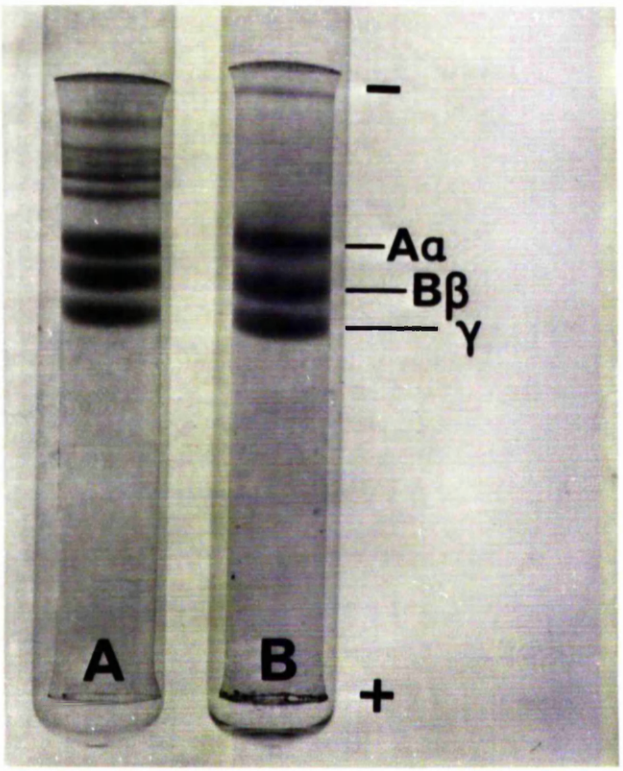
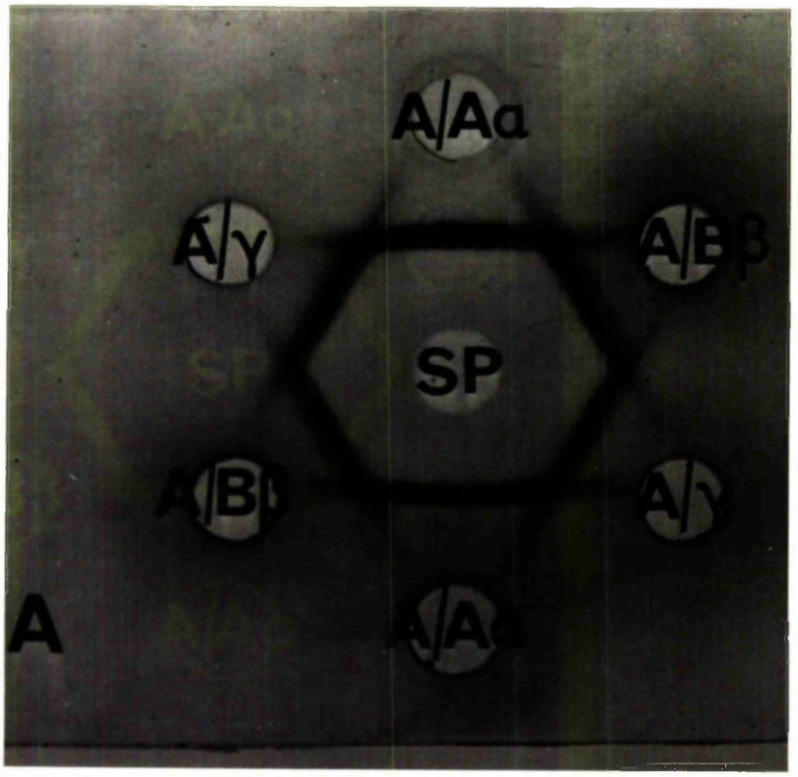


Fig. 39 SDS-polyacrylamide electrophoresis (7% w/v gels) of A. sulphite-precipitated fibrinogen from plasma (ca. 25 μg), and B. Kabi fibrinogen (11 μg). In gel B, the fibrinogen was reduced with dithiothreitol prior to electrophoresis, and the constituent polypeptide chains are indicated. Gels A and B were obtained after separate electrophoretic runs.



