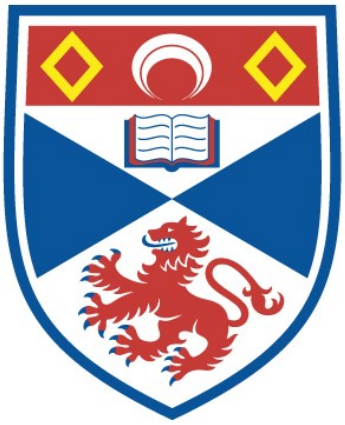
STUDIES OF THE SHAPE AND CALCIUM BINDING PROPERTIES OF FIBRINOGEN AND ITS DEGRADATION PRODUCT FRAGMENT D

David W. Britton

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1984

STUDIES OF THE SHAPE AND CALCIUM BINDING PROPERTIES OF FIBRINOGEN AND ITS DEGRADATION PRODUCT FRAGMENT D

A THESIS PRESENTED

BY

DAVID W. BRITTON B.Sc. (ST ANDREWS)

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SUMMARY

The main aims of this work were to develop and test methods for the analysis of the shape and calcium binding properties of fibrinogen and its degradation product fragment D.

The techniques of photo-sensitized labelling and cross-linking were used to obtain information about the shape of these proteins and both revealed interesting information. Photo-sensitized labelling proved particularly useful and results obtained using this technique suggested that fragment D (Ca^{++}) in solution and the fragment D domains of fibrinogen are conformationally very similar. These studies also suggested that the Aα chain of fibrinogen is highly exposed at the surface of the molecule and that it shields other portions, particularly the D-domains of the molecule.

The use of chemical cross-linking reagents revealed a gross-conformational difference between fragment D (Ca^{++}) and fragment D (EDTA) and it was further shown that this difference is a result of cleavage of the γ chain of fragment D (Ca^{++}) rather than calcium removal. This suggests that the calcium ion bound by fragment D (Ca^{++}) exercises a protective influence over a plasmin susceptible bond and that cleavage of this bond allows a gross conformational change. This is consistent with the results from photo-sensitized labelling which showed the C-terminal portion of the γ chain of fragment D to the surface exposed.

It was also shown that the technique of photo-sensitized labelling could be modified to induce cross-linking yielding a plasmin digestable product. These studies confirmed results from labelling studies which suggested that the Aα chain C-terminii protect the N-terminal portion of fibrinogen.

Calcium binding studies revealed two classes of high affinity calcium binding site in fibrinogen. Two sites of high ($Kd \simeq 10^{-5}M$) were shown to exist in the D-domains of the molecule, a third site of even higher affinity ($Kd \simeq 10^{-7}M$) was also found although the position of this site was not identified.

These results of the study may be used to reconcile some of the more extreme views of fibrinogen shape as they suggest that fibrinogen is a protected trinodular structure.

DECLARATION

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

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

CERTIFICATE

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

ACADEMIC RECORD

I first matriculated at the University of St.Andrews in October, 1976, and graduated with the degree of Bachelor of Science, Upper Second Class Honours in Biochemistry in July 1980.

I matriculated as a research student in the Department of Biochemistry and Microbiology, University of St.Andrews in October 1980.

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INTRODUCTION

1) Fibrinogen Chemistry

Fibrinogen is a large soluble glycoprotein which is present in plasma at a concentration of 3 mg/ml, constituting approximately 5% of the total plasma protein. Its main role is in the haemostatic process, acting as a precursor of fibrin monomers which in turn polymerize to form insoluble fibrin clots (Doolittle 1973). Fibrinogen has also been implicated in a number of other physiological mechanisms; defence against bacterial infection (Hawiger et al, 1975), the inflammatory response (Bernhart et al, 1971) and wound healing (Duchart and Beck, 1968). However it is in the haemostatic process that fibrinogen is of greatest importance and it is because of the role of fibrinogen as precursor of fibrin that it has been the subject of so much study.

Blomback and Yamashina (1968) showed fibrinogen to be a dimeric molecule consisting of three pairs of non-identical polypeptide chains. The three chains of fibrinogen which are known as Aα chain Bβ chain and γ chain (Blomback 1973) were shown by Gaffney and Dobos (1971) to have molecular weights of 67,000, 58,000 and 47,000 respectively. These figures give the fibrinogen half molecule a molecular weight of 172,000 and the whole molecule a weight of 344,000. This figure is in good agreement with molecular weight estimates of 340,000 obtained by analytical ultracentrifugation (Shulman, 1953; Caspray & Kekwick 1957) and light scattering measurements (Katz et al, 1952).

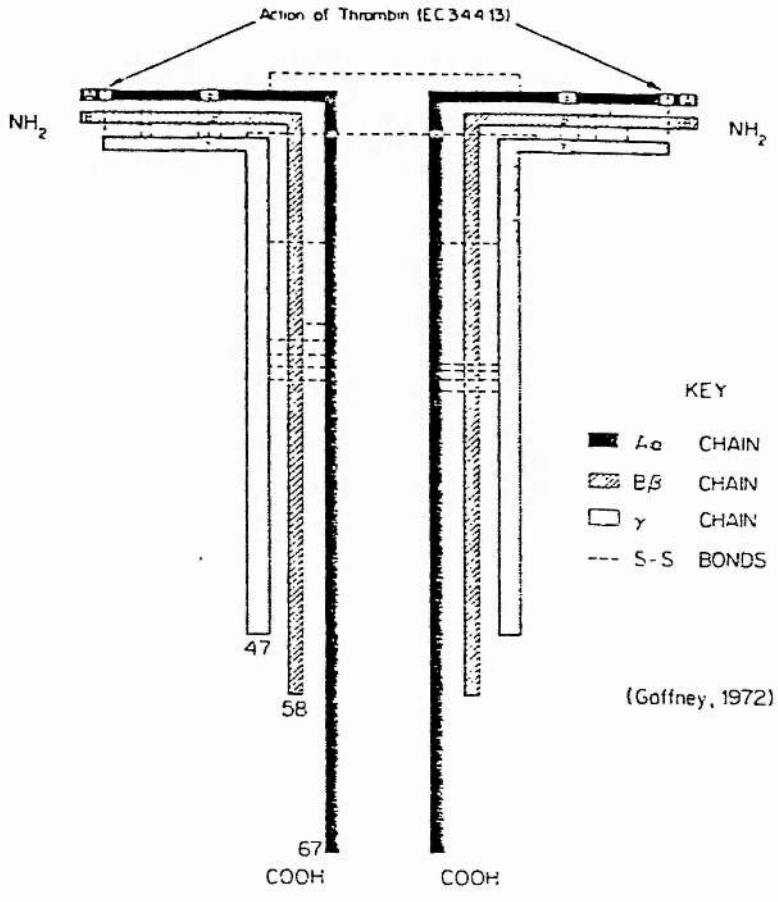


Figure 1.1 Schematic model of the fibrinogen molecule

(From : Gaffney, 1977)

Loewy et al (1961) showed that fibrinogen contained 56 - 58 cysteine residues and further showed that native fibrinogen contained no free -SH groups thereby concluding that all these residues are involved in disulphide bridges. Henschen (1964) confirmed that there were actually 58 cysteine residues in fibrinogen indicating the existence of 29 S-S bridges. Clegg and Bailey (1962) and Henschen (1963) had previously shown that the three chains of each half molecule of fibrinogen are held together by disulphide bonds and Blomback (1970) subsequently showed that the two halves of fibrinogen are held together by one disulphide bond between the two Aα chains and two disulphide bonds between the γ chains. Further analysis of the position of the disulphide bonds in fibrinogen (Blomback et al, 1976) showed that eleven occur in the central domain of the molecule which contains the N-terminii of all of the chains; this includes the three responsible for holding together the two halves of the molecule. A further eight disulphide bonds occur in each of the two outer parts of the molecule, towards the C-terminal ends of the Bβ and γ chains and the remaining two disulphide bonds are intra-Aα chain bonds found towards the C-terminal portion of this chain.

The general features of the fibrinogen molecule are outlined in Figure 1.1.

Full analysis of the primary structure of fibrinogen has been carried out and the complete sequences of the γ chain (Lottspeich and Henschen, 1977), Bβ chain, (Henschen and Lottspeich, 1977; Watt et al, 1979) and Aα chain, (Doolittle et al 1979) have been published. Analysis of the primary structure of

these chains has shown a high degree of homology, particularly between the Bβ and γ chains, the Aα chain also shows homology with each of the others although this is less than the Bβ-γ homology. These findings form the basis of the suggestion by Doolittle (1976) that all of the chains of fibrinogen have evolved from a common ancestor.

The Bβ and γ chains of fibrinogen have been shown to have carbohydrate bound to them (Mills & Triantaphyllopoulos, 1964; Pepper et al, 1974) and the sites of attachment have been shown to be Asn 52 on the γ chain (Blomback et al 1973) and Asn 364 on the Bβ chain (Topfer-Peterson et al, 1976). Variations in the carbohydrate content of these chains have been used to explain various heterogeneities in fibrinogen preparations (Finlayson & Mosesson, 1963; Mosesson & Sherry, 1966).

2) Fibrinogen in Coagulation

Fibrinogen is the natural substrate for the proteolytic enzyme thrombin (EC 3.4.21.5). This enzyme has been shown to cleave two small peptides from the N-terminii of the Aα and Bβ chains (Bailey et al, 1951; Lorand, 1952); these peptides being known as fibrinopeptide A and fibrinopeptide B respectively. After cleavage of the fibrinopeptides the remaining molecule is known as fibrin monomer, the chains of this species being denoted α, β and γ (Blomback, 1973). Blomback et al (1957) showed that fibrin monomers polymerize spontaneously to form a gel, furthermore this study showed that fibrinopeptide A is released before fibrinopeptide B and polymerization actually commences in

the lag phase before cleavage of fibrinopeptide B. The non-stabilized fibrin polymer formed in the spontaneous reaction outlined above is then cross-linked by the action of Factor XIII (Lorand, 1972) which forms γ-glutamyl-ε-lysine (Pissano et al, 1968) protein-protein cross-links in the presence of calcium (McKee et al, 1970). The first cross-links to be formed are between γ chains of adjacent fibrin monomers, leading to the production of $\gamma-\gamma$ dimers (Takagi & Iwanaga, 1970) and this is followed by cross-linking of α chains leading to the formation of $\alpha-\alpha$ polymers (McKee et al 1970). The γ chain cross-link site is located towards the C-terminal end of the molecule (Chen & Doolittle, 1970) and the α chain cross-link site is located in the central portion of the α chain (Finlayson & Mosesson, 1973).

3) Digestion of Fibrinogen

The interaction between fibrinogen and plasmin was first studied by Nussenzweig (1961) who found that plasmin digests of fibrinogen could be separated into five pools by chromatography on DEAE-cellulose. The 'terminal' core fragments D and E were found to account for approximately 70% of the weight of the intact fibrinogen molecule and were further shown to be antigenically distinct from each other. On the basis of column chromatographic and gel electrophoretic studies Marder et al (1969) proposed an asymmetric scheme of fibrinogen degradation. The major features of this scheme have gained general acceptance and are shown in Figure 1.2.

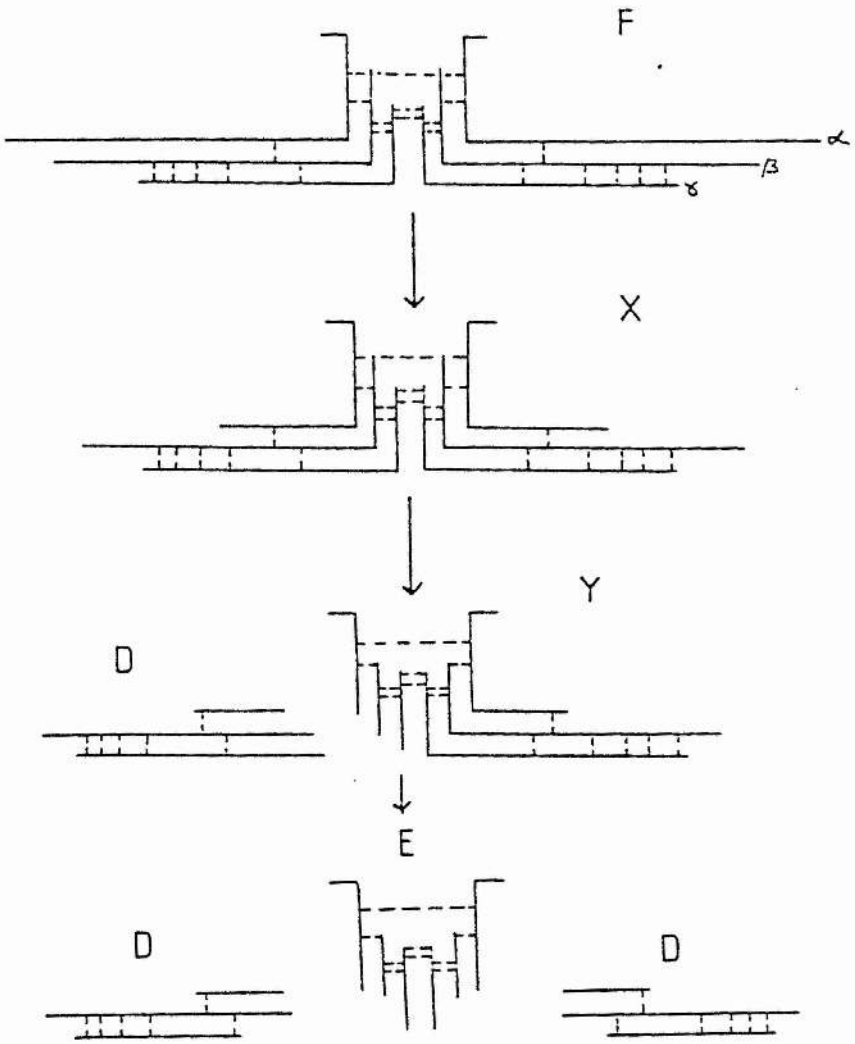


Figure 1.2 Scheme of Digestion of Fibrinogen by Plasmin

(From: Latallo (1973))

The first event in the breakdown of fibrinogen by plasmin is the removal of the C-terminal $\frac{2}{3}$ of the Aα chain (Mills and Karpatkin, 1972) to leave a molecule known as fragment X. Furlan and Beck (1972) suggested that this might occur by sequential cleavage giving rise to a family of slightly different fragment X molecules. This point which will be more fully discussed later.

The next stage in the cleavage involves the removal of peptides (m.w. 6000) from the N-terminii of the Bβ chains (Budzynski et al, 1973) and the asymmetric splitting of the three chains of fragment X. This latter cleavage releases the species known as fragment D, the remaining fragment being fragment Y. Fragment Y then undergoes cleavage to release a second fragment D leaving the core fragment E. Thus one molecule of fibrinogen gives rise to two fragment D and one fragment E species.

From this scheme it can be seen that fragment E is a dimer consisting of the disulphide bonded N-terminal portions of the Aα and γ chains and a portion of the Bβ chain from close to the N-terminus. Fragment D consists of the C-terminal portions of the Bβ and γ chains and a central portion of the Aα chain.

Studies of the plasmin digestion of fibrin have been carried out and Gaffney (1973) showed that the terminal products of digestion of non-crosslinked fibrin are electrophoretically indistinguishable from those produced by digestion of fibrinogen. However digestion of crosslinked fibrin results in the production of a higher molecular weight fragment which is known

as D-dimer (Gaffney & Brasher ,1973; Gaffney, 1973).

This brief description of the degradation of fibrinogen by plasmin has outlined the major features of the digestion, however in ths context of this work there are two points which require further discussion:-

(i) Cleavage of the Aα chain C-terminus

Mosesson and Sherry (1666) reported finding a number of pools of fibrinogen which exhibited slightly different chromatographic properties. Further analysis revealed that there were nine pools, all of which contained more than 90% clottable protein which however exhibited differences in solubility. Subsequent work (Mosesson et al, 1967) showed that these pools of fibrinogen exhibited molecular weight differences and some correlation was found between weight and solubility with the least soluble preparations having higher molecular weights. (Mosesson et al 1972).

Analysis of tryptic digests of the various pools of fibrinogen revealed that molecular weight differences were caused by digestion of the Aα chain C-terminus. Mosesson et al (1974) attributed such variations to in vivo degradation by plasmin and suggested that this played a role in the catabolism of fibrinogen. This suggestion is backed by the studies of Mills and Karpatkin (1970) who found fibrinogen purified from fresh plasma to have two Aα chain species differing by 3000 in molecular weight as determined by SDS - Polyacrylamide gel electrophoresis. This molecular weight difference was attributed to the loss of

a 27 residue peptide of molecular weight 2859 found by Cottrell and Doolittle (1976) in early plasmic digests of fibrinogen. The fact that both species of Aα chain were found in fibrinogen purified from freshly donated blood backs the suggestion of Mosesson et al (1974) that degradation of fibrinogen by plasmin occurs, in vivo.

However Semararro et al, (1977) showed that the proportion of lighter Aα chain in a fibrinogen preparation increased with the length of time that the plasma was stored prior to purification of fibrinogen. Thus, he concluded that some Aα chain heterogeneity is caused by in vitro degradation although he accepted that in vivo degradation also took place.

Therefore the variations in the Aα chain molecular weight exhibited by purified fibrinogen most probably represents a combination of in vivo and in vitro cleavage. However, the major conclusion which can be drawn from all of these studies is that the Aα chain of fibrinogen represents a highly labile portion of the molecule.

(ii) Fibrinogen Fragment D

In the scheme of digestion of fibrinogen outlined earlier the latter stages, referring to the production of fragment D are slightly over simplified. Gaffney and Dobos (1971) and Jaimeson and Gaffney (1966) reported that fragment D appeared on electrophoresis as a number of bands having molecular weights

between 73,000 and 100,000. Studies of digests (Ferguson et al, 1975; Furlan et al, 1975) revealed that each of the different species of fragment D possessed the same Aα and Bβ chain remnants and thus confirmed earlier suggestions (Pizzo et al,1972; Gaffney et al, 1972) that the lower molecular weight fragment D species were produced by plasmin degradation of the C-terminus of the γ chain remnant.

Studies by Pizzo et al,(1973) revealed that there are three species of γ chain found in fragment D preparations having approximate molecular weights of 39,000, 35,000 and 28,000, the fragment D species containing each being known as D_1, D_2 and D_3 respectively (Ferguson et al, 1975).

It was further shown that D-dimer produced by digestion of cross-linked fibrin had a γ -chain dimer the chains of which corresponded to the γ of D_1 species. Of the three fragment D species only D_1 retained the ability to form Factor XIII induced cross-links, an idea consistent with the positioning of the γ chain cross-linking site close to the C-terminus of this chain (Ferguson et al, 1975; Furlan et al, 1975).

Haverkate and Timan (1977) showed that, if digestion of fibrinogen was carried out in the presence of calcium digestion proceeded more slowly and the digestion produced a homogeneous high molecular weight fragment D (fragment D (Ca^{++}) = fgD_1=fgD(cate) m.wt 93,000). However in digestions carried out in the absence of added calcium or in the presence of EDTA a lower molecular weight form of fragment D (fragment D (EDTA) = fgD_3 (M.wt. 80,000) was found.

Subsequent studies by Haverkate et al (1979) showed that fragment D (Ca^{++}) had a potent anti-coagulent effect whilst fragment D (EDTA) has none. These workers therefore ascribed the anti-coagulent effect of fragment D to the γ chain C-terminal 12,000 molecular weight piece of the molecule, a portion which appears to be stabilized by the presence of calcium (Lawrie & Kemp, 1978; 1979)

4) Calcium and Fibrinogen

The previous discussion about the properties and heterogeneity of fibrinogen touched upon a very important aspect which remains the subject of much study and debate; the role of calcium in maintaining the structure and physiological properties of fibrinogen.

Komenko and Belitser (1963) showed that calcium limits the extent to which fibrinogen is degraded by trypsin and Ly and Godal (1973) showed that calcium had the ability to stabilise fibrinogen against denaturation by heat and alkali. However it is only comparatively recently that the full extent of the interaction between calcium and fibrinogen has been studied. In fact early studies on fibrinogen were carried out in calcium depleted solutions because Factor XIII and prothrombin, common contaminants of fibrinogen preparations, are not active in the absence of calcium.

Marguerie (1977) showed that fibrinogen was stabilized against acid denaturation by calcium and this study also revealed that the initial rate of attack on fibrinogen by plasmin was reduced

in the presence of calcium. Subsequent equilibrium dialysis studies (Marguerie et al, 1977) revealed that fibrinogen possessed a number of specific binding sites for calcium. Each fibrinogen molecule was shown to have three high affinity (Kd approx 10^{-5}M sites) and a larger number (12 - 15) of sites of lower affinity (Kd approx 10^{-3}M). It was further shown that the binding of calcium by the lower affinity sites could be abolished in the presence of magnesium suggesting that these sites are not truly specific for calcium. Marguerie et al (1977) also presented evidence which showed that one of the high affinity sites was eliminated at pH 6.0 suggesting that the three high affinity sites are not all identical.

As discussed earlier, calcium plays an important role in regulating the digestion of fibrinogen by plasmin. Haverkate and Timan (1977) showed that, if digestion was carried out in the presence of calcium a single species of fragment D resulted. This finding was supported by Purves et al (1978) who suggested that calcium ions protect the high molecular weight form of fragment D by binding to a site on the γ chain of this species. This suggestion was based on the observation that the γ chain of high molecular weight fragment D, produced by digestion in the presence of calcium, could be rapidly digested by plasmin after prolonged exposure to calcium-free buffer or chelating agents. Purves et al (1978) also showed that the presence of calcium ions lends stability to D-dimer derived from digestion of cross-linked fibrin thus contradicting the suggestion of ferguson et al (1975) that D-dimer was protected because of the occupation of the Factor XIII cross-linking sites.

Lindsey et al (1978) and Nieuwenhuizen et al (1979) also examined the high affinity binding sites of fibrinogen and came to the same broad conclusions as Marguerie et al (1977) although Niewenhuizen et al (1979) founds one high affinity site to have a different dissociation constant from the other two. These studies also extended the work of Marguerie et al (1977) showing that fragment D did indeed bind calcium as suggested by Purves et al (1978). However the ability to bind calcium was only exhibited by the high molecular weight fragment D prepared in the presence of calcium, suggesting that the calcium binding site resided in the C-terminal 12,000 molecular weight fragment of the γ-chain of fragment D (Ca^{++}).

Further, indirect evidence of the involvement of the γ chain in calcium binding was provided by Lawrie and Kemp (1979) who showed that the γ chain of fibrinogen and fragment D exhibited different mobilities on SDS polyacrylamide gel electrophoresis when run in the presence and absence of calcium.