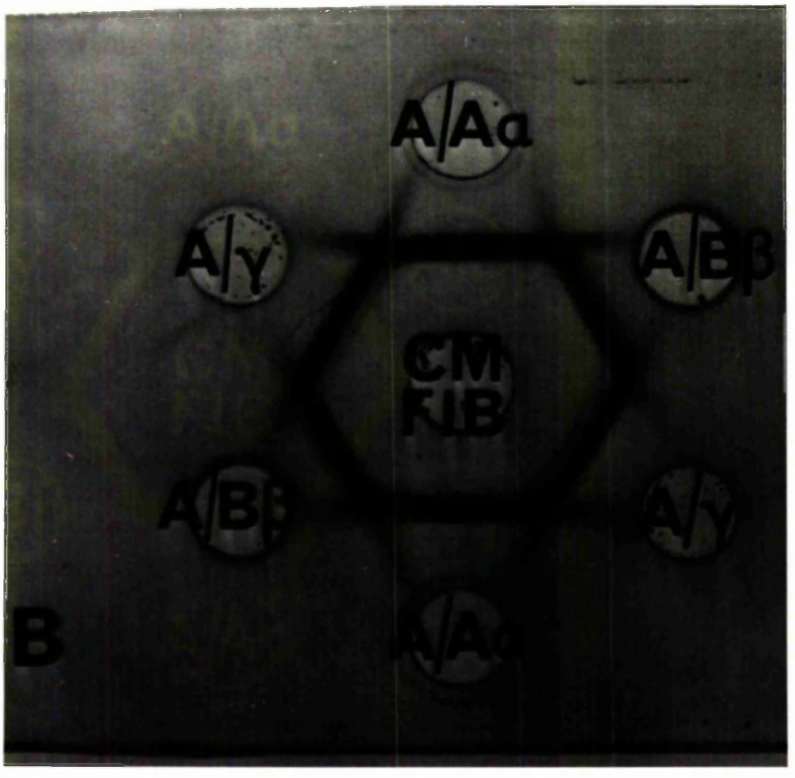


Fig. 40 Immunodiffusion with antisera to the carboxymethylated Aα (A/Aα), Bβ (A/Bβ) and γ chains (A/γ).
A. Sulphite-precipitated fibrinogen from plasma (SP) (ca. 3 mg/ml). B. carboxymethylated fibrinogen (CM FIB) (2.5 mg/ml).

electrophoretic mobility were also seen on each gel.

7.3.2 Immunological behaviour of the Bβ and γ chains

Immunological behaviour of the Bβ and γ chains anti-fibrinogen antiserum from six different commercial sources, immuno-precipitates were observed with neither Bβ nor γ chains. Moreover, these chains did not react with antisera to fibrinogen fragments D and E.

Anti-Bβ antiserum reacted with Bβ and γ chains, but not with Aα chains (Fig. 38A). Anti-γ antiserum reacted with γ chains and very faintly with Bβ chains, but not with Aα chains (Fig. 38B).

On immunodiffusion with fibrinogen fraction I-4 or plasma, anti-Bβ antiserum revealed a very faint reaction, but anti-γ antiserum did not react, even when concentrated two-fold by ammonium sulphate precipitation. Neither antisera reacted with normal human serum and no precipitin lines were produced on immunodiffusion with the series of plasmin digests of fibrinogen.

7.3.3 Examination of sulphite-precipitated fibrinogen from plasma

SDS-polyacrylamide gel electrophoresis of the sulphite precipitate of plasma revealed three major polypeptide bands of the same mobility as those of fibrinogen (Fig. 39). Additional minor bands of higher molecular weight were also evident. Immunodiffusion of the redissolved sulphite precipitate revealed that it reacted with antisera to the Aα, Bβ and γ chains of fibrinogen (Fig. 40A). Similar precipitin patterns were observed with CM-fibrinogen as antigen (Fig. 40B).

7.4 Discussion

While there have been several claims that antisera raised

against the polypeptide chains of human fibrinogen were monospecific in nature (28, 252, 285), Gollwitzer et al. (121, 123) found considerable antigenic homology between the Aα and Bβ chains of human fibrinogen, but these authors also considered the possibility of cross-contamination of the polypeptide chains. In this study, anti-Bβ antiserum precipitated with the γ chain, as well as with the immunogen, and anti-γ antiserum reacted with the immunogen, but only faintly with the Bβ chain. Therefore, it was likely that each polypeptide preparation was contaminated with intact or degraded forms of the other chain. Although absorption studies have not yet been performed, the results are similar to those of Gårdlund (111), who, despite considerable contamination of the fibrinogen chain preparations and their antisera with each other, concluded that cross-reacting antibodies to homologous structures in the chains were not present in these antisera. However, by radioimmunochemical means, an antigenic homology between the Aα and γ chains of human fibrinogen was recently described (55).