Nieuwenhuizen et al (1981) re-assessed their data about calcium binding by fibrinogen and concluded that the molecule did not possess two classes of high affinity site but instead had three identical sites. In view of the symmetry of fibrinogen this seems unlikely, as Marguerie et al (1977) pointed out that if three sites were to exist one of these would have to link two 'halves' of the molecule. Accepting current evidence (Nieuwenhuizen et al, (1979); Purves et al, (1978); Lawrie & Kemp (1979)) it would appear that two of the calcium binding sites involve only the γ-chain any third site would therefore have to be composed of

portions from more than one chain. This third site could not therefore be chemically identical to the others and so there is no reason for it to exhibit identical binding characteristics to the two sites contained in fragment D (Ca^{++}).

Recently Nieuwenhuizen et al (1983) isolated a fragment D of intermediate weight between fragment D (Ca^{++}) and fragment D (EDTA) they found that this species (Fragment $D_{(INT)}$) retained the calcium binding ability of fragment D(Ca^{++}) but had lost the anti-clotting ability of this species. Thus they suggested that the anti-clotting and calcium binding properties of fragment D (Ca^{++}) reside in different regions of the γ chain. The conclusion about the anti-clotting effect of fragment D is supported by the results of Olexa and Budzynski (1981) who isolated a peptide of molecular weight 4674 from the C-terminus of the γ chain and found this to have anti-clotting properties.

Therefore the position of the calcium binding sites of fibrinogen which reside within the fragment D domains of the molecule is well accepted and moderately well characterised. The only real debate about these sites revolves round their affinity for calcium (Nieuwenhuizen et al, 1981; Kemp et al, 1983). However the position and nature of the third site remains uncertain.

Marguerie (1977) showed that the initial rate of cleavage of fibrinogen by plasmin is reduced in the presence of calcium. Since the site of this early cleavage is the $A\alpha$ chain C-terminus (Pizzo et al, 1972; Mosesson et al, 1972) this finding implicated the $A\alpha$ chain in calcium binding. This is backed by the studies of Credo et al, 1981 who showed that the lowered

requirement of Factor XIII for calcium, which is induced by the presence of fibrinogen (Credo et al, 1978) is dependent upon the mid-sections of the Aα chains.

On the basis of studies suggesting that fragment X and fibrinogen have identical calcium binding properties (Nieuwenhuizen and Gravesen (1981) dispute the involvement of the Aα chain in calcium binding). Further studies from this group (Nieuwenhuizen et al, 1983) showed that a late fragment E product (E_3) does not bind calcium, however a similar product produced by cyanogen bromide cleavage of fibrinogen did bind calcium albeit with an affinity lower than that which these workers observed in intact fibrinogen (Nieuwenhuizen et al 1981).

On the basis of the results of Nieuwenhuizen and Gravesen (1981), Marguerie and Ardaillou (1982) re-examined the calcium binding properties of fibrinogen and fragment X. These workers found a significant difference between these species and therefore are in total disagreement with Nieuwenhuizen and Gravesen (1981).

Therefore it can be seen that two totally different, and irreconcilable proposals exist concerning the position of the third high affinity calcium binding site of fibrinogen. Some of these differences however, may be attributable to the lability of the fibrinogen molecule coupled with the known difficulties in studying high affinity calcium binding sites. (Potter et al 1973).

5) The Shape of Fibrinogen

Much effort has been put into the analysis of the size and shape of fibrinogen over a number of years (Hall-Slayter,1959; Koppel et al, 1966; Marguerie et al, 1975). However, to date no single model has been proposed which has met with general acceptance. This may,in part be attributed to the fact that intact fibrinogen has not yet been crystallized and so, definitive x-ray crystallographic analysis has not yet been carried out. Tooney and Cohen (1972) did manage to crystallize fibrinogen which had been partially degraded by a bacterial protease however this in itself suggests that they were examining a molecule which had undergone a significant conformational change.

In view of this difficulty in crystallizing fibrinogen most of the information about the shape of the molecule has been obtained by electron microscopy or physcio-chemical analysis.

Early electron microscopic studies (Hall and Slayter, 1959) were carried out using the technique of shadow casting and on the basis of these studies Hall and Slayter proposed that fibrinogen was a trinodular structure of length 47.5 nm and diameter 9 nm (Figure 1.3(a)).They also observed that the two outer nodules were slightly larger than the central one. Support for this type of model has come from a number of workers using different techniques. Fowler and Erickson (1979) carried out electron microscopic studies using the techniques of shadow casting and negative staining and in both cases found trinodular shapes. On the basis of the known sequence data Doolittle et al (1977) developed computer simulated models which predicted that fibrinogen should have a trinodular shape.

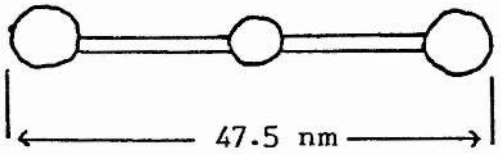
Doolittle et al (1978) subsequently showed that the sequences linking domains were helix permissive and suggested that by building up a hydrophobic core, these regions would form a 'coiled-coil' structure, this suggestion is of particular importance in considering other models.

More recently Telford et al (1980) using antibodies and Price et al (1981) using antibody fragments identified the central nodule of the Hall and Slayter model as corresponding to fragment E whilst the two outer ones were analogous to fragment D.

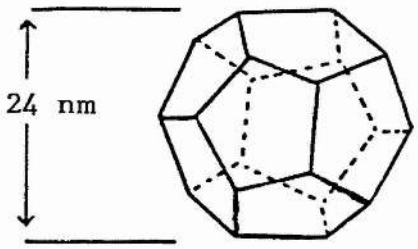
Koppel et al (1966) carried out electron microscopic studies of fibrinogen using the technique of negative staining. On the basis of this work they concluded that fibrinogen actually had a cage-like structure which they interpreted as a pentagonal Dodecahedron (Figure 1.3(b)).Similar studies carried out by Pouitt et al (1972) and Belitser et al (1975) supported this idea that fibrinogen was a swollen spherical structure.

Bachman et al (1975) carried out electron microscopic studies of fibrinogen using a freeze-etching technique, the first time such a technique was applied to the study of a protein in solution. These workers observed rod-like molecules of length 47.5 nm and diameter 9 nm, dimensions which are almost identical to those observed by Hall and Slayter (1959). Some of these rod-like molecules were bent and this the authors interpreted as suggesting flexibility and/or denaturation. (Figure 1.3(d)).

(a) Hall & Salyter (1959)



(b) Koppel (1966)



(c) Hudry-Clergeon (1975)
Marguerie et al (1975)

(d) Bachmann (1975)

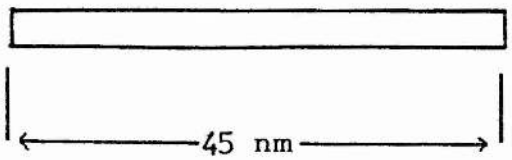


Figure 1.3 Proposed models of fibrinogen shape

In looking at the results from electron microscopic studies it is worth bearing in mind the work of Bang,1963 and Krakow et al, 1972 who showed that the fibrinogen molecule exhibited an abnormally high degree of hydration. This could explain some of the differences in results obtained by the different techniques.

In shadow-casting, dry protein is examined and the protein therefore needs to be fixed prior to dehydration and pulverisation. Normally the fixation procedure will produce intra-molecular binding without any conformational changes. However it is possible that,in the case of a molecule like fibrinogen which has a high degree of hydration the fixation procedure may not be strong enough to preserve the biological structure (Tranqui et al 1979). It can be seen that if this were the case the coiled-coil region proposed by Doolittle could be affected since its existence is partially dependent on hydrophobic interactions.

The process of negative staining in the case of highly hydrated proteins has not really been fully examined. Johnson and Horne (1970) showed that with 'normal' samples (ie degree of hydration from 1-1.2) the stain dehydrates faster than the sample allowing the sample to be examined in a partially dehydrated state. However the penetration of stain into a highly hydrated structure like fibrinogen could represent a source of artefacts (Tranqui et al 1979).

The technique of freeze-etching involves less rigourous sample preparation and the protein is certainly analysed in a fully hydrated state. However the technique does require that the sample is examined in a solution of low salt concentration ($< 10^{-3}$M) and it is possible that the fibrinogen molecule could undergo changes under such conditions.

Early hydrodynamic studies by Scherega and Laskowski (1957) indicated that fibrinogen had an unusually high frictional coefficient (a parameter of size and shape) and the two possible explanations which they proposed were that the molecule was either elongated or highly hydrated. This raises an important point about all hydrodynamic studies, the interpretation of data is greatly influenced by assumptions made about the degree of hydration of the molecule, this of course is particularly significant in the case of fibrinogen which has an unusually high degree of hydration.

Marguerie et al (1975) and Marguerie and Stuhrmann (1976) applied the technique of neutron small-angle scattering to fibrinogen and,on the basis of these studies concluded that fibrinogen was an oblate ellipsoid, more graphically described as a flattened disc bent into a banana shape (Figure 1.3(c)). This model requires a high degree of hydration (6g H_2O/g protein) and so is consistent with observed hydrodynamic data. On the basis of this work Marguerie (1979) suggested that the 'bent' molecules observed by Bachmann (1975) actually represent the true native form of fibrinogen rather than denatured molecules.

Serrallach et al (1979) attempted to fit each of the models of fibrinogen shape suggested by electron microscopy to experimentally derived hydrodynamic data. These workers were however unable to obtain good agreement with any of the rigid models even when the degree of hydration was varied within very wide limits (up to 9g H_2O/g protein). Agreement could only be obtained by fitting a model of a highly flexible molecule to the data. A high degree of flexibility within the fibrinogen molecule was also proposed by Lederer (1979) on the basis of small angle X-ray scattering data. Further, Lederer proposed that the 'bent banana' observed by Marguerie (1979) merely represented one specific conformation of a highly flexible molecule.

From the points raised in this discussion it is obvious that although no general agreement about the shape of fibrinogen has yet been reached each of the models do have points in their favour. Part of the problem in this field may be explained in terms of the techniques used, electron microscopy generally involves examining a sample which has undergone much preparation and there is no guarantee that changes do not take place during this. Physico-chemical analysis may suffer from over-simplification in that effects of different regions of the molecule may be averaged out in interpretation.

Another possible source of differences has been suggested by Mueller and Burchard (1978) and Kemp (1983) who pointed out that the picture obtained in shape analysis may be greatly affected by the presence or absence of calcium, an idea which on the basis of data from digestion studies (Marguerie 1977; Haverkate and Timan, 1977) seems perfectly feasible.

6) Aims of this Study

The discussion about fibrinogen presented here has shown that, although much is known about the molecule there still remains a number of points which have not been fully resolved. Particular areas of debate concern the shape and calcium binding properties of the molecule and it is not inconceivable that the former is greatly influenced by the latter.

Therefore the main aim of this work was to develop and test methods which could be used to gain information about the shape of protein in solution. These techniques would then be applied to fibrinogen and its degradation product fragment D in an effort to derive information about the shape of these species which could be compared to the various current models of fibrinogen shape.

The calcium binding properties of fibrinogen and fragment D were also to be examined and efforts were to be made to examine the effects of calcium binding on the shape of these species.

INTRODUCTION

Since the analysis of the shape of fibrinogen is to form the major part of this study the points made in the general introduction about the lability of the C-terminal portions of the Aα chains of fibrinogen are particularly signhificant.

The work of Tooney and Cohen (1972) suggested that a conformational change occurs on cleavage of the Aα chain C-terminii and Semerraro et al (1977) suggested that such cleavage may occur both in vivo and in vitro. Therefore to analyse the shape of fibrinogen it is of great importance to obtain as homogeneous and intact a preparation as possible. In view of the work of Semerraro (1977) it would seem that the best policy would be to adopt a rapid purification scheme to reduce in vitro cleavage to a minimum.

Obtaining a highly intact fibrinogen preparation is also important in the analysis of calcium binding since Marguerie and Ardaillou (1982) have suggested that the Aα chain C-terminii are involved in one of the high affinity calcium binding sites of fibrinogen.

In view of the susceptibility of fibrinogen to in vitro degradation fragment D(Ca^{++}) which has been shown to be resistant to digestion in the presence of calcium (Haverkate & Timan, 1977) is probably a better species to carry out developmental work on analytical techniques.

Therefore the main aims of this section are to purify homogeneous fibrinogen and fragment D (Ca^{++}) and to characterise these species.

The production of fragment D (Ca^{++}) from fibrinogen involves degradation by the enzyme plasmin, this protein can be purified as a by-product of fibrinogen purification and this will also be attempted.

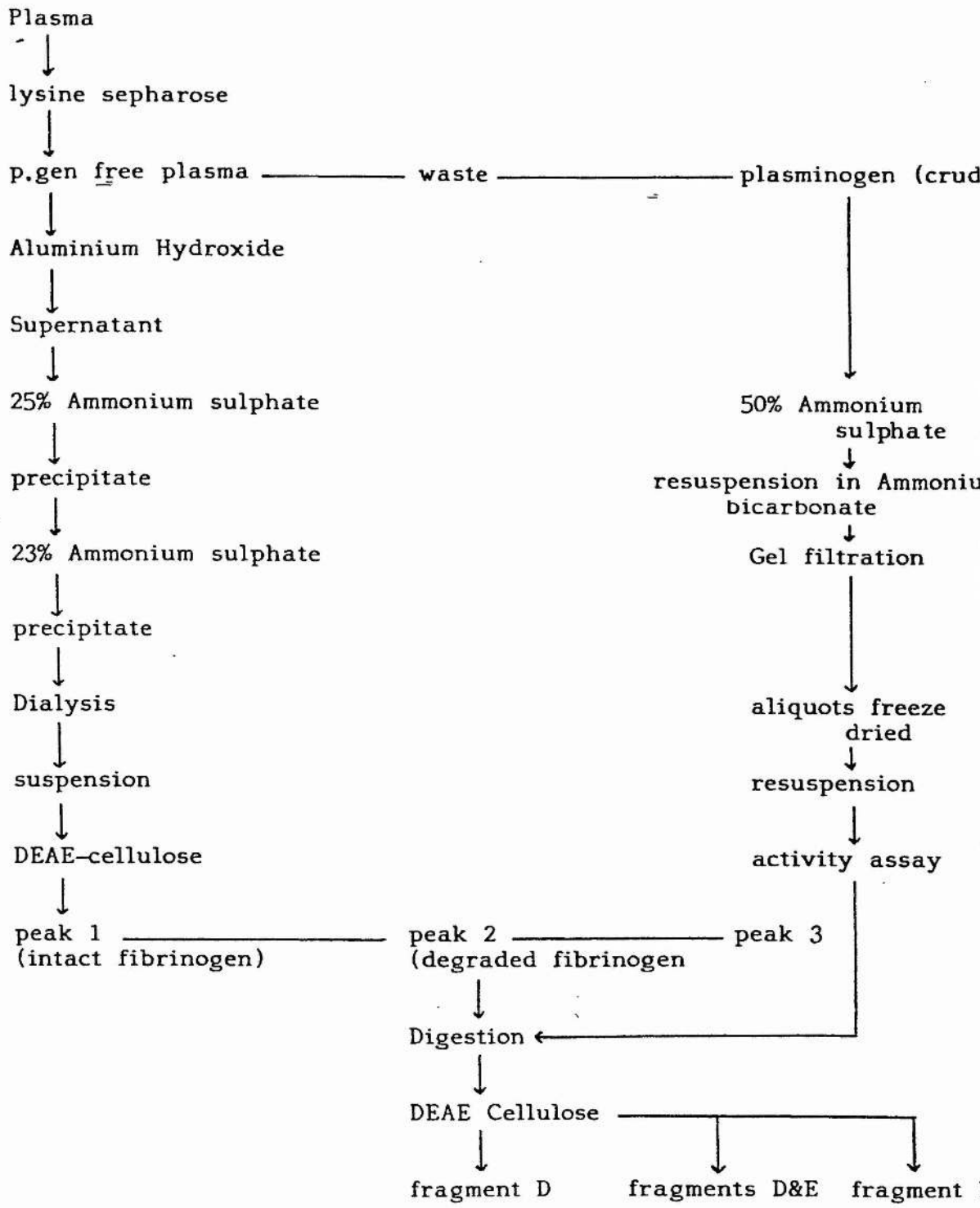


Figure 2.8 Purification Schemes used in this Study

METHODS

A. PREPARATIVE

1) Purification of Fibrinogen

Fibrinogen was purified from outdated, fresh frozen plasma donated by the Blood Transfusion Service, Ninewells Hospital, Dundee. Purification was by a method adapted from that of Lawrie et.al. (1979).

Plasma was thawed at 37°C and, if any precipitate could be seen after thawing this was removed by centrifugation at 2500 rpm for 15 minutes. Plasminogen was removed from the plasma by passage through a column (25 cm x 1 cm) of lysine-agarose as described by Deutsch and Mertz (1970). The plasminogen free plasma was then stirred for 10 minutes at 37°C with a 1/10 volume of aluminium hydroxide prepared as described by Ikemori et.al. (1975). The aluminium hydroxide was removed from the mixture by centrifugation at 2500 rpm for 10 minutes and the supernatant was collected.

Fibrinogen was precipitated from the supernatant by the addition of saturated ammonium sulphate (BDH Analar) solution to a final concentration of 25% saturation. The precipitate was collected by centrifugation and washed with 23% saturated ammonium sulphate. After centrifugation, the precipitate was resuspended in 0.05M Tris-HCl pH 8.6 containing 0.05M NaCl and 1mM $CaCl_2$. Four 45 minute dialyses against at least 100 volumes of this buffer were carried out at room temperature.

After dialysis the sample was centrifuged to remove any insoluble material before application to a column (40 cm x 2.5 cm) of DEAE cellulose (Whatman) which had been precycled as per manufacturers instructions before equilibration with 0.05M Tris-HCl pH 8.6 containing 0.05M NaCl and 1 mM $CaCl_2$.

The first protein peak was eluted from the column with starting buffer and two further peaks were eluted with starting buffer first made 0.1M and then 1M in NaCl.

2) Purification of Plasminogen

Plasminogen was purified from outdated, fresh frozen plasma using a method based on those of Deutsch and Mertz (1970) and Rickli and Cuendet (1971).

Plasminogen was absorbed onto a column of lysine-agarose and the resulting plasminogen-free plasma was used for the purification of fibrinogen as outlined earlier. After elution of plasminogen free plasma the lysine-agarose was washed with 0.05M Tris-HCl pH 8.6 containing 0.3M NaCl. Plasminogen was then eluted by passage of 0.1M ε-amino caproic acid through the column. All fractions found to contain protein were pooled and solid ammonium sulphate was added to a final concentration of 50% saturation.

The precipitate was collected by centrifugation and resuspended in 0.05M ammonium bicarbonate before application to a column (40 cm x 1.5 cm) of sephadex G-25 (Pharmacia) equilibrated with the same buffer. All protein containing fractions

from this column were pooled and the pool was then divided into small aliquots which were lyophilised and stored at -4°C until required.

3) Activation of Plasminogen

For use in digestions, lyophilised plasminogen was resuspended in 0.05M Tris-HCl pH 7.4 containing 0.012M NaCl. Streptokinase (Sigma - 2000 u/ml) was then added to a final concentration of 10% (v/v) and the mixture was incubated at 37°C for 15 minutes.

4) Preparation of Fragment D(Ca^{++})

Fragment D (Ca^{++}) was prepared from peak 2 fibrinogen as follows, the fibrinogen was dialysed overnight against 0.05M Tris-HCl pH 7.5 containing 2 mM $CaCl_2$. Plasmin, prepared and activated as outlined earlier was added at a concentration of 0.05% (w/w) and digestion was allowed to proceed for four hours at 37°C.

The digest was then applied to a column (25 cm x 1 cm) of DEAE cellulose and eluted with 0.05M Tris-HCl pH 7.5 containing 2 mM $CaCl_2$, after elution of one protein peak with this buffer the remaining protein was eluted using an NaCl gradient of 0 to 0.3M NaCl.

The fragment D containing fractions were then pooled and freed from any contaminating plasmin by passage through a column (15 cm x 1 cm) of lysine agarose.

5) Preparation of Fragment D (EDTA)

Fragment D (EDTA) was prepared as described above, the only difference being that all buffers used contained 5 mM EDTA instead of 2 mM $CaCl_2$.

B. ANALYTICAL

1) Polyacrylamide gel electrophoresis

In this work, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was used for determination of molecular weight of proteins, assessment of purity of protein preparations and quantitation of cross-linking reactions. It was carried out using a method adapted from that of Weber and Osborne (1969) and the stock solutions used are listed below:

Gel buffer	0.1M Tris-HCl (Sigma)
	6M Urea
	0.2% (w/v) SDS (BDH)
	pH adjusted to 7.4 with HCl
Acrylamide/Bisacrylamide -	
	10/3 Solution in gel buffer
	5/5 solution in gel buffer
Chamber Buffer	0.1M Tris-HCl
	0.2% (w/v) SDS
	pH 7.4

A 10/3 acrylamide/bisacrylamide is a 10% (w/v) solution of acrylamide (BDH) of which 3% (w/w) is bisacrylamide (BDH), similarly for a 5/5 solution. In this work 3% gels were also used and these were prepared using a dilution of the 10/3 acrylamide/ bisacrylamide solution.

Gels 10 cm long were polymerised in glass tubes of 11 cm length and 4 mm inner diameter after addition of ammonium persulphate solution (BDH) and NNN'N'-tetramethylethylenediamine(TEMED) (Sigma) to the acrylamide/ bisacrylamide solution at the final concentrations given in Table 2(i).

Acrylamide/Bisacrylamide	10/3	5/5	3/3
Ammonium Persulphate	0.05% (w/v)	0.06% (w/v)	0.075% (w/v)
TEMED	0.1% (v/v)	0.125% (v/v)	0.15% (v/v)

Table 2(i) Final Concentrations of Components for Polymerisation of Gels

After addition of persulphate and TEMED, gel solution was quickly dispensed into the glass tubes which were sealed at the bottom with parafilm. A small amount of distilled water was then layered over the top of the gel solution to prevent a meniscus from forming. The gels were allowed to set and the distilled water was then removed and replaced with gel buffer, the gels were then stored overnight at 4°C before use.

Normally between 10 µg and 20 µg of protein was loaded onto each gel however before loading protein samples were desalted either by a two hour dialysis against chamber buffer or by a centrifugal method based on that of Neal and Florini (1973). Desalting columns (2.5 cm x 1 cm) of sephadex G-25 were prepared,these were equilibrated with chamber

buffer and the void volume of the column was removed by centrifugation at 2200 rpm for 10 seconds. The sample was then applied to the column and the centrifugation was repeated. Experiments using radioactively labelled protein showed that 75 - 80% of the total protein was retrieved after this procedure and that the protein was fully equilibrated with chamber buffer.

Samples for reduction were then added to an equivalent volume of reducing medium which contained 8M urea, 3% (w/v) SDS and 5% (v/v) β-mercaptoethanol (BDH). The mixture was then either boiled for 5 minutes or left at 37°C for an hour. Non-reduced samples were mixed with an equal volume of a solution of 8M urea 3% (w/v) SDS before application to gels.

All samples were then mixed with 1 drop of glycerol and 10 ul of tracking dye (0.05% (w/v) bromophenol blue) and applied to gels. The gels were placed in a Shandon disc gel electrophoresis tank and a current of 5 mA per gel was applied. Electrophoresis was allowed to proceed until the tracker dye was near the end of the gel, the gels were then removed from the tubes and the position of the marker dye on each one was marked with indian ink. The gels were then placed in a staining solution containing 2% (w/v) Coomassie brilliant blue (Sigma), 45% (v/v) methanol and 7.5% (v/v) acetic acid. After staining for 2 hours gels were destained by extensive washing with a solution of 25% (v/v) methanol and 7.5% (v/v) acetic acid.

After sufficient destaining had taken place gels were scanned using a vitatron TLD 100 densitometer and the mobility of each protein band was then calculated using the formula :-

$$\text{mobility} = \frac{\text{distance moved by protein band}}{\text{distance moved by tracker dye}}$$

In each electrophoretic run gels loaded with a mixture of reduced, standard protein samples were included, the standards used were:- phosphorylase b, bovine serum albumen, ovalbumen and myoglobin (all purchased from Sigma, London). By plotting the mobility of the standards against $\log_{10}$ of their known molecular weights a callibration graph could be obtained and from this the molecular weights of unknown proteins could be estimated.

It must be noted that SDS PAGE only provides accurate estimates of the molecular weights of reduced proteins therefore any values quoted in this work for non-reduced or cross-linked samples should be regarded as apparent molecular weights.

2) Detection of Protein

Throughout this work the presence of protein in solution was detected and monitored by measuring the absorbance of solutions at 280 nm. The $E_{1\%}$ values of each of the major proteins used in this work are known (Marder et al, 1969; Robbins 1965) and these are given in Table 2(ii).

Protein	$E_{1\%}$ at 280 nm
Fibrinogen	15.1
Fragment D	20.8
Fragment E	10.2
Plasminogen	17.0

Table 2(ii) $E_{1\%}$ Values of Major Proteins used in this Study.

3) Assay of Plasmin (ogen) activity

Plasminogen and plasmin were both assayed using the chromogenic synthetic substrate S-2251 (Kabi diagnostica). This has the structure:-

H - D - Val - Leu - lys - pNa

and the p-nitroaniline (pNa) group may be cleaved either by the action of plasmin of plasminogen-streptokinase complex. The release of pNa may be followed by monitoring the increase in absorbance at 405 nm using a spectrophotometer.

The basis of each assay is shown below:

Plasminogen Plg + Streptokinase (excess) $\longrightarrow$ Plg - Sk

H-D-Val-leu-lys-pNa + $H_2O \xrightarrow{Plg-Sk}$ H-D-Val-leu-lys-OH+.Na

Plasmin H-D-Val-ley-lys-pNa + $H_2O \xrightarrow{Plasmin}$ H-D-Val-leu-lys-OH+pNa

Plasminogen was assayed as follows. An equal volume of streptokinase (5000 u/ml) was added to the sample in a cuvette.

This mixture was incubated at 37°C for 10 minutes after which 100 µl of S-2251 was added and the volume in the cuvette made up to 1 ml by addition of 0.05M Tris-HCl pH 7.4 containing 0.012 m NaCl which had also been incubated at 37°C. The cuvette was then placed in a cell carriage heated to 37°C in a spectrophotometer and the increase in absorbance at 405 nm was monitored on a chart recorder.

To assay for plasmin activity the volume of the sample was made up to 900 µl by addition of 0.05 M Tris-HCl pH 7.4 containing 0.112 M NaCl which had been previously heated to 37°C. 100 µl of S-2251 was then added and this reaction was monitored as before.

The assays of plasminogen and plasmin as outlined here do not yield absolute units of activity since the substrate concentrations used are below those recommended by the manufacturer for standardized assays. However, in this work the values of activity were only to be used to compare activities of different preparations in an effort to attain reproducability in fibrinogen digestions and therefore it was unnecessary to determine activities in standard units.

4) Test of Clottability of Protein Samples

A sample of the protein to be tested was taken and its absorbance at 280 nm was accurately measured. 1/10 volume of Thrombin (Sigma, 50 units/ml) in 2mM $CaCl_2$ solution was then added and the mixture was left for 15 minutes. After this time any clot which had formed was gently squeezed and the absorbance at 280 nm of the supernatant was measured. The percentage clottability was then calculated from the absorbance values before and after clot formation.

5) Test for Presence of FactorXIII

Thrombin was added to samples as described previously and the resulting mixture was incubated at 37°C for 2 hours. After this time any clot which had formed was removed and placed in a solution of 8M urea containing 3% (w/v) SDS and 5% (v/v) β-mercaptoethanol. The clot was then left at 37°C until complete dissolution had occurred and samples of the solution were then applied to SDS gels.

The presence of FactorXIII was indicated by the dissappearance of the band corresponding to the γ chain of fibrinogen and the concomitant appearance of a band of higher molecular weight corresponding to a γ-γ dimer.

6) Ouchterlonie Immunodiffusion

1% (w/v) agarose in 0.05 M Tris-HCl pH 8.6 containing 0.05M NaCl was prepared and stored at 4°C in 10 ml aliquots until required.

The agarose was melted by boiling, poured into 10 ml immunodiffusion plates and left to harden. Rosette patterns of wells were then cut out of each plate and samples of the solutions to be tested were placed in the outer wells, the central well being filled with anti-serum.

Diffusion was allowed to proceed for 48 hours after which the plates were washed with 0.9% w/v NaCl and dried by squashing under layers of filter paper. The plates were then stained for 15 minutes before extensive washing with destain. Staining and destaining solutions were identical to those used for SDS gels.

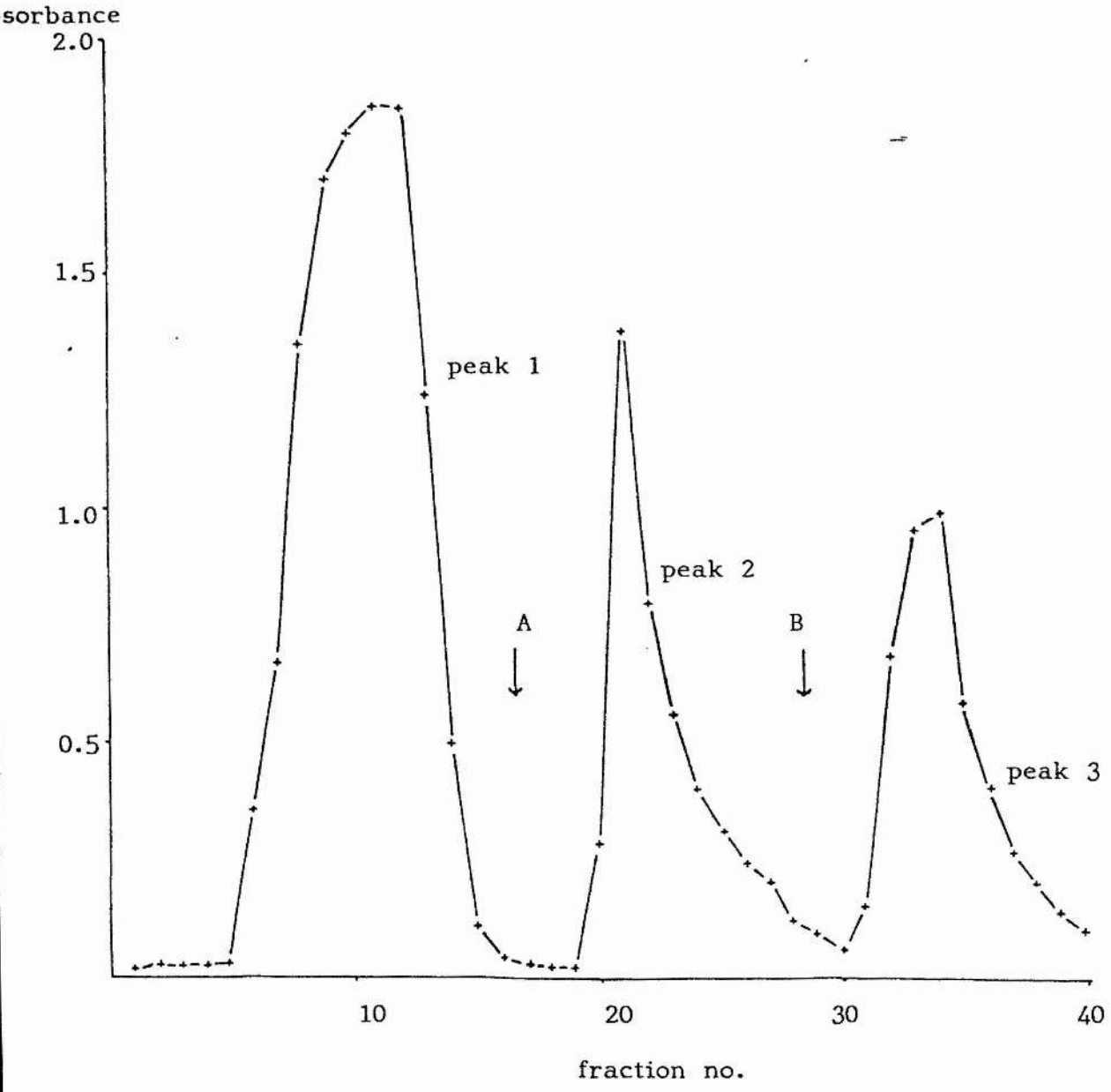


Figure 2.1 Elution profile for the purification of fibrinogen on DEAE-cellulose

Elution buffer 0.05M Tris-HCl pH8.6 containing 0.05M NaCl, 1mM $CaCl_2$

A, [NaCl] raised to 0.1M

B, [NaCl] raised to 1.0M

RESULTS

1) Purification of Fibrinogen

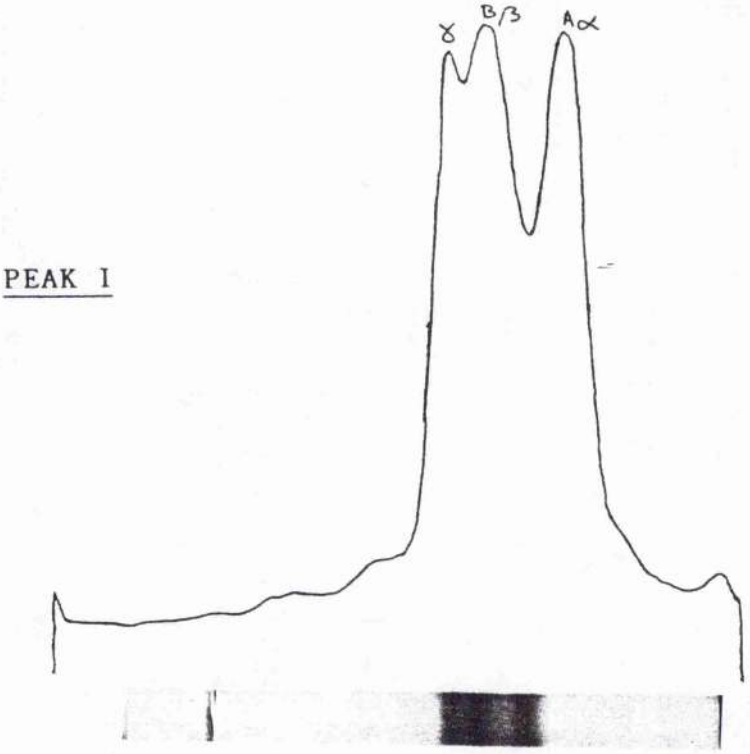
Figure 2.1 shows the elution profile of the DEAE cellulose column used in the final stage of the fibrinogen purification scheme outlined earlier.

Both peak 1 and peak 2 were found to contain fibrinogen with the material of peak 1 being 98% clottable whilst that of peak 2 was 96% clottable. The appearance of γ-γ dimers after incubation of peak 2 material with Thrombin indicated that this material was contaminated with Factor XIII however peak 1 showed no such contamination when similarly tested. These findings are in complete agreement with those of Lawrie et.al. (1979).

Peak 3 was found to contain high molecular weight material which was non-clottable, this material was thought to be fibrinectin although this was not positively confirmed.

The major differences between each of the fibrinogen containing peaks are best shown by examination of SDS gels of material from each of the peaks run after reduction. Figure 2.2 shows photographs of such gels and the corresponding densitometer scan of each of them.

It can be seen that, after reduction, peak 1 fibrinogen exhibits three bands of approximately equal intensity corresponding to the $A\alpha$, $B\beta$ and γ chains of fibrinogen. However, on examination of the gel of peak 2 fibrinogen it is obvious that the



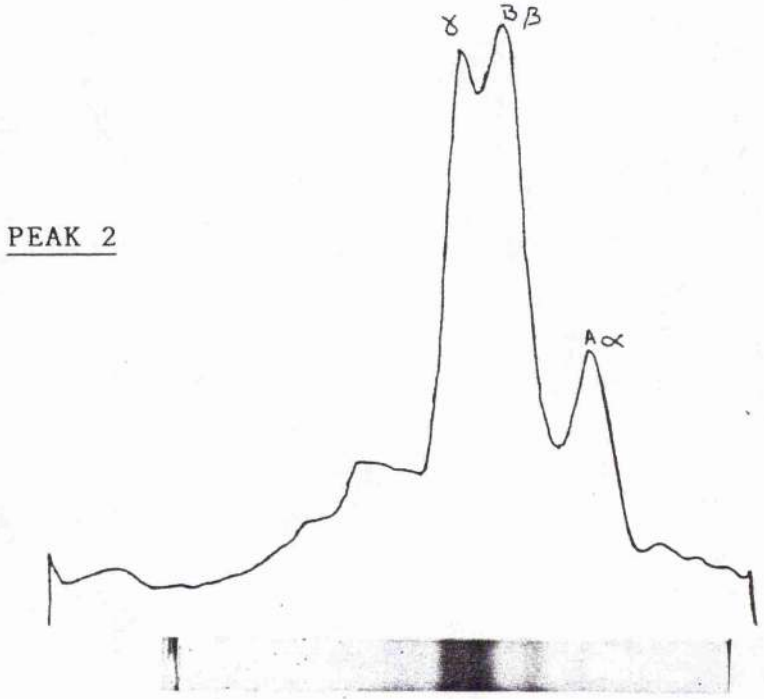


Figure 2.2 Photographs and scans of SDS Polyacrylamide gels of Peak 1 and Peak 2 Fibrinogen

Aα chain band is very much less intense than the other two bands. This could only be accounted for by digestion of the fibrinogen having taken place and this suggestion is consistent with the appearance of lower molecular weight material on the gel of reduced peak 2 fibrinogen. In view of the work of Semerraro (1977) and Mosesson et al (1974) it appears to be most likely that this digestion has occurred from the C-terminus of the Aα chain.

The gel of peak 2 fibrinogen also shows some contamination with higher molecular weight material, there is no evidence of this in peak 1 fibrinogen.

Therefore in conclusion, the purification scheme used in this work yields two pools of fibrinogen,the first being highly intact and free from contamination whilst the second has probably undergone digestion of the Aα chain C-terminus and shows some contamination with other material.

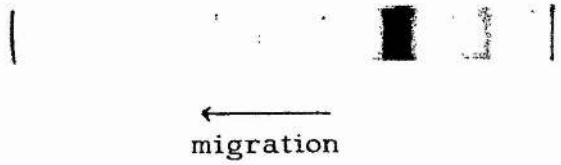


Figure 2.3 Photograph of SDS Polyacrylamide gel (5% acrylamide) of purified plasminogen

2) Purification of Plasminogen

Figure 2.3 shows a photograph of an SDS gel of plasminogen purified as outlined earlier, it clearly shows that there is one predominant band of apparent molecular weight 79,000, this figure corresponds well to the expected molecular weight of plasminogen. The major band has a faint shoulder of M.wt.(app) 73,500 which may represent some degraded plasminogen. The gel also shows a faint band of M.wt.(app)130,000, which appeared as a contaminant of all the plasminogen preparations in this work.

Activity assays were routinely carried out on plasminogen preparations and, when considered as activity per unit weight of protein different preparations showed very similar activities. Typical results are given in Table 2(iii), this table shows values obtained before and after the standard activation procedure and these are quoted as Δ A/min per mg of protein. These values were obtained using the S-2251 assay system as outlined earlier

	Before Activation	After Activation
Plasminogen	49.8	13.4
Plasmin	0	6.42

Table 2(iii) Kinetic Analysis of Purified Plasmin(ogen)

Two main points can be taken from these figures, firstly the purified plasminogen showed no measurable plasmin activity

migration

Figure 2.4 Photograph of SDS Polyacrylamide gel (5% acrylamide) of activated plasmin

this is useful since it alleviates any concern about the plasminogen preparation losing activity by autolysis during storage. The second point raised by these results is that not all of the plasminogen is activated by the method used in this work. This finding is backed by the photograph in Figure 2.4 which shows a gel of activated plasmin after reduction. It can be seen from this photograph that,as well as the bands of Mw 58,000 and 22,000 which correspond well to the expected weights of reduced plasmin (60,000 and 25,000 (Castellino 1979)) there is also a band of 78,000 which is probably non-activated plasminogen.

However,subsequent studies showed that after the activation procedure used in this work there was sufficient plasminolytic activity present to digest fibrinogen to core fragments. In view of this and the fact that S-2251 assays showed that this level of activation was consistently achieved no further attempts were made to increase the degree of activation.

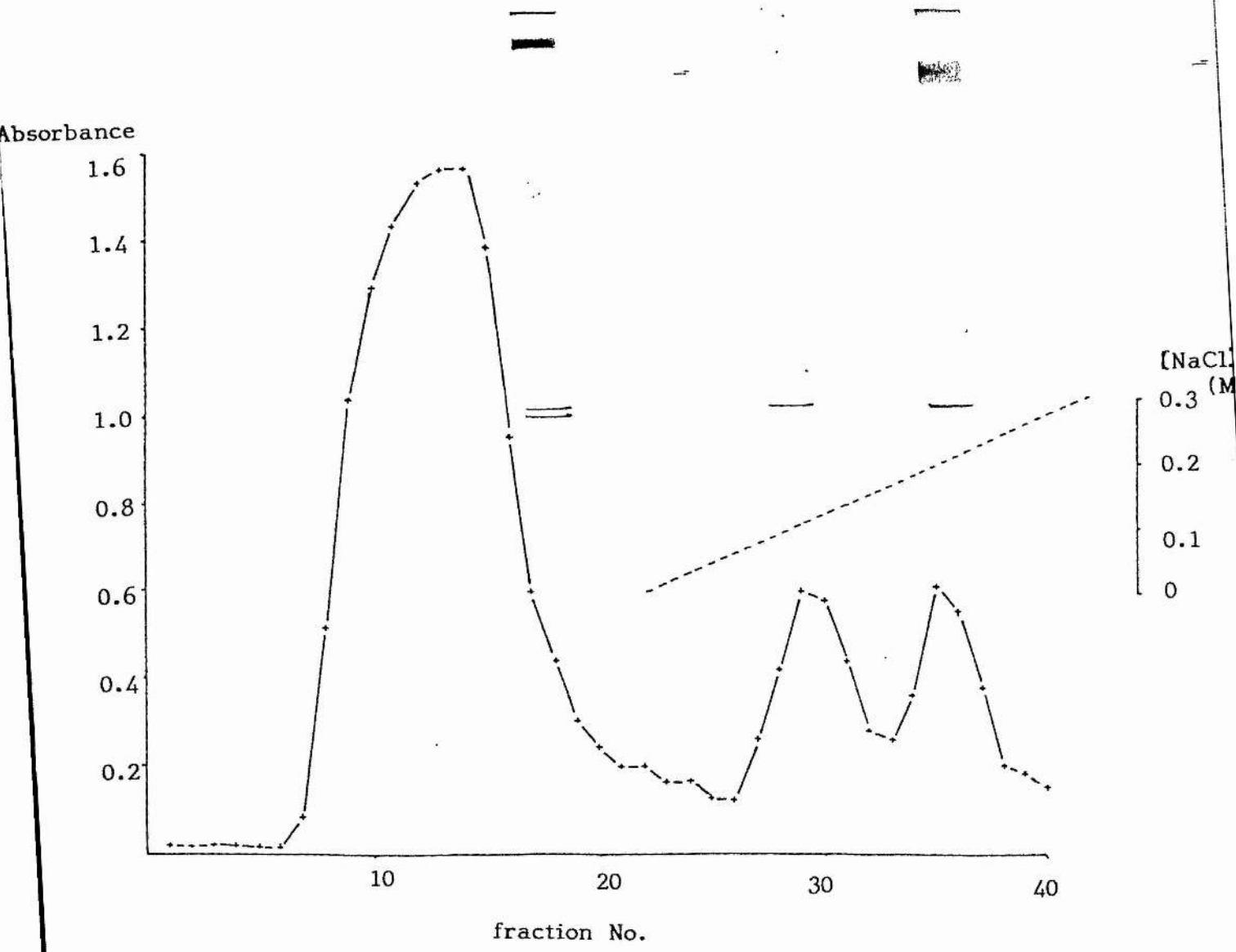


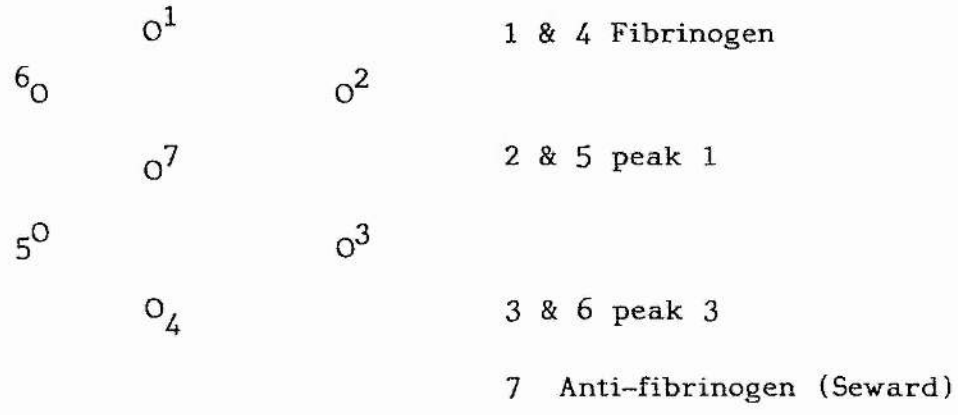
Figure 2.5 Elution profile for the separation of fragments D and E on DEAE-cellulose

3) Preparation of Fragment D (Ca^{++})

Figure 2.5 shows the elution profile of the DEAE cellulose column used for the separation of digests of fibrinogen, photographs of SDS gels of non-reduced material from each peak are also shown.