Anti-Bβ antiserum, but not anti-γ antiserum, was found to react weakly with intact fibrinogen. Similar findings have been described by other workers (28, 121, 252, 285, 309) and may indicate that most of the antigenic determinant sites on these chains are sterically hindered in the whole molecule. Against expectations, these sites did not appear to be exposed during plasmin degradation of fibrinogen. On the other hand, it should be noted that anti-γ antiserum, when assessed by radioimmunoassay, exhibited a small, but measurable, binding capacity for fibrinogen (55, 285). In accordance with the findings of others (28, 252), anti-fibrinogen antiserum did not react with any of the polypeptide chains of fibrinogen. However, Gollwitzer et al. (123) have observed precipitation reactions with the Aα and Bβ chains of bovine fibrinogen, using antiserum to bovine

Aα and Bβ chains of bovine fibrinogen, using antiserum to bovine

fibrinogen. In addition, antiserum to fragment D was reported to produce precipitin lines on immunodiffusion of the Bβ and γ chains (59), but no such results were found in this study.

The intersecting precipitin lines found upon gel diffusion of CM-fibrinogen and antisera to each of the three chains of fibrinogen indicated that the antigenicity of each chain was largely non-identical in nature. Furthermore, cleavage of the disulphide bonds of fibrinogen caused expression of the antigenic determinant sites of the Bβ and γ chains and this was also observed for sulphite-precipitated fibrinogen from plasma. By this means, immunological examination of the subunit polypeptides of fibrinogen may be performed on small volumes (0.1 ml) of plasma, and such a technique may serve as a convenient method for immunological probing of the constituent chains of fibrinogen in such cases as acquired and congenital dysfibrinogenaemias.

Note.

The immunodiffusion experiment on the sulphitolysed chains of plasma fibrinogen was suggested by Dr. P. J. Gaffney, National Institute for Biological Standards and Control, London.

FUTURE DEVELOPMENTS

Antiserum to the carboxymethylated Aα chain of human fibrinogen reacted with the Aα chain, whether in isolated form or as part of the fibrinogen molecule. Immunoelectrophoresis with this antiserum demonstrated the early release of at least two independent antigenic fragments from the Aα chain of fibrinogen during plasmin degradation. Activation of the plasma fibrinolytic system, both *in vitro* and *in vivo*, also led to the release of Aα-related antigens from plasma fibrinogen.

Based on these findings, quantitative estimation of the Aα-related antigens in human serum may represent an assay procedure for measuring the extent of *in vivo* fibrin(ogen)olysis. This assay should be a more sensitive indicator of fibrin(ogen)olysis than the current FDP assay, which measures only those major fibrinogen derivatives in serum, which have become non-clottable. Using anti-Aα antiserum, no such constraint would be imposed on the assay system, which would measure the earliest-released polypeptides from the Aα chain of fibrin(ogen).

It remains to be seen if the measurement of Aα-related antigens in human serum can detect the occurrence of low grade hypercoagulability through the secondary activation of the fibrin(ogen)olytic system. The assay may also provide additional information in the controversy "fibrinolysis vs. fibrinogenolysis", particularly in the cases of systemic activation after exercise and venous occlusion.

PRESENTATIONS AND PUBLICATIONS

Presentations

Immunological detection of COOH-αc chains of fibrinogen - a useful future?
Haemostasis Club - Fundamental and Clinical Aspects of Fibrinolysis.
NIBSC, Holly Hill, Hampstead, London, 24th Nov., 1976.

Immunological characterisation of an early cleavage fragment of human fibrinogen.
West of Scotland Blood Club, University Department of Medicine,
86 Castle Street, Glasgow, 18th May, 1977.

Immunobiology of fibrinogen.
Haemostasis Club - Fibrinogen.
McSweeney Lecture Theatre, St. Thomas' Hospital, London SE1,
6th Dec., 1978.

Publications

Conkie, J. A. & Davidson, J. F. (1977) An early plasmin cleavage fragment from the Aαc chain of human fibrinogen and its possible location. First Florence Conference on Haemostasis and Thrombosis, Florence, p. 40 (Abstract).

Conkie, J. A. & Davidson, J. F. (1977) An early plasmin cleavage fragment from the Aαc chain of human fibrinogen and its possible location. In Proceedings of the First Florence Conference on Haemostasis and Thrombosis, (in press).

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