It can be seen that peak 1 contains only one protein species of apparent molecular weight 87,000 on an SDS gel. This band is also present although to a much lesser extent on the gels of peak 2. Very often these gels also showed a very faint second band of M.wt. (app) 45,000 although this is not clear on the gel in Figure 2.5. The gel of peak 3 only shows this lower molecular weight band.

Material from peaks 1 and 3 was tested by immunodiffusion against anti-fibrinogen in the following set up:



The results of this experiment are shown in Figure 2.6 (overleaf)

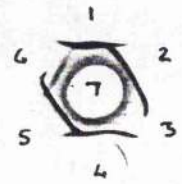


Figure 2.6 Pattern of immunodiffusion of material from DEME-cellulose column. (Anti-serum = Seward anti fibrinogen)

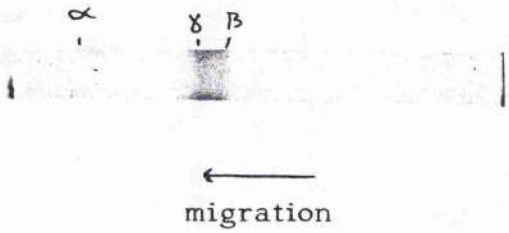


Figure 2.7 Photograph of SDS polyacrylamide gel (5% acrylamide) of peak I from DEAE-cellulose column

It can be seen that both peaks 1 and 3 exhibit a reaction of partial identity with fibrinogen showing that, as expected, the material of both peaks is fibrinogen derived. There is however a reaction of non-identify between the material from each of the peaks showing that they share no common antigenic determinants. In view of this and the apparent molecular weights it must therefore be concluded that peak 1 contains fragment D and peak 3 contains fragment E, further it can be concluded that peak 2 contains both these species.

Figure 2.7 shows a photograph of an SDS gel of reduced peak 1, this shows three distinct bands corresponding to molecular weights of 43,000, 39,000 and 13,000 respectively thus confirming that peak 1 contains intact fragment D (Ca^{++}).

4) Preparation of Fragment D (EDTA)

A number of digestions of fibrinogen and fragment D (Ca^{++}) were carried out in the presence of EDTA. In these studies it proved impossible to obtain a homogeneous fragment D (EDTA) preparation and there were large variations in the proportions of different species present. It also proved difficult to separate the different fragment D species on SDS gels and normally gels of non-reduced material showed a very broad band corresponding to a number of fragment D species, the existance of which could be shown by analysis of gels of reduced samples.

DISCUSSION

Although each of the purification schemes used in this work have been described separately, they were such that fibrinogen, fragment D and plasminogen could all be purified from the same plasma sample. Figure 2.8 shows the inter-related nature of the various purification schemes.

Since the object of this work was to develop methods for the study of fibrinogen the purification of good quality fibrinogen was very important to this study. The scheme used in this work allowed purification of very high quality fibrinogen from plasma within 10 hours. The major drawback of this purification scheme is the very low yield of fibrinogen was improved by the collection of a second pool of slightly degraded material, although this material was unsuitable for shape analysis or calcium binding studies it proved suitable for the production of fragment D.

INTRODUCTION

1) Basic Photochemistry

Photochemical reactions occur as a result of the absorbtion of UV radiation or visible light by a molecule (Wells, 1972; Turro & Lamola, 1977). The absorbtion of a quantum of radiation by a molecule results in the promotion of an electron from its normal ground state to an orbital of higher energy. The excited molecule thus produced is unstable and the excess energy which it carries may be dissipated by the molecule taking part in a chemical reaction. This is however only one possibility, the excess energy may also be lost directly as fluoroescence, phosphorescence or heat.

In this study, it is however the participation of excited species in chemical reactions which is of greatest interest. Such an excited molecule may react in one of two ways; stable products may be produced directly by the rearrangement of excited molecules or, more commonly, reaction can proceed in a number of steps involving reactive intermediates. These reactive intermediates are radicals, which may be formed in inter or intra-molecular events initiated by the photo-excited molecule. Stable products are thus usually formed in secondary reactions which can proceed via a number of different routes.

Photo-chemical systems therefore allow great scope for control over reactions for the following reasons:

(i) Radiation is selectively absorbed by matter, depending both upon the wavelength of the excitation energy and the molecular structure of the species under study. Therefore by careful selection of excitation wavelength it is possible to excite only one molecule in a complex mixture.

(ii) The microenvironment of the reaction system greatly influences the creation of reactive species and thereby controls the secondary reactions which take place.

The second factor outlined above is particularly true of photo-sensitized systems which form the basis of this work. In a photo-sensitized reaction a species, referred to as a sensitizer bring about changes in other, non-absorbing species present in the reaction mixture. These changes may occur as a result of direct transfer of excitation energy or by electron transfer, and lead to the production of reactive species which can subsequently take part in secondary reactions giving rise to stable products, the sensitizer may or may not itself be changed in these processes.

2) Photochemical Modification in Analysis of Biochemical Species

The use of group specific and affinity controlled reagents has yielded a great deal of information about structure-function relationships in a number of macromolecular species (Vallee & Riordan, 1969; Shaw, 1970; Cohen, 1970), the prime example of this being the studies on active sites of enzymes using electrophilic reagents.

However, the obvious limitation of these techniques is that the molecule under study must possess a specific structure which will readily take part in labelling. Therefore for gross study of proteins it is desirable to have a labelling system which will react more indiscriminately. This requirement is met, at least in part, by photoaffinity labelling which has proved to be useful in topographical studies of membranes (Staros & Richards, 1974; Staros et al, 1975). As a technique in biochemistry, photoaffinity labelling has two major advantages:-

(i) The initiation and duration of reaction are easily controlled.

(ii) The labelling process occurs via highly reactive, short lived intermediates under mild conditions.

The second point mentioned is of particular interest in the study of fibrinogen in view of the known lability of the C-terminus of its Aα chain (Semeraro et al, 1977).

The most common approach to photoaffinity labelling has been to derivatize ligands such that they are stable in the dark but spontaneously decompose on illumination giving rise to reactive species which bind irreversibly to a portion of protein which constitutes the normal binding site of the ligands. This approach has been well reviewed (Bayley & Knowles, 1977; Jori & Spikes, 1978; Chowdhry & Westheimer; 1979) and has yielded a great deal of information about biological membranes.

However, another possible approach to photoaffinity labelling is to utilize sensitized photochemical reactions where a target molecule is excited and this target molecule then mediates the labelling of a biomolecule with a marker molecule. The usefulness of this type of technique has been illustrated by the dye-sensitized photo-oxidation of amino acids brought about by irradiation of a mixture of protein and suitable sensitizing dye in the presence of air (Spikes & MacKnight, 1970). By careful selection of the dye and reaction conditions it is possible to achieve selective modification of certain amino acids exposed at the surface of the protein (Westhead, 1965; Ray & Koshland, 1962; Jori et al, 1970).

Although sensitized photo-oxidation offers great possibilities it has never been fully exploited, this may be attributed to the fact that the modification induced in the amino acid residues is minor and so, difficult to detect. However Brandt (1980) suggested that the technique could be extended and, that under suitable conditions, it was possible to link small, non-coloured molecules onto a protein in a photo-sensitized reaction.

It has been shown (Foote, 1968; Spikes, 1977) that a sensitized photochemical reaction of this nature could proceed by either of two mechanisms. One pathway involves radical formation in the substrate which is thought to occur by electron or hydrogen abstraction from substrate by light excited dye molecules and/or dye radicals (Type I reaction). The other mechanism involves energy transfer from the excited dye molecules to molecular oxygen and subsequent reaction between singlet oxygen and substrate (Type II reaction).

Brandt (1980) showed that if tryptophan is used as substrate, oxygen has no effect on coupling. Therefore the reaction must proceed by a Type I mechanism. The reactions involved in such a mechanism are outlined below.

Initially the dye (D) absorbs light and enters an excited state:

$$D + h \longrightarrow D^*$$

From this excited state the dye can then form radicals (Lindgvsit, 1960):-

$$D^* + D \longrightarrow D^{\cdot}_{red} + D^{\cdot}_{ox}$$

Molecules which do not themselves absorb can be converted to radicals by reaction with D* or reactive species generated by D*. A well documented example of this is the generation of radical ions or neutral radicals in biological macromolecules (M) or small molecules like tryptophan following electron of hydrogen abstraction (Foote, 1968; Spikes, 1977) for instance:-

$$D^* + M \longrightarrow D^{\cdot}_{red} + M^{\cdot}_{ox}$$

$$D^* + Try \longrightarrow D^{\cdot}_{red} + Try^{\cdot}ox$$

The products thus formed in primary photochemical reactions are unstable and can react in coupling reactions of the type discussed in this work. There are three main possibilities for secondary reactions which are of interest:-

$$M^{\cdot} + D^{\cdot} \longrightarrow M\text{-}D$$

$$M^{\cdot} + Try^{\cdot} \longrightarrow M\text{-}Try$$

$$M^{\cdot} + M^{\cdot} \longrightarrow M\text{-}M$$

All three of these reactions are readily quantifiable in the system used in this study; the first may be estimated by measuring 499nm the absoerbance of the protein solution at 499nm after separation from excess dye, the second may be quantified by using radioactive tryptophan and measuring the amount of radioactivity bound by the protein and the third reaction may be assessed by examining the protein on SDS PAGE.

In this study it is the second reaction, that of binding of labelled tryptophan by protein which is of major interest and the

main aims of the work are:-

(i) To optimise conditions for the photo-sensitized labelling of protein with tryptophan.

(ii) To assess the usefulness of such photo-senstizied labelling as a monitor of protein conformation.

(iii) To use photo-sensitized labelling to study shape of fibrinogen and its degradation product fragment D should the technique prove suitable.

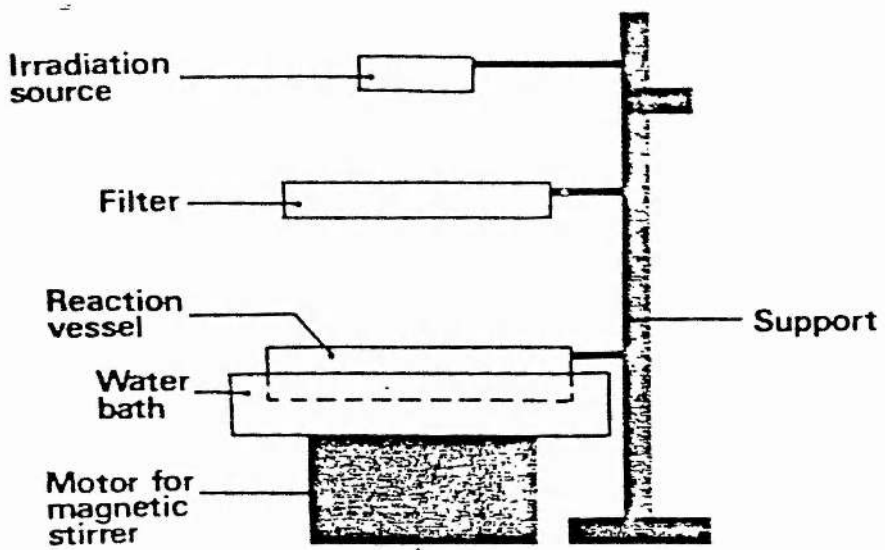


Figure 3.1 Schematic Illustration of Apparatus for Sample Irradiation

(From: Brandt, 1977)

METHODS

1) Purification of Fluorescein

Fluorescein (Fisons Scientific) was further purified by precipitation from a 0.01 M solution of sodium hydroxide by addition of glacial acetic acid as described by Brandt (1974). This procedure was repeated three times, after which the molar extinction coefficient of the purified fluorescein in 0.01 M sodium hydroxide was 8.4×10^4 M^{-1} cm^{-1} at 491 nm. A 3 mM stock solution of fluorescein in 0.01 M sodium hydroxide was prepared and stored in darkness at 4°C until required.

2) Irradiation Procedures

These were based on the technique developed by Hemmendorf et.al (1981). In initial developmental experiments an Osram 275 watt IRR tungsten lamp was used. In later experiments an Osram 500 watt Photoflood lamp was used since its greater intensity allowed more labelling to take place in a shorter time with reduced heating of sample. Light intensities were measured using a Macam photometer with a wide range photocell.

Samples contained between 0.1 mg and 1 mg of protein in 0.05 M Tris-HCl pH 7.5 containing 2 mM $CaCl_2$. Fluorescein purified as described was added to give a final concentration of 1 mM and L-tryptophan (BDH, England) was added at final concentrations between 3 nM and 75 nM with the proportion of 3H-tryptophan (Amersham International, England, 16.7 uCi/mg) varying from 100% (w/w) to 4% (w/w).

The experimental apparatus used for irradiation is shown in Figure 3.1. All samples were irradiated in a vial of diameter 2 cm at

distances between 13.5 cm and 30 cm from the light source. The sample volume was always adjusted to 1.5 ml with buffer prior to irradiation, giving a sample thickness of 4 mm. During irradiation samples were placed in an ice bath and stirred continuously by a magnetic stirrer. In all experiments a glass beaker containing a solution of 0.5 m sodium nitrite 1 cm thick was placed between the light source and the sample to filter out all wavelengths below 400 mm.

After irradiation the protein had to be separated from unbound tryptophan and fluorescein. In early experiments this was achieved by gel filtration on a column (70 cm x 2 cm) of sephadex G-25 (Pharmacia) using 0.05 M Tris-HCl pH 7.5 containing 2 mM $CaCl_2$ as the elution buffer. In other experiments it was achieved by extensive dialysis of samples against the same buffer.

In experiments where labelling was carried out in the presence of 6M urea all buffers used subsequently also contained 6M urea.

3) Determination of Degree of Coupling

Protein estimations were carried out using the method of Bradford (1976). Radioactivity was measured by adding an aliquot of protein solution to 6 ml of toluene scintillant made up as described by Cooper (1977) and the amount of radioactivity in each fraction was then counted using a Packard Tri-Carb 300 CD scintillation counter. All measurements of protein and radioactivity were carried out in triplicate.

Estimates of the distribution of radioactivity between the constituent chains of the proteins tested was carried out by examining reduced samples of the proteins on polyacrylamide gels, staining and destaining being carried out as described elsewhere. The gels were then cut into 1 mm slices which were solubilized in 0.5 ml of 30% (w/w) hydrogen peroxide (BDH, England) in sealed vials at 75°C for 8 hours.

After solubilization 4 ml of scintillant was added to each vial and radioactivity was then measured as described above.

4) Amino Acid Analysis

All buffers used in amino acid analysis were of pronalysis grade (May & Baker, London).

Samples for amino acid analysis were hydrolysed in 6M HCl in sealed tubes for 24 hours at 110°C under nitrogen. Samples were then dried by rotary evaporation and stored in a dessicator over NaOH pellets for 18 hours prior to resuspension in 0.2 N citrate pH 2.2 containing 100 μM nor-leucine (BDH, London) before analysis.

Analysis was carried out using a Joel JLC-5 AH twin column, multi-sample analyser. Basic amino acids were eluted from the short (12 cm x 0.9 cm) column with 0.35 N citrate buffer pH 5.25 containing 0.2% (v/v) benzyl alcohol. All other amino acids were eluted from the long (65 cm x 0.9 cm) column; the early more acidic residues being eluted with 0.2 N citrate pH 3.28 containing 7% (v/v) methanol and the more basic residues with 0.2 N citrate pH 4.25.

All amino acids except proline were detected by monitoring absorbance at 570 nm after their reaction with ninhydrin reagent, proline was detected at 440 nm. Standard colour values for each amino acid after reaction with ninhydrin were obtained by chromatography of standard amino acid mixtures (Sigma Chemical Co. London)

Nor-leucine was included in every analysis at a final concentration of 100 μM as internal standard. Thus, knowing the colour value for each amino acid and the concentration of the internal standard, the concentration of each residue could be calculated directly from its peak area on the trace from the analyser.

5) Digestion of Labelled Fragment D (Ca^{++})

Labelled fragment D (Ca^{++}) was dialysed against three changes of 0.05 M Tris-HCl pH 7.5 containing 25 mM EDTA, it was then digested for four hours using plasmin prepared and activated as outlined earlier.

6) Digestion of Labelled Fibrinogen

This was carried out in an identical manner to the digestion of normal fibrinogen outlined in the previous chapter.

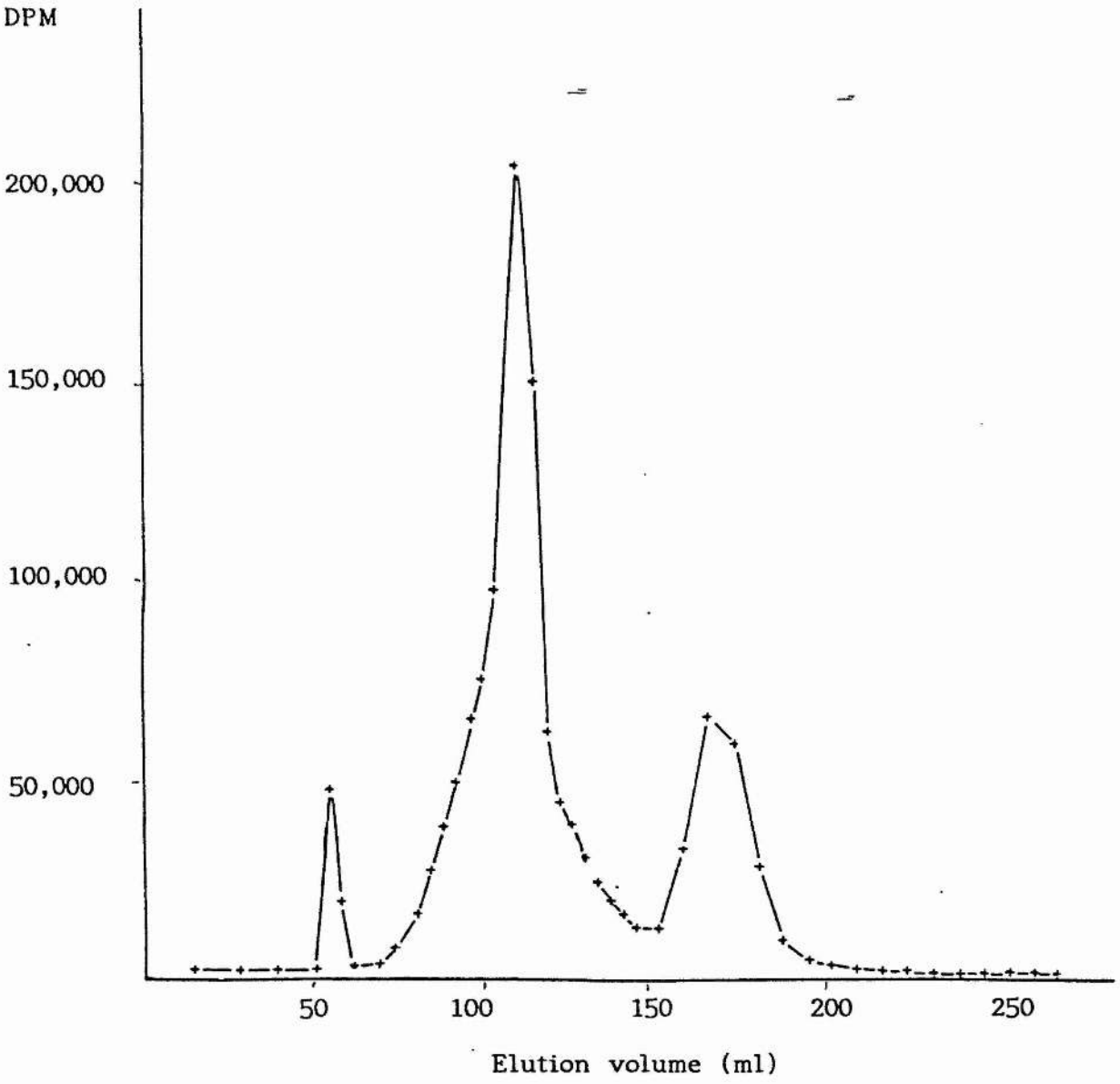


Figure 3.2 Elution profile of irradiated material from Sephadex G-25

RESULTS

1) Labelling of Fibrinogen Fragment D

Fibrinogen fragment D prepared as outlined earlier was illuminated as described and applied to a column of Sephadex G-25. The elution profile of this column is shown in Figure 3.2.

The first peak was shown to contain protein when fractions from each peak were lyophilised and applied to 5% Acrylamide gels containing SDS.

The protein containing fractions had no absorbance at 491 nm, the wavelength of maximum absorbance for fluorescein or at 499 nm where it has been suggested by Brandt (1974) that protein bound fluorescein can absorb.

Thus it was concluded that the protein had in fact been linked to tryptophan and that no fluorescein had been bound.

The second and third radioactive peaks were not fully characterised, however neither was found to contain protein and it was also shown that neither absorbed at 491 nm. Therefore it must be assumed that they both contain only tryptophan with the most likely explanation being that the second peak is polymeric whilst the third is normal tryptophan.

2) Test of Binding of Tryptophan to Fragment D

The previous experiment showed that protein and some of the tryptophan co-elute from the gel filtration column however there is no evidence that there is covalent binding between the two. Therefore the purpose of this experiment was to see if the label could be dialysed from the protein.

A sample of the protein peak was taken and made 6 molar with respect to urea. The sample was then dialysed overnight against three changes of 0.05 M Tris-HCl pH 7.5 containing 0.2 M NaCL.

Following dialysis the concentration of the protein was measured and triplicate aliquots were counted for radioactivity. It was found that the ratio of protein to radioactivity remained unchanged.

Therefore the tryptophan bound to the protein could not be removed by denaturation of the protein or by an increase in the ionic strength of the buffer and so it must be assumed that there is a covalent linkage between the protein and the tryptophan.

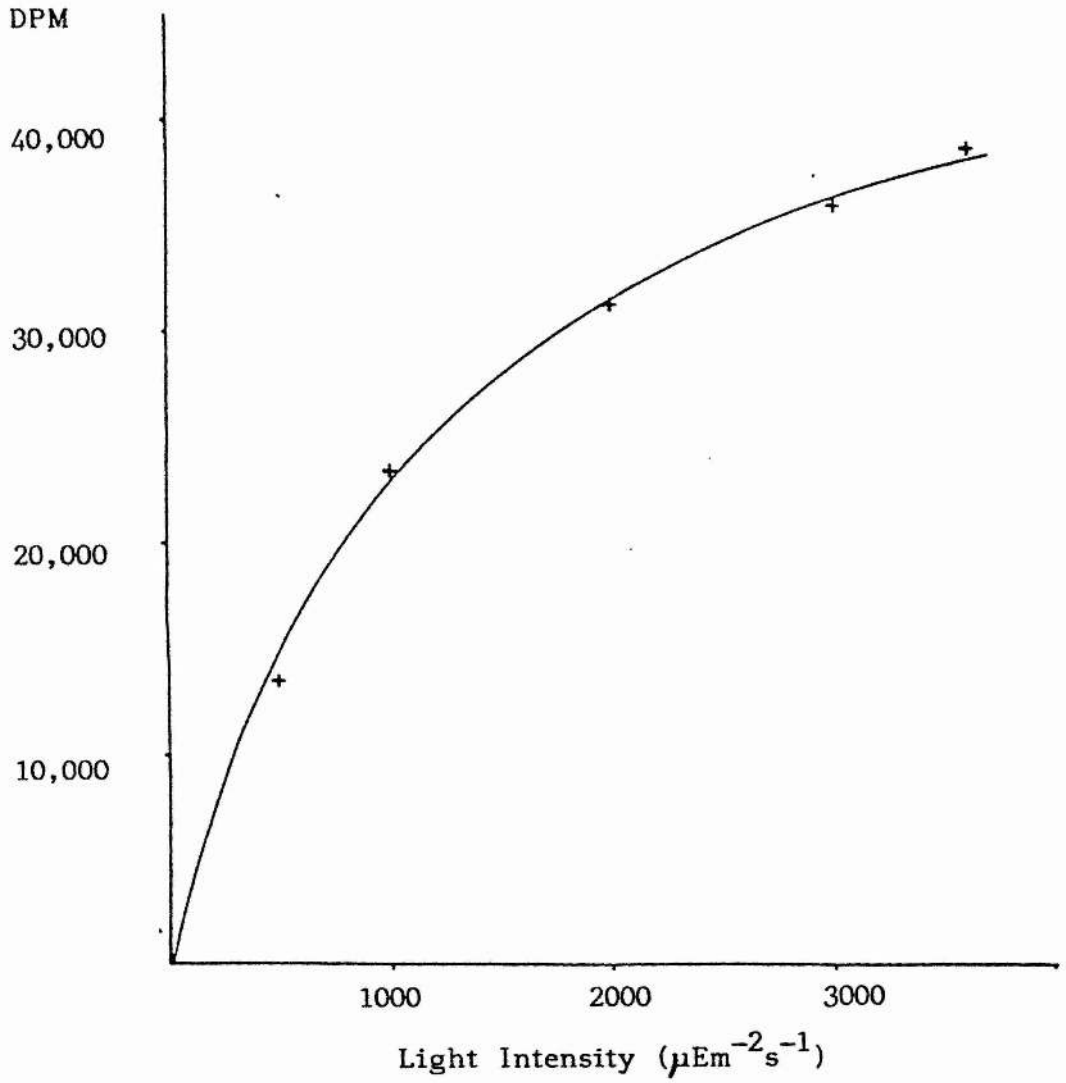


Figure 3.3 Effect of increasing light intensity on uptake of label by protein

3) Examination of Light Dependence of Labelling

Having established in previous experiments that covalent linking of ^{3}H-tryptophan to protein could be induced in the next stage in the study of the labelling technique was examination of the light dependence of the reaction.

Identical incubation mixtures to those used in previous experiments were prepared in subdued light and the vials were covered with metal foil. The foil covered vials were then subjected to irradiation under identical conditions to those used previously. Protein was separated from the mixture by gel filtration and it was found that there was no radioactivity associated with the protein peak. It was therefore concluded that the labelling is in fact light dependent.

The relationship between illumination and labelling was then examined. An Osram 500 watt Photoflood bulb was placed at different distances from a series of identical protein samples, resulting in irradiation of samples at different light intensities. Irradiation was for 1 minute and unbound fluorescein and tryptophan were then removed from the protein by extensive dialysis against 0.05 M Tris-HCl pH 7.5 containing 2 mM $CaCl_2$. The results of this experiment are shown in Figure 3.3.

This graph clearly shows that uptake of label by protein increases as light intensity is increased. At lower light intensities the rate of increase in label bound is much greater than the rate of increase in light intensity. Therefore small differences in light intensity as determined by light-sample distance would result in large differences in the amount of label bound by identical samples. This clearly shows that it is desirable to perform irradiation at maximum possible light intensity both to maximise

binding of label by protein and to improve reproducability.

With the apparatus used for the experiments in this work the minimum light-sample distance obtainable was 13.5 cm which gave a light intensity of 3.6 mE M^{-2} s^{-1} at the surface of the sample. Therefore this was the light intensity used for all subsequent irradiations.

The total amount of light applied to the sample during irradiation could, of course, be increased by increasing the illumination period. However it was decided to maintain illumination period at 1 minute to minimise any danger of thermal induced changes in protein caused by the heat generated by the light source.

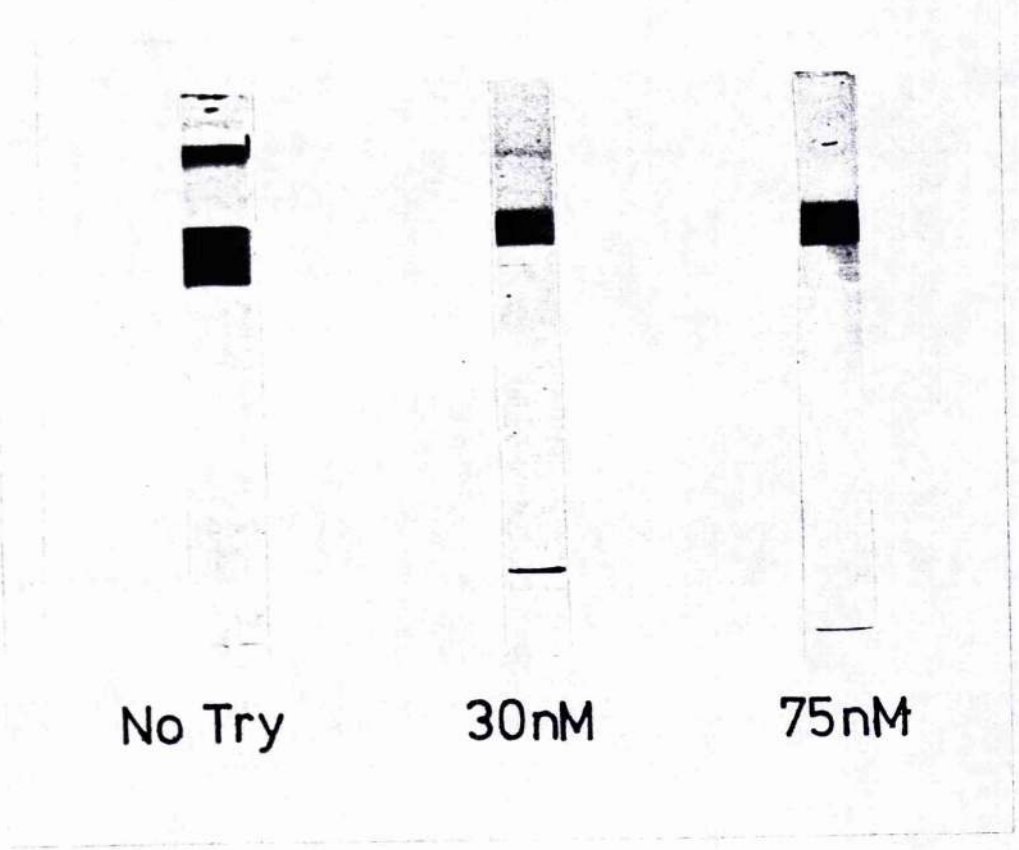


Figure 3.4 Effect of irradiation of protein in presence of different concentrations of tryptophan

4) Optimisation of Labelling : Cross-Linking Ratio

Examination of protein on SDS PAGE after previous labelling experiments showed that, although labelling was taking place there was also an appreciable amount of cross-linking of protein occurring. Since labelling and cross-linking are competing reactions cross-linking will reduce the availability of any given region for labelling and this could lead to errors in the assessment of distribution of label. Thus if the technique of photo-sensitized labelling is to be used to assess conformation it is necessary to minimise the amount of cross-linking taking place.

As labelling and cross-linking are competing reactions the two ways to reduce cross-linking are to decrease the concentration of protein present or to increase the concentration of tryptophan. In the system under study here, it is, of course, necessary to have sufficient protein for anlysis on SDS PAGE after labelling and so it is not really practicable to use less than 0.1 mg of protein for each experiment. However, envisaging development of the technique to the point where digestion could be carried out after labelling it was thought that this would then become insufficient. Therefore to avoid changing the system during the work the quantity of protein to be used in each experiment was set at 0.6 mg giving a final concentration of 0.4 mg/ml.

Figure 3.4 shows the effect of irradiation of fragment D at this chosen concentration in the presence of differing concentrations of tryptophan. It can be seen that, at a final concentration of 30 nM tryptophan very little inter-molecular cross-linking has occurred (less than 5% total protein as assessed by uptake of coomassie

blue stain). Therefore in all labelling experiments tryptophan was present at a final concentration of 30 nM.

Obviously it would be desirable to eliminate cross-linking entirely however this would require final tryptophan concentrations of around 75 nM and at this concentration the level of ^{3}H-tryptophan would be below 5% (w/w) and this would result in labelled protein having a low specific activity which would make analysis of distribution by gel electrophoresis very difficult. Therefore the concentration of tryptophan of 30 nM used in labelling experiments in this work represents a compromise between reducing cross-linking and obtaining labelled protein with sufficiently high radioactivity for subsequent analysis.

5) Distribution of Label within Fibrinogen Fragment D

Fibrinogen fragment D (Ca^{++}) which had been labelled as outlined earlier was reduced and run on SDS PAGE. A photograph of a typical gel is shown below:

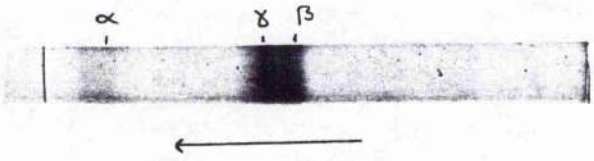


Figure 3.5 SDS Polyacrylamide Gel (5% Acrylamide) of Reduced Photo-Sensitized Labelled Fibrinogen Fragment D (Ca^{++})

It can be seen that this shows no appreciable difference from gels of untreated fragment D (Ca^{++}) shown earlier (Fig 2.7)

Gels of labelled fragment D (Ca^{++}) were sliced and the radioactivity in each slice was counted. As well as normal electrophoretic runs some samples were run for longer periods to improve separation of the β and γ chains and others were run for shorter periods to maintain the α chain as a tight band, the radioactivity of which could then be compared with the total radioactivity of the β and γ bands. The pattern of distribution of label between the three chains showed little variation over a large number of experiments and the results obtained are summarised in Table 3(i). Values are given as the percentage of the total label in the sample present on each chain and are quoted as mean values from seven experiments ± 1 standard deviation:

α	19,0% $\pm$ 2.1
β	28.6% $\pm$ 1.3
γ	52.0% $\pm$ 2.5

Table 3(i) Distribution of Label between the Chains of Fragment D (Ca^{++})

If the labelling technique is shown to be useful in assessing information these results would suggest that the γ chain is highly exposed at the surface of fragment D (Ca^{++}) whilst the β chain is much more protected in the interior of the molecule. Both these suggestions are consistent with the well characterised susceptibility of the γ chain and resistance of the β chain to proteolytic attack (Furlan, et al, 1975).

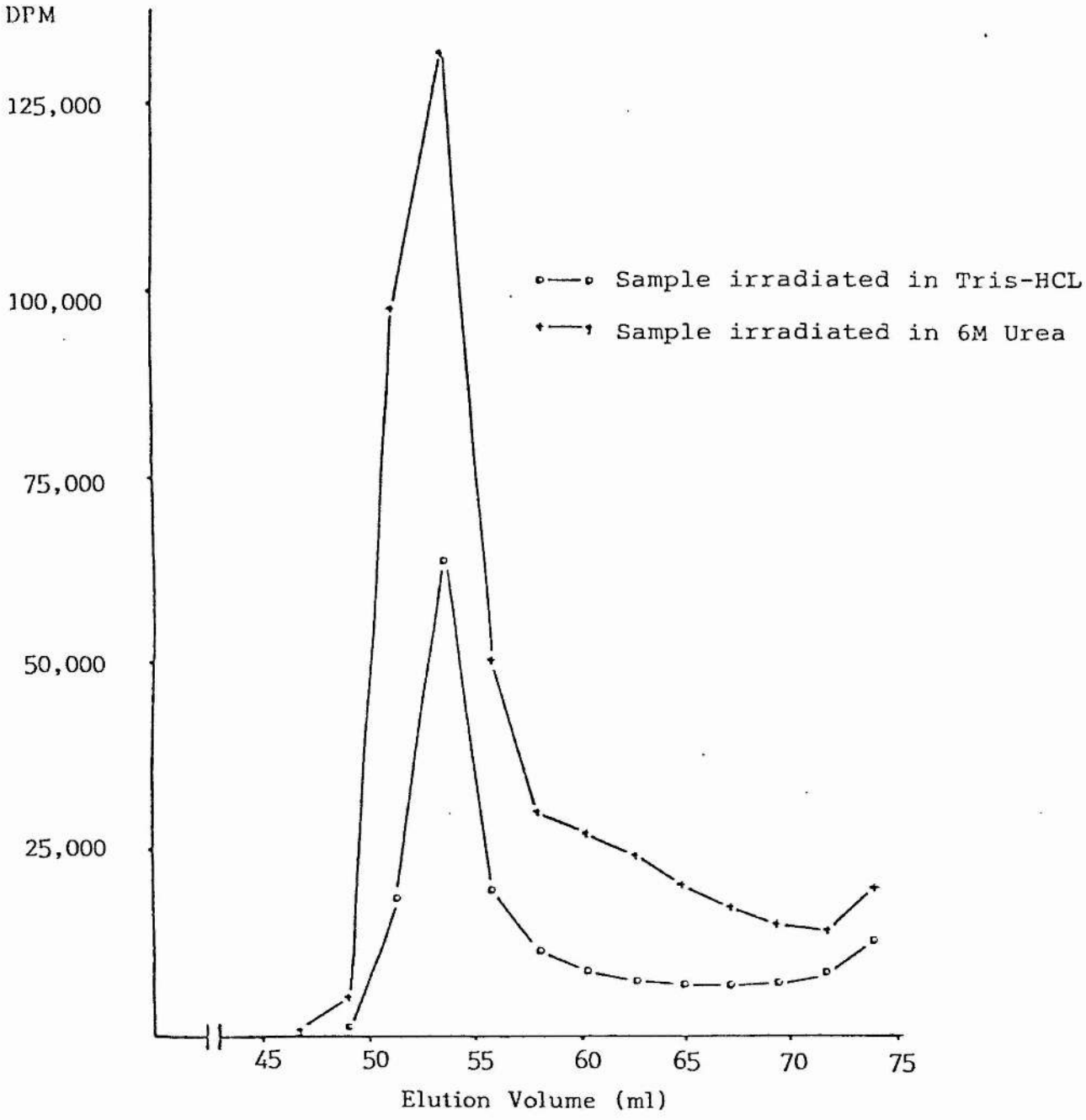


Figure 3.6 Elution profile of irradiated protein from Sephadex G-25

6) Labelling in the Presence of Urea

Identical aliquots of fragment D (Ca^{++}) in 0.05 M Tris-HCl pH 7.5 containing 2 mM $CaCl_2$ were taken and one was made 6M with respect to urea. Both were then irradiated under identical conditions and applied to gel filtration columns. Examples of the elution profiles of protein containing peaks are shown in Figure 3.6.

It can be seen from Figure 3.6 that protein labelled in the presence of urea bound much more tryptophan. Over a series of experiments it was found that fragment D (Ca^{++}) labelled in the presence of 6M urea bound 2.7 times the amount of label bound by identical samples labelled without urea.

The distribution of label between the chains of fragment D (Ca^{++}) labelled in the presence of urea was then examined as outlined earlier and the averaged results from 6 experiments are presented in the same way as before in Table 3(ii).

α	18% $\pm$ 2.8
β	36.5% $\pm$ 2.1
γ	45.5% $\pm$ 2.1

Table 3(ii) Distribution of Label between the Chains of Fragment D (Ca^{++}) Labelled in the Presence of 6M Urea

When data from this experiment was compared to data from the previous experiment using Student's t-test a significant increase in the amount of label bound by the β chain and a decrease in the amount bound by the γ chain was found. (β chain $p < 0.0002$, γ chain $p < 0.05$).

Therefore denaturation clearly affects the distribution of label and so it can be concluded that the labelling technique does in fact monitor protein conformation.

The fact that there is no difference in the amount of label bound by the α chain is not entirely unexpected since it bound a disproportionately high amount of label when fragment D (Ca^{++}) was labelled under non-denaturing conditions. Thus it would appear to be highly exposed in native fragment D (Ca^{++}) and therefore denaturation would not be expected to significantly alter its degree of exposure.

7) Digestion of Labelled Fragment D (Ca^{++})

Fibrinogen fragment D (Ca^{++}) which had been subjected to photo-sensitized labelling was dialysed against buffer containing 25 mM EDTA and was then digested with plasmin for four hours. A gel of a typical digestion is shown below:

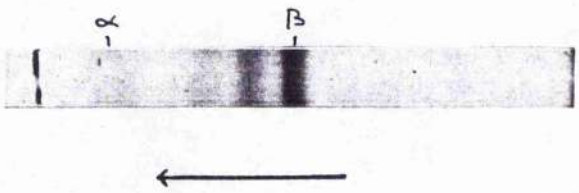


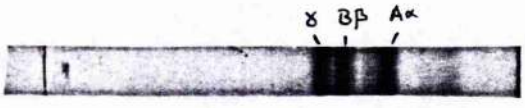
Figure 3.7 SDS Polyacrylamide Gel (5% Arcylamide) of Reduced Digest of Labelled Fragment D (Ca^{++})

It can be seen that this gel shows a number of bands corresponding to molecular weights between 39,000 and 28,000. Furlan et.al. (1975) and Nieuwenhuizen et.al (1983) have reported the presence of chains between these molecular weights in digests of fragment D (Ca^{++}) carried out in the absence of calcium and all have been designated as γ chain remnants.

As digestions of fragment D (Ca^{++}) in the presence of EDTA did not produce a single γ chain, the sum of all of the radioactivity lying between the end of the β chain band and the start of the α chain band was taken to represent γ chain. Since the β chain is unaffected by the digestion, it is possible, by using the absolute amount of radioactivity which it carries to estimate how much label the population of γ chains would have carried when intact.

Using these assumptions it was found that the digestion of the γ chain had resulted in the loss of 39% of the label that it carried when intact. The smallest γ chain remnants present in the digest have a molecular weight of 28,000 which means that they have lost at most, an 11,000 portion of protein. This 11,000 C-terminal portion represents a loss of 28% of the total γ chain protein.

Therefore it must be concluded that the C-terminal portion of the γ chain of fragment D (Ca^{++}) binds a disproportionately high amount of label.



migration

Figure 3.8 Photograph of SDS polyacrylamide gel (5% acrylamide) of fibrinogen reduced after Photo–sensitized labelling

8) Labelling of Fibrinogen

Fibrinogen was labelled under the conditions outlined earlier and was separated from unbound fluorescein and tryptophan by the centrifugal desalting technique outlined in the previous chapter. This allowed the sample to be analysed on polyacrylamide gels more quickly which was thought to be preferable in this case in view of the known lability of the C-terminus of the Aα chain of fibrinogen (Semerraro et.al.1977; Mosesson et.al.1974).

Figure 3.8 shows a photograph of an SDS polyacrylamide gel of reduced, labelled fibrinogen and, on examination it may be seen that intact Aα chain is still present. The pattern of distribution of label was examined as outlined earlier and averaged results of four experiments are given in Table 3(iii).

Aα	58.3% $\pm$ 3.1
Bβ	20.5% $\pm$ 2.4
γ	21.2% $\pm$ 2.6

Table 3(iii) Distribution of Label between the Chains of Fibrinogen

These results show that the Aα chain of fibrinogen binds most of the label whilst the Bβ and γ chains only bind minor amounts. This is consistent with the published results of Hemmendorf et.al. (1981) and, if the technique of photo-sensitized labelling is shown to be surface oriented would also be consistent with suggestions made by a number of workers (Gaffney & Dobos, 1971; Blomback, M., 1976; Furlan & Beck, 1975) who, using different techniques concluded that the Aα chain of fibrinogen is more heavily exposed at the surface of the protein than either of the other chains.

9) Comparison of Labelling of Fibrinogen and fragment D

Equivalent amounts of fibrinogen and fragment D were labelled under identical conditions and the samples were freed from fluorescein and unbound tryptophan by dialysis.

In this experiment dialysis was chosen because it allowed both samples to be subjected to identical treatments. Dialysis against 0.05 M Tris-HCl pH 7.5 containing 2 mM $CaCl_2$ was carried out in darkness for 24 hours at 4°C

Aliquots of each protein were taken and protein estimations and radioactivity measurements were carried out in triplicate.

It was found that fibrinogen bound 0.414 µg of tryptophan per mg of protein whilst fragment D (Ca^{++}) bound 0.427 µg of tryptophan per mg of protein. These results represent averaged values of seven experiments and for both proteins the experimental spread was greater than was hoped for, Standard deviations for both sets of data were in the region of 0.05.

	Irradiated Fibrinogen	Peak I Fibrinogen
Asp	122.8	126.1
Thr	60.2	61.9
Ser	89.7	86.8
Glu	115.3	114.7
Pro	49.6	51.8
Gly	102.2	99.5
Ala	55.5	52.4
Val	38.7	41.4
Met	17.6	16.9
Ile	31.7	35.1
leu	61.0	58.6
tyr	35.3	36.7
Phe	32.7	31.5
Try	21.7	20.8
lye	78.1	77.7
His	25.2	26.0
Arg	59.4	59.0

Table 3(iv) Amino Acid Compositions of Irradiated and Non-Irradiated Fibrinogen

10) Amino Acid Analysis of Labelled Fibrinogen

Amino acid analysis of labelled fibrinogen was carried out after irradiation to determine whether any changes in individual amino acids or amino acid composition could be detected. Samples for analysis were irradiated for 5 minutes instead of the usual period of 1 minute to maximise any such changes. Table 3(iv) gives averaged amino acid analysis data from 4 hydrolyses of irradiated and non-irradiated fibrinogen.

There was no significant difference in the retention time of any amino acid after irradiation and it can therefore be assumed that no major modification of any single residue has occurred.

Comparing the two sets of data it can be seen that they are very similar. There is certainly no evidence to back the claim made by Hemmendorf et.al. (1981) that, after irradiation, reduced levels of histidine and tyrosine were to be found. This apparent difference could be due to the higher light intensity used by Hemmendorf in his work.

The major conclusion to be drawn from these results however, is that, under the conditions used in this work, photo-sensitized labelling has no effect on the amino acid composition of fibrinogen. The technique as used here would therefore appear to be non-destructive and consequently unlikely to seriously disrupt the conformation of any protein under study.

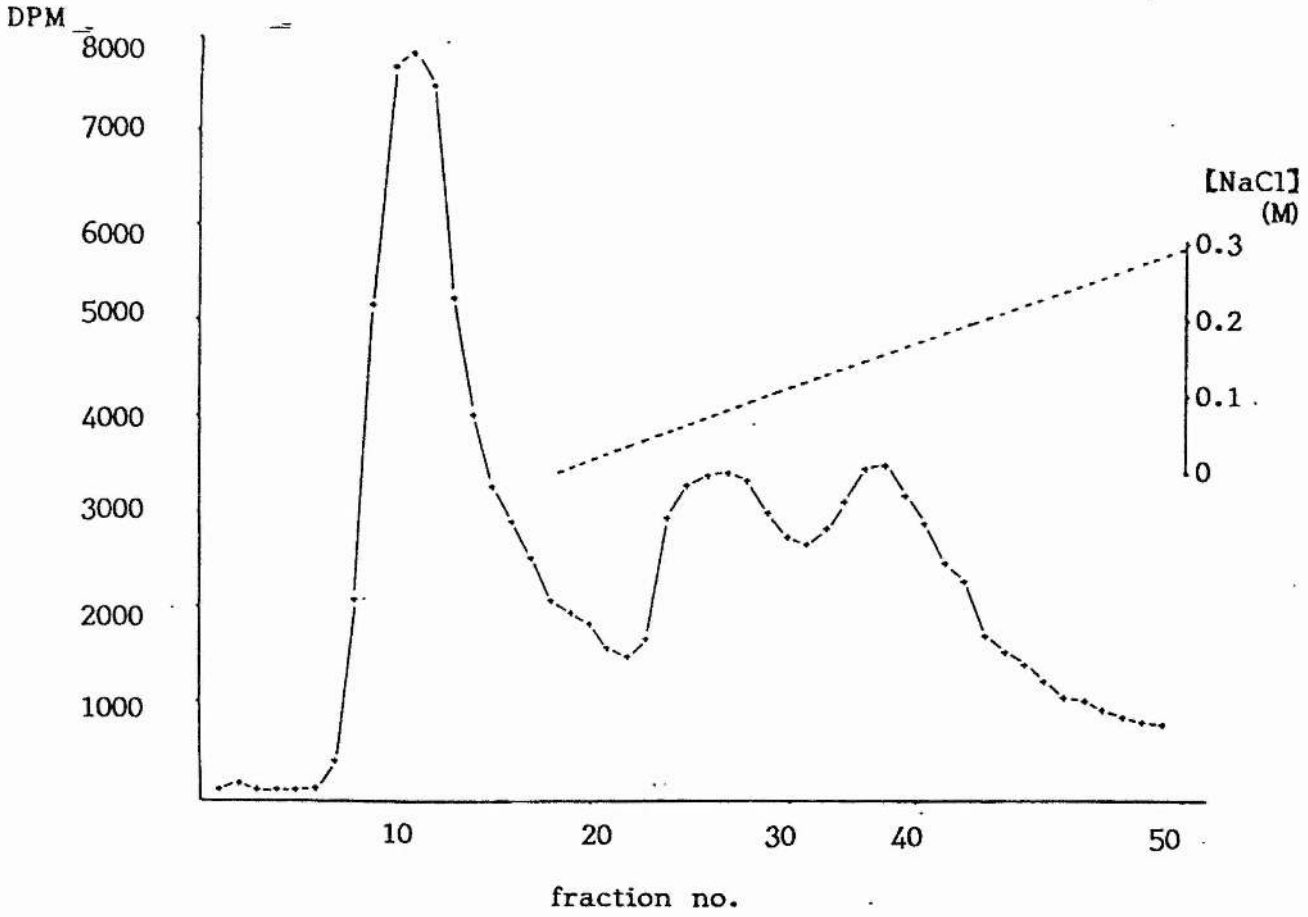


Figure 3.9 Elution profile of digest of ^{3}H-Fibrinogen from DEAE-cellulose column

11) Digestion of Labelled Fibrinogen

Fibrinogen which had been labelled as outlined earlier which showed typical distribution of label and a level of labelling close to the average value of 0.414 µg try/mg protein quoted earlier was digested with plasmin. The products of the digestion were separated on a column (20 cm x 1 cm) of DEAE-cellulose the eluant of which was immediately passed through a column (10 cm x 1 cm) of lysine agarose. The elution profile of the combined columns is shown in Figure 3.9.

It can be seen that this profile is very similar to the profile obtained when digests of unlabelled fibrinogen were applied to similar columns (see Figure 2.5) and this similarity was confirmed by examination of gels of each peak which were foundto be identical to those in Figure 2.5.

The first peak contains pure fragment D (Ca^{++}) and estimations carried out on the material from this peak showed that the fragment D (Ca^{++}) had bound 0.303 µg of tryptophan per mg of protein, this value is an average from 4 experiments and the agreement between them was good (Standard deviation = 0.022). This represents a statistically significant reduction from the value of 0.427 µg tryptophan bound per mg of protein found when fragment D (Ca^{++}) is labelled in solution.

The pattern of distribution of the label between the chains of the fragment D (Ca^{++}) was then analysed and the pattern found is given in Table 3(v) overleaf.

α	19% $\pm$ 0.7
β	31% $\pm$ 3.1
γ	50% $\pm$ 3.5

Table 3(v) Distribution of Label between the Chains of Fragment D (Ca^{++}) derived from Labelled Fibrinogen

Comparison of this data with that given in Table 3(i) for protein labelled as fragment D (Ca^{++}) using Students t-test showed no significant difference in the two sets of data.

DISCUSSION

At the start of this section the stated aims were:

(i) To optimise conditions for the photo-sensitized labelling of protein.

(ii) To assess the usefulness of such labelling as a probe of conformation.

(iii) To examine fibrinogen using photo-sensitized labelling if the technique proved suitable.

Towards the first two objectives studies were carried out using the fibrinogen degradation product fragment D because of the known stability of fragment D to proteolytic attack in the presence of calcium ions (Haverkate & Timan, 1977).

Initial experiments showed that binding of label to protein could be induced by illumination in the presence of fluorescein and it was further shown that the linkage between the protein and the tryptophan label was covalent. Subsequent work showed that the conditions of the experiment could affect the degree of labelling and this work led to the establishment of a set of conditions which maximised the labelling of protein whilst minimising the competing cross-linking reaction. Thus the first aim had been achieved, a system had been developed which induced consistent labelling of protein.

Studies on fibrinogen fragment D (Ca^{++}) showed that the distribution of label between the chains of the protein remained consistent over a number of experiments and that this distribution was independent of the total amount of label bound by the protein.

When labelling was carried out on fragment D (Ca^{++}) which was denatured by addition of urea prior to irradiation it was found that these samples bound 2.7 times the amount of label bound by identical samples labelled without denaturation. It was also found that the pattern of distribution of label was altered significantly when labelling was carried out in the presence of urea.

These results are what would be expected if the technique is conformation dependent. Obviously if this were the case maximal uptake of label would be by random coil protein where, if labelling were also totally random, one would expect the amount of label bound by each chain to be directly proportional to the protein mass of the chain. In the case of this experiment however, the presence of disulphide bonds imposes conformational constraints on the denatured fragment D (Ca^{++}) and so the case of totally random distribution cannot apply. However the change in distribution, certainly by the β and γ chains does reflect a move towards such a distribution with the proportion of label bound by the β chain rising from 28% to 36% whilst the prorportion bound by the γ chain falls from 52% to 45%. Thus it would appear that the labelling technique is, in fact, conformation dependent. Further, indirect evidence for this conclusion is provided by the observed distribution of label in non-denatured fragment D (Ca^{++}). Furlan et.al. (1975) showed that the γ chain of fragment D is susceptible to proteolytic attack whilst the β chain is highly resistant and this is consistent with the observed distribution of label in fragment D (Ca^{++}) which

suggests that the γ chain is highly exposed at the surface of the molecule whilst the β chain is protected in the interior.

Results obtained from the labelling of fibrinogen are in good agreement with currently accepted ideas about fibrinogen shape and, when the data from this work is analysed using the method of Hemmendorf et.al. (1981) an $A\alpha$: $B\beta$: γ labelling ratio of 2.8 : 1 : 1.03 is obtained, this is very close to the figures published in that work of 2.7 : 1 : 1. Thus the technique appears to yield a highly consistent distribution of label.

Results from amino acid analysis and the fact the digestion of fibrinogen after labelling proceeds as normal suggests that the labelling technique does not significantly alter the structure or conformation of protein under study.

Thus it would appear that the technique of photo-sensitized labelling is of use in assessing protein shape.

Having established the usefulness of photo-sensitized labelling as a probe of protein conformation the results obtained from the labelling of fibrinogen can now be examined in an effort to derive information about the shape of the protein. The most striking point about the distribution of label in fibrinogen (Table 3(iii)) is that the $A\alpha$ chain binds almost three times more label than either of the other two chains. This agrees entirely with the views expressed by a number of workers (Gaffney & Dobos, 1971; Blomback.M, 1976; Furlan & Beck, 1975) who, using different techniques all concluded that the $A\alpha$ chain of fibrinogen and, more particularly the C-terminus of the $A\alpha$ chain is predominantly surface oriented.

More information about the shape of fibrinogen was obtained by studying its digestion product fragment D (Ca^{++}). The first point to be noted in these studies is that the pattern of distribution of label between the chains of fragment D (Ca^{++}) labelled as fragment D (Ca^{++}) in solution shows no significant difference to the distribution in fragment D (Ca^{++}) derived from labelled fibrinogen. This suggests that the fragment D domain of fibrinogen undergoes no major conformational changes during digestion of the protein and any findings about the shape of fragment D (Ca^{++}) in solution are therefore directly applicable to fibrinogen. However material labelled as fragment D (Ca^{++}) bound 25% more label than fragment D (Ca^{++}) produced from digestion of labelled fibrinogen, suggesting that the fragment D domains of fibrinogen are, to some extent protected by some other portion of the molecule. Earlier results in this work would suggest that it is the C-terminus portion of the Aα chain which fulfils this protecting role.

Knowing the molecular weights of each of the chains of fibrinogen and fragment D (Ca^{++}) it is possible, using data about the total label bound by each protein and the distribution of that label, to ascertain the amount of label bound by 1 mole of each chain. From this value the amount of label per unit length of each chain may be calculated, assuming of course that length is directly proportional to weight. The numerical value of label per unit length may then be taken as an index of exposure for each chain. Data derived in this way is given in Tables 3(vi) and 3(vii) for fibrinogen and fragment D (Ca^{++}) derived from labelled fibrinogen respectively.

Species	% label bound	Label/mole	Label/unit length
Fibrinogen	100%	144,500 mg	
A α	58%	41,905 mg	0.625
B β	20%	14,450 mg	0.249
γ	21%	15,173 mg	0.322

Table 3 (vi) Analysis of Distribution of label bound by fibrinogen

Species	% label bound	Label/mole	Label/unit length
Fragment D (Ca^{++})	100%	27,300 mg	
α	19%	5,460 mg	0.546
β	28%	7,644 mg	0.182
γ	53%	14,469 mg	0.371

Table 3 (vii) Analysis of distribution of label bound by fragment D (Ca^{++})

This treatment of results reveals a number of interesting points about both species and their relationship to each other. Dealing first with the data for fragment D (Ca^{++}) this reveals that although the γ chain binds most of the label the α chain actually has a higher exposure index indicating that, as a whole, it is more surface oriented than the γ chain. The points made earlier about the β chain are reinforced by these figures, the major proportion of it must be protected in the interior of the molecule. Therefore the picture of fragment D (Ca^{++}) which emerges from this work is one where the bulk of the surface of the protein is composed by γ chain with almost all of the α chain also solvent exposed.

When these findings are taken in conjunction with the results of digestions of labelled fragment D (Ca^{++}) in the presence of EDTA more conclusions about the exposed surface of fragment D (Ca^{++}) may be drawn. Although the results of these digestions were not entirely satisfactory they do suggest that the C-terminus of the γ chain is more highly exposed than the rest of the chain.

Even if all of the γ chains in the population had been digested to 28,000 molecular weight remnants, the observed loss of label would mean that the 11,000 molecular weight portion lost would have an exposure index of 0.513 indicating that it was highly exposed. However as the gel in Figure 3.7 clearly shows not all the γ chains have been digested to that extent, therefore the true figure for the exposure index of the C-terminus of the γ chain must in fact be even higher than 0.513. This allows the conclusions drawn earlier about fragment D (Ca^{++}) to be extended and thus it can be said that it is the C-terminus

of the γ chain of fragment D (Ca^{++}) which is heavily surface oriented and an obvious extension of these findings is that the N-terminus must therefore be more protected.

The figures for fibrinogen in Table 3(vi) merely reinforce the point made earlier about the high degree of surface exposure at the Aα chain. However considering the figures for both proteins together a number of points may be made. One striking point is that the two moles of fragment D (Ca^{++}) produced from every mole of fibrinogen only carry 37% of the label of the fibrinogen yet they represent 58% of the protein mass. Therefore the fragment D domains of fibrinogen must be protected regions in the molecule and, as mentioned earlier, it is most likely that it is the C-terminus of the Aα chain which shields them.

Perhaps the most important point shown by these results is the evidence that almost all of the label bound by the γ chain of fibrinogen is bound by the portion which represents the γ chain of fragment D (Ca^{++}). This suggests that this is the only portion of the γ chain of fibrinogen which is solvent exposed. This finding is particularly interesting in view of the role of this portion of the γ chain of firbinogen in calcium binding (Lawrie & Kemp, 1979) and in forming inter-molecular cross-links under the action of Factor XIII in fibrin formation (Haverkate et.al.,1979). Thus the technique of photo-sensitized labelling appears to provide direct evidence that this cross-linking site is exposed in fibrinogen in solution.

INTRODUCTION

Chemical cross-linking reagents have been used in the investigation of structure and functional relationships in a number of proteins (Peters, 1977). The location of cross-links induced by chemical cross-linking reagents is dependent upon the three dimensional shape of the protein under study as this will determine the distance between suitable reacting residues in the same, or different protein molecules.

Being a large hexameric protein, fibrinogen would appear to lend itself particularly well to this kind of study particularly if, after cross-linking it could be digested thus revealing information about different parts of the molecule and their relation to each other. Furthermore, having shown in the previous section of this work that the fragment D domain of fibrinogen and fragment D (Ca^{++}) in solution appear to be very similar conformationally, cross-linking studies of fragment D (Ca^{++}) may yield information which is directly applicable to fibrinogen.

In this work two different types of chemical cross-linking reagents have been used, the bis-imidates which react with lysine residues (Kennedy 1976) and tetranitromethane (TNM) which reacts with tyrosine residues (Sokolovsky et.al. 1966). The bis-imidates are particularly suited to this type of study since they form a group with the general structure:-

$$^{+}H_2N = COCH_3 - (CH_2)_n - COCH_3 = NH_2^{+}$$

where the number of CH_2 groups may be controlled giving rise to a series of reagents which will cross-link over different interresidue

distances. The bis-imidates used in this work were dimethyl suberimidate (DMS) where n = 8 and dimethyl adipimidate (DMA) where n = 6, giving cross-link distances of 11 Å and 8 Å respectively. Since the reaction between the imidate and lysine results in no net change of charge it is unlikely that the reaction will disrupt the tertiary structure of the protein.

Since TNM reacts with tyrosine residues, it was thought that it would be more likely to penetrate hydrophobic domains of the protein causing more intra-molecular cross-linking than the bis-imidates which react with lysine residues which are generally more abundant on the surface of proteins.

The major problem in using chemical cross-linking reagents is that, should a second residue not be suitably positioned, after reaction with one residue the cross-linking reagent will be hydrolysed leaving the residue which initially reacted blocked. Thus the modification induced in the protein by reaction with a cross-linking reagent is fairly large, with, in the case of bis-imidates most of the lysine residues of the protein being converted to amidine derivatives (Kennedy 1976) and, in the case of TNM most of the tyrosines being nitrated. It seems likely that, particularly in the case of bis-imidates such wholesale modification of fibrinogen would interfere with subsequent plasmic digestion of the protein. TNM produces a special problem in the case of fibrinogen in that the cross-linking reaction requires overnight incubation at 37°C. With a protei such as fibrinogen with its well documented lability of Aα chain C-termina portions (Semerarro,1977;Mosesson et al 1974) these reaction conditions

are not really suitable. Considerations such as these have limited the extent of previous studies of fibrinogen using chemical cross-linking (Furlan & Beck 1975; Mihalyi 1963; Doolittle 1973).

Therefore it was decided to investigate the point made in the previous chapter that the technique of photo-sensitized labelling can, if conditions are not suitably controlled cause polymerisation to occur. Recalling the possible coupling reactions following the primary photochemical reaction outlined in the previous chapter:-

$$M^{\cdot} + D \longrightarrow M - D \qquad (1)$$

$$M^{\cdot} + Try^{\cdot} \longrightarrow M - Try \qquad (2)$$

$$M^{\cdot} + M^{\cdot} \longrightarrow M - M \qquad (3)$$

D = dye M = Macromolecule

It can be seen that these are all competing reactions, and in the previous section conditions for reaction (2) were optimised. However, if the amount of tryptophan in the reacting mixture were reduced then the system should favour reaction (3), bearing in mind that results from the previous section showed that reaction (1) does not occur to any large extent.

Furthermore results of amino acid analysis in the previous chapter showed that photo-sensitized labelling was non-destructive, therefore any cross-linking reaction based on that technique would not be expected to damage protein and should yield a product suitable for digestion.

Therefore this section may be considered in two parts each with different, although connected aims. The aim of the first part being to study the terminal degradation products

fragment D produced from fibrinogen by plasmin digestion both in the presence of Ca^{++} and EDTA using classical chemical cross-linking techniques.

The aim of the second part is to develop a system for photo cross-linking fibrinogen in the hope that such a technique will cross-link the protein without the major modifications induced by chemical cross-linking reagents thus yielding a cross-linked product which may be further examined by plasmin digestion.

METHODS

1) Cross-Linking with Bis-Imidates

DMS was purchased from the Sigma Chemical Company, DMA was a gift from Dr.J.Coggins, Dept.of Biochemistry,University of Glasgow.

Cross-linking was carried out using a method based on that of Furlan et.al. (1977). A 30 mM stock solution of the required bis-imidate in 0.1M triethanolamine buffer pH 8.5 containing 2 mM $CaCl_2$ was prepared immediately prior to use. The necessary volume of this stock solution required to give the desired final concentration of bis-imidate was added to a solution of protein in the same buffer. Cross-linking was carried out using bis-imidate concentrations from 1 mM to 20 mM and protein concentrations between 0.25 mg/ml and 3.0 mg/ml.

Reaction was allowed to proceed for 30 minutes at 30°C, samples were then desalted, either using the centrifugal method outlined earlier or by dialysis for 2 hours against chamber buffer Cross-linked protein was then analysed using SDS PAGE on gels containing 5% acrylamide.

2) Cross-Linking with Tetranitromethane

TNM was purchased from the Sigma Chemical Company and cross-linking was carried out using a method based on that of Furlan & Beck (1975).

TNM was dissolved in 95% ethanol to a final concentration

of 50 mg/ml. Suitable aliquots of this solution were added to solution of protein in 0.05 M Tris-HCl pH 7.5 containing 2 mM $CaCl_2$ to give final TNM concentrations between 0.05 mg/ml and 2.0 mg/ml. Final protein concentrations were between 0.25 mg/ml and 3.0 mg/ml.

Reaction was allowed to proceed at 37°C for 12 hours. The protein was then precipitated by addition of an equal volume of 10% (w/v) trichloroacetic acid (TCA) followed by centrifugation at 2000 rpm for 10 minutes. The precipitate was then washed with 5% (w/v) TCA, centrifuged as before and resuspended in a solution of 6M Urea containing 3% SDS. This solution was incubated at 37°C for 2 hours prior to sample application to gels for SDS PAGE.

3) Photo-Sensitized Cross-Linking

Optimum conditions for photo-sensitized cross-linking were determined using information gained from experiments in the development of photo-sensitized labelling outlined in the previous chapter.

Protein concentrations of between 0.4 mg/ml and 2.0 mg/ml were used, fluorescein was present at a final concentration of 1 mM and illumination was for 1 minute. No tryptophan was included in these incubations. Samples were freed from contaminating fluorescein by dialysis, and analysis was by SDS PAGE on gels containing 3% acrylamide in the case of non-reduced samples and 5% acrylamide for reduced samples.

Digestions were carried out as outlined earlier.

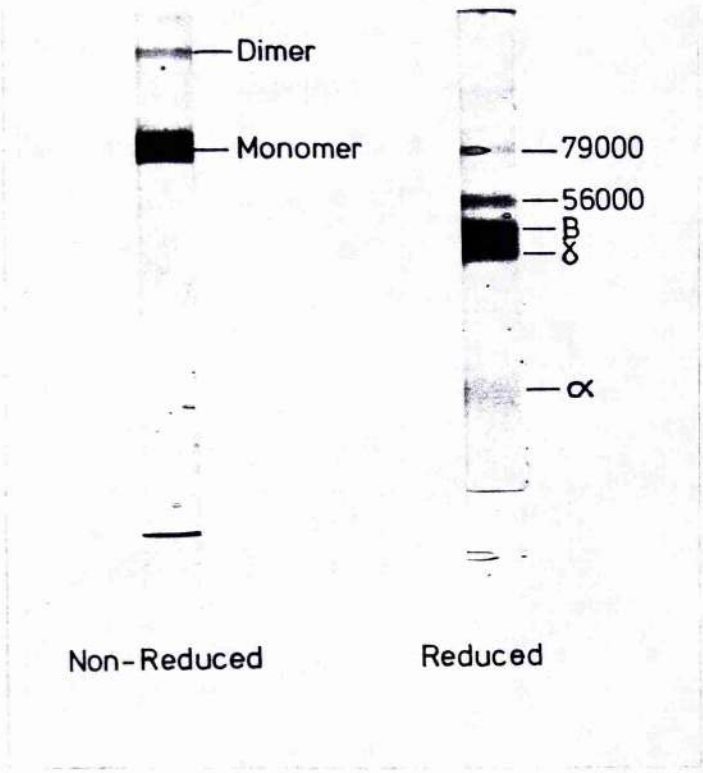


Figure 4.1 SDS Polyacrylamide gels (5% Acrylamide) showing effect of cross-linking on fragment D (Ca^{++})

RESULTS

1) Cross-Linking of Fragment D (Ca^{++})

Fibrinogen fragment D (Ca^{++}) was treated with each of the three chemical cross-linking reagents at varying concentrations. It was found that qualitatively there was no difference in the pattern of cross-linking induced by any of them although it was noted that the reagents producing shorter cross-links caused less cross-linking to occur.

Figure 4.1 shows 5% acrylamide gels of fragment D (Ca^{++}) treated with 1 mM DMS.

The gel of DMS treated fragment D (Ca^{++}) run without reduction clearly shows that inter-molecular cross-linking has occurred as there is a band of lower mobility which can only be ascribed to dimeric fragment D (Ca^{++}). However after reduction there appears to be much more evidence of cross-linking having taken place with the gel showing very obvious bands having apparent molecular weights of 56,000 and 79,000 respectively. It must therefore be assumed that when fragment D (Ca^{++}) is treated with chemical cross-linking reagents the bulk of the cross-linking which takes place is intra-molecular.

Figure 4.2 shows the effect of increasing the concentration of cross-linker on the cross-linking pattern of fragment D (Ca^{++}). The two most obvious points are the appearance of a band having

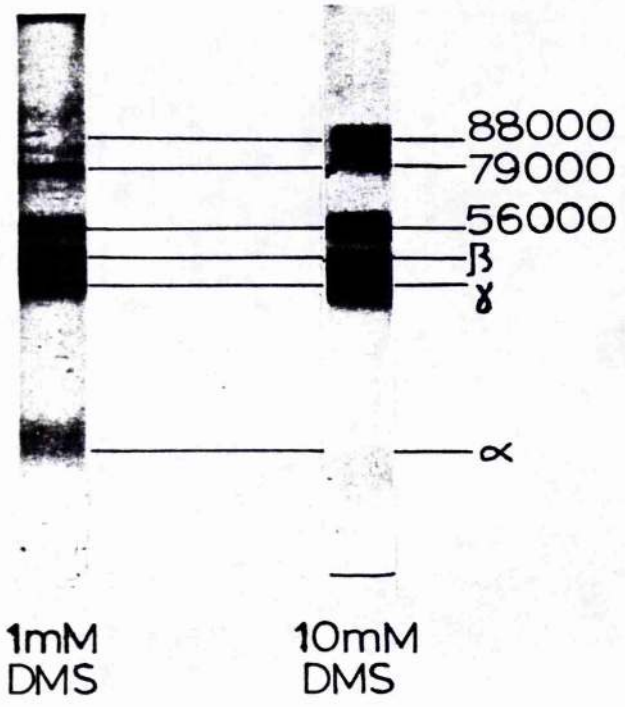


Figure 4.2 SDS Polyacrylamide gels (5% Acrylamide) showing effect of increasing cross-linker concentration on the pattern of reduced chains of fragment D (Ca^{++})

apparent molecular weight of 88,000 and the almost complete disappearance of monomeric α-chain.

It was also found that increasing cross-linker concentrations only increased the amount of dimer formation to the point where dimeric fragment D represented 16% of the total protein applied to gels.

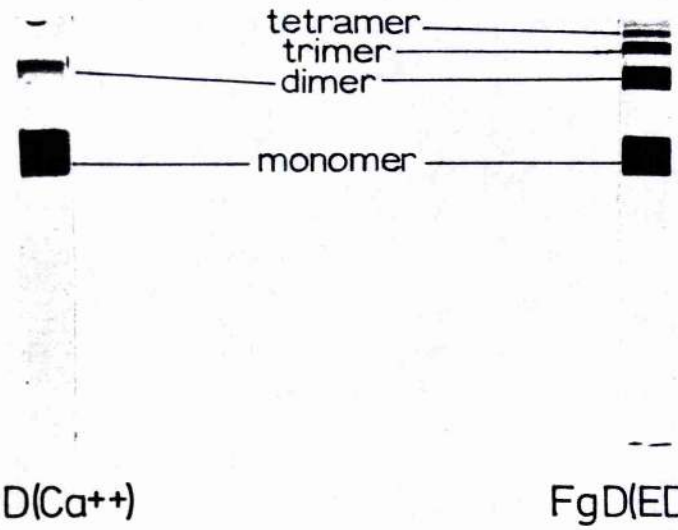


Figure 4.3 SDS Polyacrylamide gels (5% Acrylamide) showing effect of cross-linker on fragment D (Ca^{++}) and fragment D (EDTA)

2) Comparison of Cross-Linking of Fragment D Species

The cross-linking patterns of fragment D (Ca^{++}) and fragment D (EDTA) were compared over a wide range of protein and cross-linker concentrations and it was found that fragment D (EDTA) consistently showed a much higher level of inter-molecular cross-linking than fragment D (Ca^{++}). This is illustrated by Figure 4.3 which shows the pattern on 5% acrylamide gels of each of the fragment D species treated with DMA at protein concentrations of 1 mg/ml.

It was previously shown that when fragment D (Ca^{++}) is treated with chemical cross-linkers the bulk of the cross-linking which takes place is intra-molecular. However the pattern of cross-linking with fragment D (EDTA) is grossly different with very much more inter-molecular cross-linking taking place leading to the formation of dimers, trimers and tetramers. This suggests that there is large conformational difference between fragment D (Ca^{++}) and fragment D (EDTA) with fragment D (EDTA) adopting a less rigid, more open conformation which permits closer contact between the constituent chains of adjacent molecules allowing the production of ologomers when fragment D (EDTA) is treated with chemical cross-linking reagents.

3) Effect of EDTA and Urea on Cross-Linking

Having shown a conformational difference between fragment D (Ca^{++}) and fragment D (EDTA) which has undergone cleavage at the C-terminus of the γ-chain the purpose of this work was to investigate whether the observed conformational change was a result of digestion or calcium removal.

Solutions of fragment D (Ca^{++}) were dialysed against three changes of 0.1M TEA pH 8.5 containing 25 mM EDTA, stock bis-imidate solutions were prepared using the same buffer immediately prior to use and cross-linking was carried out as outlined previously.

Over the range of protein and bis-imidate concentrations used the patterns of cross-linking shown by fragment D (Ca^{++}) cross-linked in the presence of 25 mM EDTA were indistinguishable from those obtained in control experiments carried out in the presence of calcium. (see Figure 4.1).

Haverkate and Timan (1977) showed that in the presence of 2M urea and absence of added calcium fragment D (Ca^{++}) was susceptible to attack by plasmin. Therefore the above experiments were repeated using the same buffers made 2M in urea. Once again the pattern of cross-linking remains unchanged therefore the reported increased susceptibility to attack is not accompanied by a gross change in conformation.

The results from this work therefore suggest that fragment D (Ca^{++}) has a compact, stable conformation which is maintained even in the absence of calcium. Thus the conformational difference between fragment D (Ca^{++}) and fragment D (EDTA) shown previously must be the result of the cleavage of a plasmin susceptible bond towards the C-terminus of the γ -chain of fragment D (Ca^{++}). This cleavage however can only take place after the removal of calcium which can therefore be regarded as exercising a protective influence over this susceptible bond.

4) Photo-Sensitized Cross-Linking of Fragment D (Ca^{++})

Samples of Fragment D (Ca^{++}) were irradiated in the presence of fluorescein without tryptophan and, on analysis of the protein on SDS PAGE after removal of fluorescein by dialysis it could be seen that cross-linking of the protein had taken place.

The pattern of the protein samples on 5% gels after reduction was qualitatively similar to that obtained when the protein was treated with chemical cross-linkers (Figure 4.1). However when the samples were analysed without reduction it could be seen that more inter-molecular cross-linking had occurred with the band corresponding to dimeric fragment D amounting to up to 20% of the total protein applied to the gel as measured by uptake of coomassie blue stain.

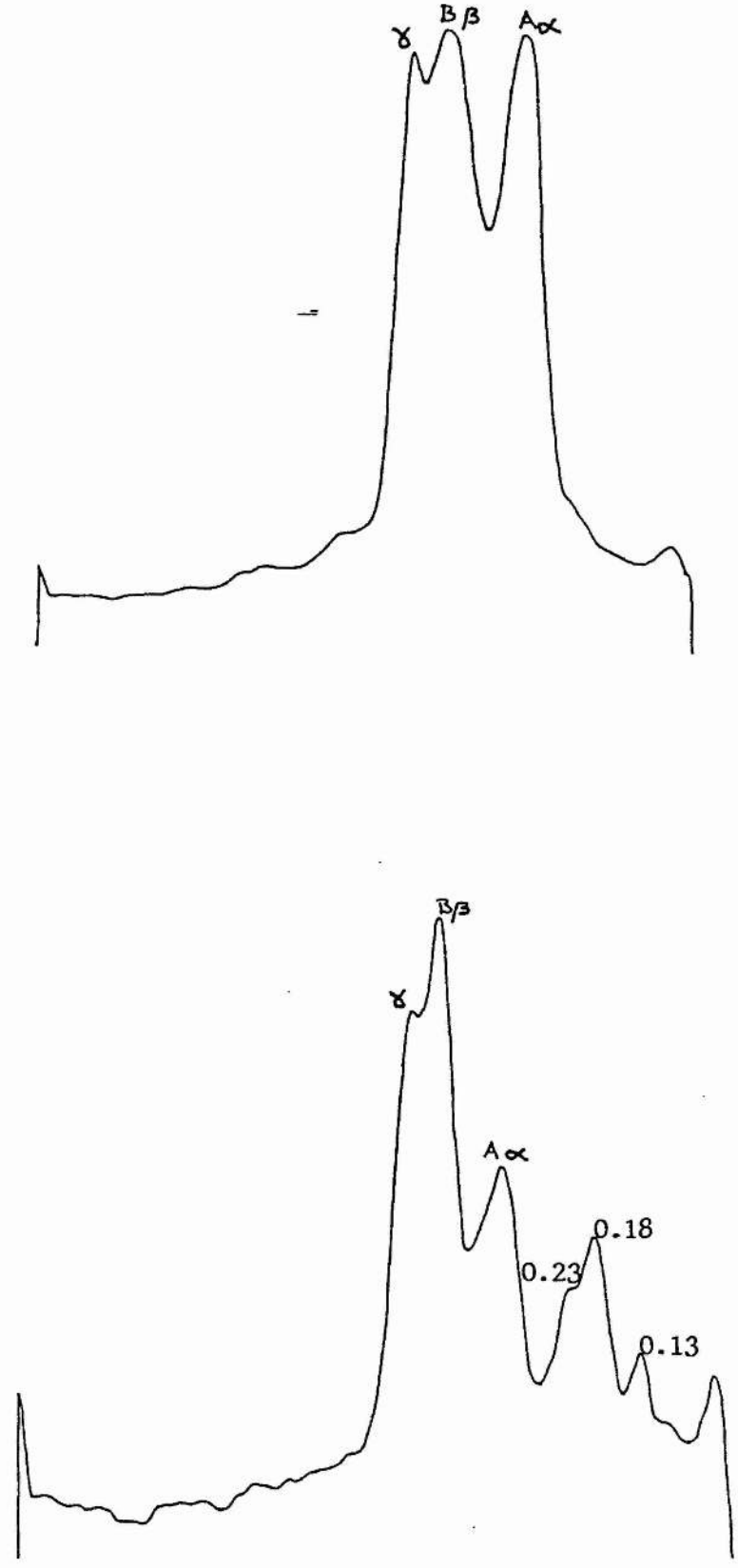


Figure 4.4 Scans of SDS polyacrylamide gels of reduced Peak I fibrinogen before and after photo-sensitized cross-linking

5) Photo-Sensitized Cross-Linking of Fibrinogen

Samples of Peak I fibrinogen were irradiated in the presence of fluorescein and absence of tryptophan, they were then freed from fluorescein by dialysis and analysed by polyacrylamide gel electrophoresis in the presence of SDS.

Analysis of non-reduced samples of irradiated fibrinogen revealed the presence of a band corresponding to dimeric fibrinogen and over the series of experiments this band was found to amount to between 18% and 23% of the total protein applied to the gel as determined by intensity of staining with coomassie blue stain.

Samples were then reduced and applied to 5% gels and typical scans of gels of fibrinogen before, and after photo-sensitized cross-linking are given in Figure 4.4 . The most obvious feature of these results is the large decrease in intensity of the band corresponding to monomeric $A\alpha$ chain, this is of course entirely consistent with the results from the photo-sensitized labelling of fibrinogen which showed that $A\alpha$ chain was heavily exposed at the surface of fibrinogen. There is also a significant reduction in intensity of the γ -chain band relative to the $B\beta$ chain. There would appear to be three major cross-linked species present having mobilities of 0.23, 0.18 and 0.13 respectively, true molecular weight estimations for these species cannot be obtained since they must contain cross-links however these mobilities correspond to apparent molecular weights of 89,000, 102,000 and 113,000 respectively.

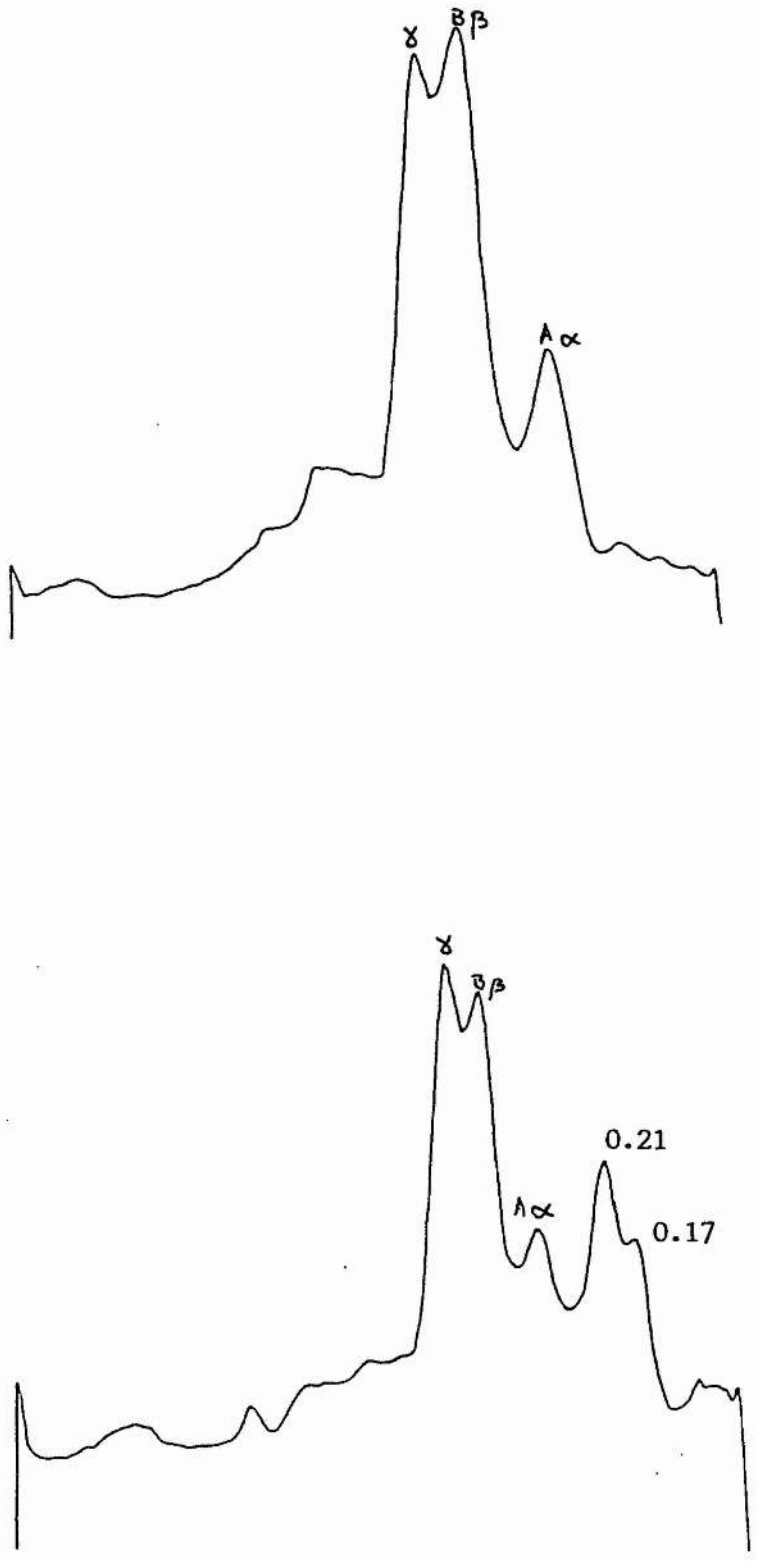


Figure 4.5 Scans of SDS polyacrylamide gels of reduced, degraded fibrinogen before and after photo-sensitized cross-linking

6) Photo-sensitized Cross-Linking of Degraded Fibrinogen

Samples of fibrinogen which had been shown on SDS PAGE to have undergone degradation of the Aα chain were then subjected to photo-sensitized cross-linking.

Analysis of staining intensities of these samples by polyacrylamide gel electrophoresis showed that dimeric fibrinogen accounted for between 16% and 22% of the total protein applied to gels.

Analysis of the pattern of reduced samples on gels (Figure 4.5) shows a number of differences from the previous experiment. The peak corresponding to monomeric Aα chain on the scan of cross-linked fibrinogen is small. However it is also small in the non-treated sample and it can be seen that the reduction in the Aα peak relative to the Bβ and γ peaks in the cross-linked sample is very much less than in the previous experiment. These scans also show that in this case the Bβ-chain exhibits a greater reduction in intensity than the γ-chain, this being the reverse of the previous experiment.

The scan of cross-linked degraded fibrinogen after reduction only reveals two major cross-linked species and these have mobilities of 0.21 and 0.17 corresponding to apparent molecular weights of 84,000 and 96,000 respectively.

7) Digestion of Photo- Cross-Linked Fibrinogen

Samples of intact and degraded fibrinogen which had been subjected to photo-sensitized cross-linking were digested with plasmin. On analysis of these digests by polyacrylamide gel electrophoresis in the presence of SDS they were found to be indistinguishable from digests of non cross-linked fibrinogen.

Fragment D was then purified from these digests and analysed, once again it appeared normal and reduction showed no evidence of abnormal bands.

DISCUSSION

The examination of fibrinogen fragment D using chemical cross-linking reagents revealed a number of interesting points. The first of these involved the reagents used for cross-linking and was the observation that reagents which react with different residues, which are generally found in different regions of protein produced the same pattern of cross-linking. This suggests that TNM only reacts with tyrosine residues which are exposed at the surface of proteins and this suggestion is consistent with the findings of Furlan and Beck (1975) who found that treatment of fibrinogen with TNM produced inter-molecular cross-linking involving the Aα chain.

This common pattern of cross-linking produced by different reagents was in the context of this work fortuitous as subsequent experiments studying fragment D (Ca^{++}) in the presence of EDTA could not have been carried out using TNM since overnight incubation at 37°C in the presence of EDTA would result in extensive digestion even if only trace quantities of plasmin were present.

The most striking point about fragment D illustrated by those studies is the gross conformational difference between fragment D (Ca^{++}) and fragment D (EDTA). The cleavage of the C-terminal portion of the γ-chain of fragment D (Ca^{++}) appears to be associated with an opening up of the molecular resulting in a fragment D (EDTA) species which has a less compact conformation. Further, the results of this work show that this change is a direct

result of digestion and can not be produced simply by removal of calcium even under mildly denaturing conditions. Nieuwenhuizen et al (1981) suggested that EDTA could bind to fragment D inducing a conformational change which makes the protein more liable to proteolytic attack. In this work no evidence for such a change could be found and so the conclusion which must be drawn is that any such change must be small, certainly when compared with the conformational change which occurs after cleavage of the δ -chain.

Eisele and Mihalyi (1975) studied the kinetics of digestion of fragment D (Ca^{++}) and concluded that the digestion of the γ -chain which takes place could, theoretically occur in either a random or sequential order. The findings presented here which show that, although calcium ions prevent digestion, their removal does not induce any great conformational change, suggest that the digestion of fragment D (Ca^{++}) occurs in a sequential manner with the initial, rate limiting, cleavage taking place at a bond which is protected by the presence of calcium.

Looking at the patterns of intra-molecular cross-linking induced in fragment D (Ca^{++}) by chemical cross-linking reagents the observed disappearance of monomeric α -chain at higher levels of cross-linker backs up the suggestion from photo-senstized labelling that the α -chain of fragment D (Ca^{++}) is highly exposed at the surface of the molecule. In view of this and, taking account of the findings of the previous section of this work I would ascribe the band having apparent molecular weight 55,000 to an $\alpha - \delta$ dimer. The band of apparent molecular weight 79,000 I believe

to be a $\gamma - \gamma$ dimer both of these suggested identities are backed by the reduction in intensity of the band corresponding to monomeric γ -chain in samples where only two cross-linked species are found. It is the 79,000 band which I believe to be responsible for inter-molecular cross-linking, because after its appearance on gels its intensity increases as concentration of cross-linker is increased. However it reaches a maximum intensity which is not changed by any subsequent increase in the concentration of cross-linker. In this behaviour it mimics the band corresponding to dimeric fragment D (Ca^{++}) on gels of non-reduced samples which can never be made to account for more than 16% of total protein regardless of any further increases in concentration of cross-linking reagent.

The band of apparent molecular weight 88,000 must I believe involve the β -chain in view of the reduction in intensity of β chain relative to the γ chain in samples where this species is found.

Therefore the information about the conformation of fragment D (Ca^{++}) which may be deduced from this work is in good agreement with the findings of the previous section of this thesis and the picture which emerges is of a fragment D (Ca^{++}) molecular which has a compact, stable conformation where the and chains constitute the bulk of the surface exposed regions of the molecule.

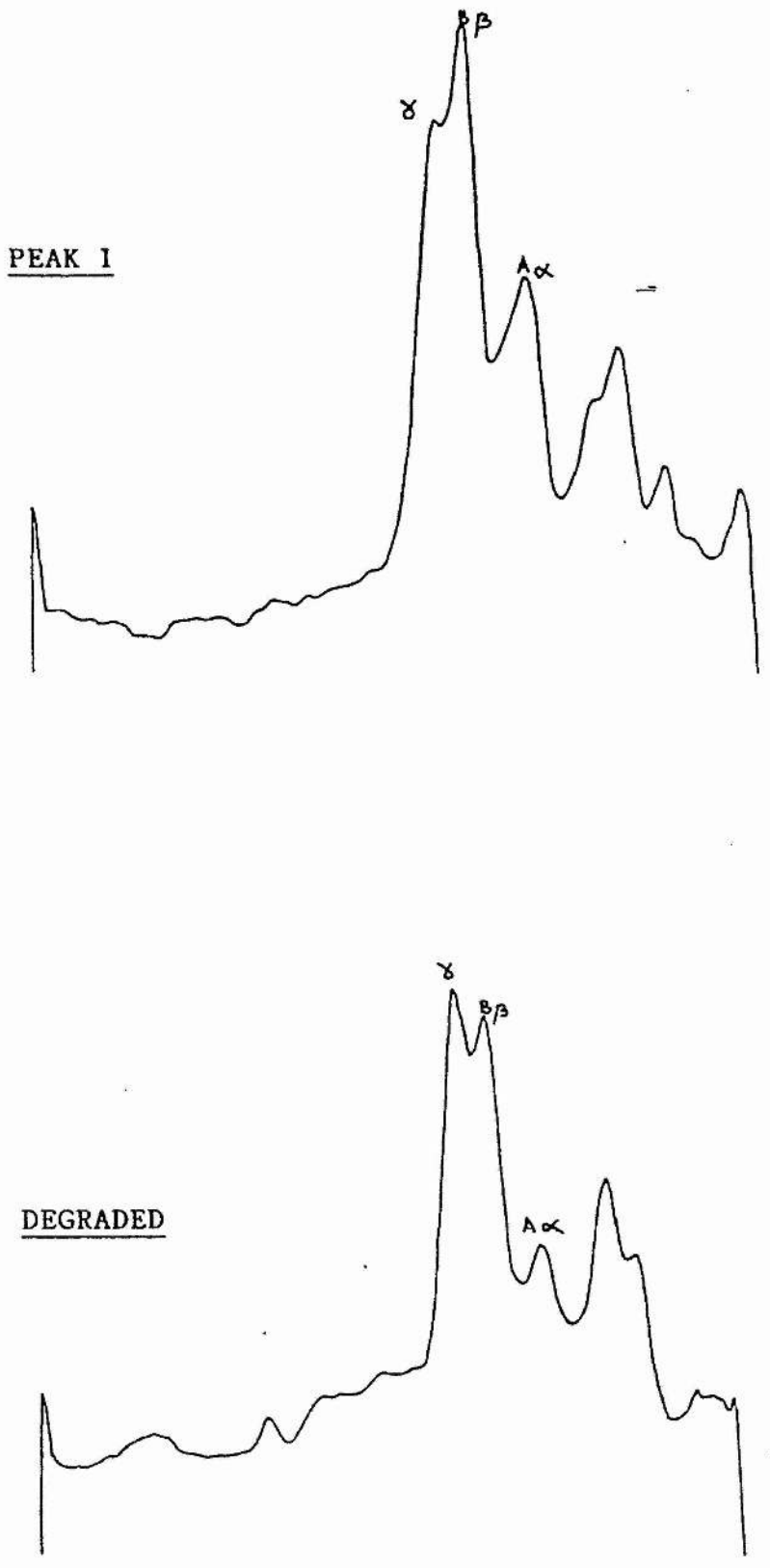


Figure 4.6 Scans of reduced fibrinogen samples after photo-sensitized cross-linking

The initial point to be made about the photo-sensitized cross-linking experiments is that the technique did induce cross-linking whilst yielding a product suitable for digestion. Therefore photo-sensitized cross-linking represents an approach which could be of great use in protein chemistry particularly in view of the contributions already made by studies using chemical cross-linking reagents (for review see Peters and Richards, 1977).

The experiments carried out on fragment D (Ca^{++}) using the technique of photo-sensitized cross-linking showed that the cross-linking which takes place occurs between regions which undergo cross-linking when the protein is treated with chemical cross-linkers. This is not surprising in view of the fact that the lysine residues with which bis-imidates can react are surface oriented and earlier conclusions which suggested that this is also the case with tyrosine residues which are accessible to TNM. The fact that photo-sensitized cross-linking can induce more inter-molecular cross-linking than chemical cross-linkers is not unexpected since the photo-sensitized reaction is much more random and does not involve only one single species of amino acid as is the case with chemical cross-linking reagents.

The examination of intact and partially degraded fibrinogen revealed a number of interesting points although caution must be exercised in the interpretation of data about degraded fibrinogen since the degree and extent of the cleavage of Aα chain C-termini was not quantified.

However, it would appear that cleavage of the C-terminus of the Aα chain of fibrinogen does not lead to a large conformational change analogous to that induced in fragment D by cleavage of the γ chain C-terminus, since the degraded fibrinogen does not exhibit any more inter-molecular cross-linking than intact fibrinogen. Therefore, although the C-terminus of the Aα chain constitutes a large proportion of the surface of fibrinogen it does not appear to impose a great conformational constraint over the rest of the molecule.

Furthermore this study confirms that the fragment D domains of fibrinogen must be protected since no evidence could be found of photo-sensitized cross-linking leading to the formation of fragment D dimers or of fragment D being the site of any intra-molecular cross-links.

By studying together, the pattern of photo-sensitized cross-linked fibrinogen, both intact and degraded, after reduction (Figure 4.6) it is possible to tentatively identify the major cross-linked species which are formed. In view of the high degree of surface exposure of Aα chain shown in the previous section it would seem likely that this chain would be very prone to cross-linking in this system and this idea is consistent with the observed reduction in intensity of the band corresponding to monomeric Aα chain in photo-sensitized cross-linked intact fibrinogen. Therefore the most likely identity of the band of mobility 0.13 which appears only with intact fibrinogen is an Aα - Aα dimer. The band of mobility $\simeq$ 0.17 is also likely to involve Aα chain since it is more prominent in the intact sample, in view of this, and the observed reduction in intensity of monomeric γ chain in this sample this band probably corresponds to an Aα-γ dimer.

The third major cross-linked species of mobility $\simeq$ 0.22 is likely to involve Bβ chain as there is a fall in intensity of monomeric Bβ chain concomitant with the increase in intensity of this species in photo-sensitized cross-linked degraded fibrinogen. In view of this and the low apparent molecular weight of this species (84,000 - 89,000) this species appears most likely to be a Bβ-γ dimer.

Since the bands which have been tentatively identified as Aα-γ and Bβ-γ dimers represent a larger proportion of the total protein in cross-linked degraded fibrinogen and there was no increase in inter-molecular cross-linking in this sample these must represent intra-molecular cross-links. There was no evidence in either sample of fragment D being involved in any cross-linking therefore it must be concluded that the sites which cross-link are towards the N-terminus of the Bβ and γ chains. Therefore since these cross-linked species are more intense in degraded fibrinogen it must be concluded that the C-terminal portion of the Aα chain protects the N-terminal portions of the Bβ and γ chains in intact fibrinogen.

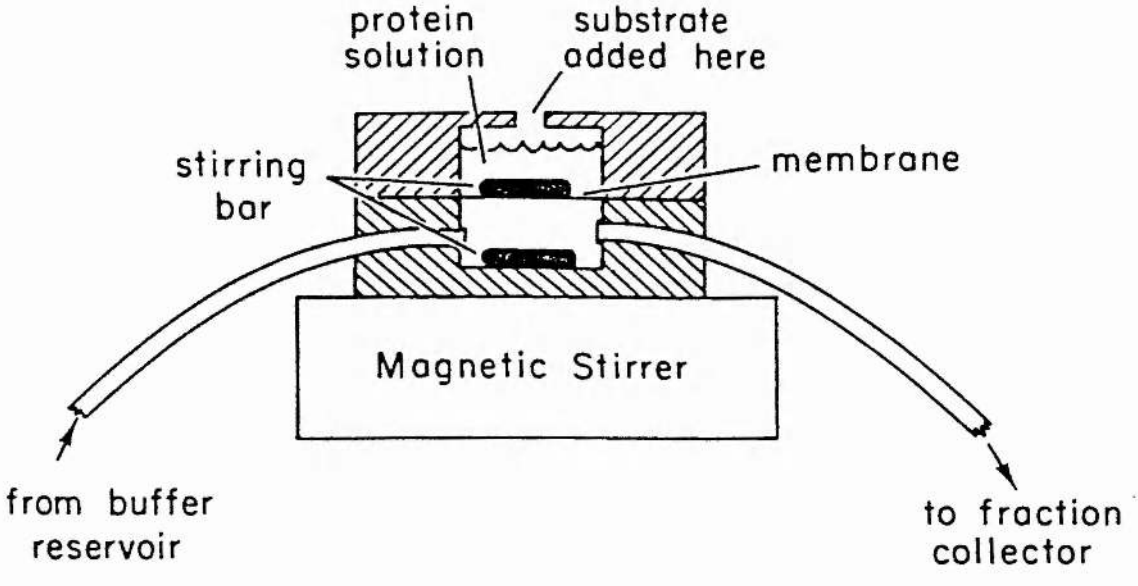


Figure 5.1 Diagram of Apparatus for Measurement of Ligand Binding by Rate of Dialysis

(From: Colowick and Womack 1969)

INTRODUCTION

Most of the information about calcium binding by fibrinogen which has been published to date has been obtained in studies using equilibrium dialysis (Marguerie et al, 1977; Nieuwenhuizen et al, 1979; Lindsey, et al, 1978). However, in view of the lability of fibrinogen, particularly the C-terminus of the Aα chain, the technique of equilibrium dialysis is not particularly suitable for analysis of fibrinogen as it involves prolonged dialyses of up to 48 hours duration.

Therefore, in this work it was decided to use the technique of "rate of dialysis" to re-assess data about the calcium binding properties of fibrinogen and its degradation product fragment D.

The technique of rate of dialysis was originally developed by Colowick and Womack (1969) to measure the binding of sugars and nucleotides to yeast hexokinases. The principle behind the technique is that when a ligand forms a complex with a macromolecule in solution a measure of the concentration of remaining free ligand may be obtained by measuring the rate of dialysis of that ligand across a suitable membrane. If the ligand is radioactive and of suitably high specific activity, the rate of dialysis may be measured rapidly without appreciably affecting the total amount of ligand in the reacting mixture. The rate of dialysis of labelled ligand may be conveniently measured using the apparatus in Figure 5.1.

In studying the binding of calcium to fibrinogen, labelled calcium is added to a calcium-free fibrinogen solution in the upper chamber. Some of this $^{45}Ca^{++}$ is bound by the protein, the rest remains free in solution and the rate of dialysis of this free calcium is measured by monitoring the steady state level of radioactivity in the effluent from the lower chamber. Subsequent additions of unlabelled calcium result in progressively larger fractions of the isotope in the free state resulting in step-wise increases in the steady state concentration of $^{45}Ca^{++}$ in the effluent. Finally a swamping excess of unlabelled calcium is added, this results in an increase in the $^{45}Ca^{++}$ concentration of the effluent to a value which corresponds to that expected when no appreciable fraction of labelled calcium is bound by fibrinogen.

From this data it is possible to assess the proportion of calcium in the bound (B) and free (F) forms after each addition. It is then possible to estimate the concentration of calcium binding sites (B_T) and the dissociation constant (Kdiss) of these sites from the equation:-

$$B = B_T - K\,diss\ (B/F)$$

: using a Scatchard plot of B versus B/F.

In the examination of calcium binding by fibrinogen the situation is complicated by the existance of more than one class of calcium binding site having different affinities for calcium (Marguerie, 1977; Nieuwenhuizen et al,1979; Lindsey et al,1978) 1978). This results in Scatchard plots exhibiting curvature, the traditional interpretation of such plots has involved drawing tangents

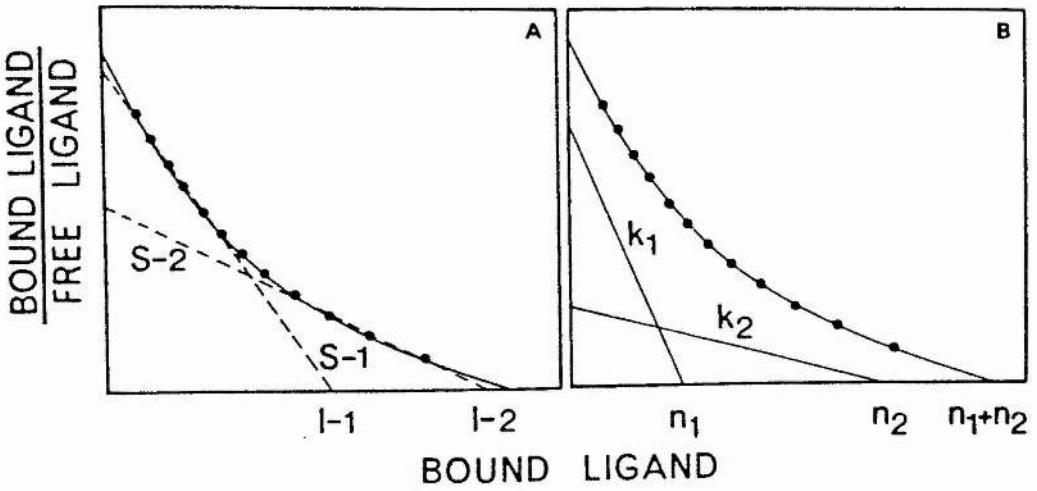


Figure 5.2 Scotchard Plots of Data Constructed to Simulate Binding to a Two-Component System.

A = Intuitive (incorrect) interpretation

B = Curve fitting analysis

(From: Norby et.al. 1980)

to the extremities of the curve and using these to derive parameters for each of the classes of binding site. However Norby et al (1980) showed that such an approach is not valid and can lead to gross errors in derived parameters. Figure 5.2 shows how such a treatment yields inaccurate values which differ greatly from those obtained when data is properly analysed using a curve fitting computer programme which reduces a curve to its component straight lines.

Therefore the purpose of this work was to use the technique of rate of dialysis coupled with computerised data analysis to re-examine the calcium binding properties of fibrinogen and its degradation product fragment D in the hope that information so derived may, when examined in conjunction with results from cross-linking and photo-sensitized labelling allow the formulation of a rational model of fibrinogen shape.

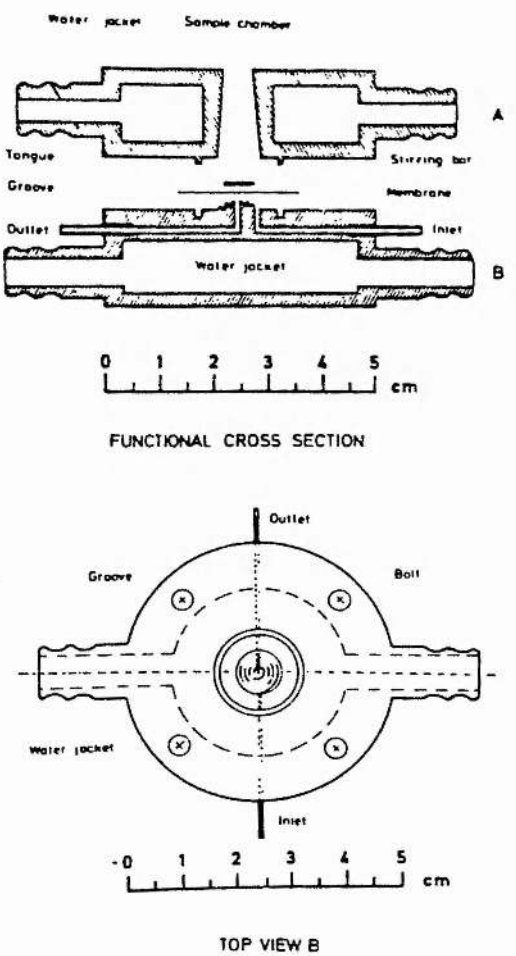


Figure 5.3 Diagram of Flow Dialysis Apparatus

Top: Functional Cross Section

A = Upper Compartment
B = Lower Compartment

Below: Top View of the Lower Compartment (B)

(From : Feldman, 1978)

MATERIALS AND METHODS

1) The Dialysis Cell

The cell used for the measurement of rate of dialysis was identical to that used by Feldman (1978) and is shown in Figure 5.3. This design of cell has a number of advantages over that used by Colowick and Womack, the small (< 0.1 ml) volume of the lower chamber and its large surface area/volume ratio ensures rapid equilibration with the upper chamber. Moreover, the design of the lower chamber circumvents the difficulties in achieving instantaneous and complete mixing in small volumes. This design also has a water jacket which allows experiments to be carried out at a carefully controlled temperature.

2) Dialysis Membranes

Spectropore dialysis membrane (Pierce and Warriner, UK) was acetylated using a method based on that of Craig and Konigsberg (1961). A length of membrane was soaked in distilled water, it was then placed in a solution of pyridine/acetic anhydride (9:1). After removal from the acetylating solution the membrane was washed with 1% acetic acid and stored at 4°C in 50% ethanol. Immediately prior to use membrane was soaked in Calcium-free buffer.

3) Preparation of Calcium-Free Buffer

Calcium determinations on all solutions were carried out using atomic absorbtion spectroscopy by Mr.A.McBain, Victoria Hospital, Kirkaldy. Solutions were prepared at ten times the desired final concentrations, passed through a column (25 cm x 1 cm)

of chelex-100 resin (Biorad Laboratories) and diluted with similarly treated distilled water immediately prior to use.

4) Preparation of Calcium-Free Protein

The technique of rate of dialysis requires that the protein used in experiments is freed from the ligand under study before the experiment is started. Therefore preparation of calcium-free protein is very important in this study and a number of methods were used to free protein of calcium;

(i) Protein solution was made 5 mM in EDTA and left at room temperature for thirty minutes. Three twelve hour dialyses against calcium free buffers were then carried out at 4°C.

(ii) Protein was subjected to four twelve hour dialyses against calcium-free buffer without pre-treatment with EDTA.

(iii) Protein was treated as described in (ii) and then mixed for one hour with Calex (immobilized parvalbumen) resin prepared and treated as described by Lehky et al (1977). The calex was then removed by centrifugation.

5) Removal of EDTA from Fibrinogen Solutions

The removal of EDTA from fibrinogen solutions was examined by making solutions 5 mM in EDTA of which 0.0001% (w/w) was ^{14}C-EDTA (Amersham International, 108 mCi/mmol). The solution was then placed in a dialysis sac and dialysed against 500 volumes of buffer. Aliquots of protein solution were removed at timed intervals and duplicate radioactivity and protein estimations were carried out.

6) Test of Ligand Binding

Volumes of protein solution (5 - 10 mg/ml) between 0.3 ml and 0.5 ml which had been subjected to one of regimes for calcium removal outlined earlier were placed in the upper chamber of the dialysis cell. The flow of calcium-free buffer through the lower chamber which was controlled by means of an adjustable hydrostatic head was then started and the flow rate was adjusted to 2.7 ml/min. 5 µl of labelled calcium chloride (New England Nuclear) was then added to the upper chamber and collection of 1.35 ml fractions of the effluent from the lower chamber was started.

Once a steady state level of radioactivity in the effluent had been obtained aliquots of unlabelled $CaCl_2$ were added to the upper chamber with suitable intervals between each for the attainment of a new steady state level of radioactivity in the lower chamber effluent. Normally additions were made every five fractions and in a typical experiment there were 5 x 5 µl additions of 0.5 mM $CaCl_2$ followed by, 5 x 10 µl additions of the same solution, 3 x 5 µl additions of 10 mM $CaCl_2$, concluding with the addition of excess calcium (10 µl of 2M $CaCl_2$).

Radioactivity was measured by addition of 0.5 ml aliquots from each fraction of the lower chamber effluent to 4 ml of toluene scintillant prepared as described by Cooper (1977) and counting in an Intertechnique Scintillation Spectrometer model SL 30.

7) Analysis of Experimental Data

Data from rate of dialysis experiments was analysed using the computer programme "Ligand" developed by Munson and Rodbard (1980). By analysis of an experimentally derived Scatchard plot using this programme estimates of "binding parameters" (affinity constants and binding capacities) for a ligand reacting simultaneously with any number of binding sites may be obtained.

The operator selects a model having a given number of classes of binding site and the programme then analyses the experimental data, deriving the parameters of the given classes of sites, by breaking down the experimental curve into the desired number of straight lines. The programme then assesses and compares the statistical validity of all given models allowing selection of the model which best fits experimental data.

Another valuable feature of this programme is that it allows for the incorporation of a parameter defined as "non-specific binding", in the original programme this parameter was used to allow for systematic binding of ligand to any contaminant present in receptor preparations. However in this work this has been used to account for the binding of calcium to the low affinity (Kd 10^{-3}M) calcium binding sites of fibrinogen.

RESULTS

1) Preparation of Calcium-Free Buffers

The results of Atomic Absorbtion Spectroscopic analysis of the two buffer systems used in this work before and after passage through a chelex-100 chelating column are given in Table 5(i).

Buffer	Ca^{++} before chelex-100 (uM)	Ca^{++} after chelex-100 (uM)
0.05M Tris pH 7.5	1.9	1.0
0.15M NaCl	3.75	-
0.05M Tris pH 7.5 containing 0.15M NaCl	0.5	1.8
0.05M Imidazole	-	-
0.05M Imidazole containing 0.15M NaCl	3.1	-

Table 5(i) Calcium Concentrations of Buffer Solutions as Measured by Atomic Absorbtion Spectroscopy

(-) indicates value outwith sensitivity of instrument

These results clearly show that both Tris-HCl and NaCl are contaminated with calcium however this does not appear to be the case with imidazole. Further, these results appear to indicate that the Tris-HCl buffer actually has an affinity for calcium since, unlike with NaCl the contaminating calcium is not removed by passage through a chelating column

In view of these findings an imidazole buffer system was used for all subsequent experiments on the rate of dialysis system. The buffer was made up at 10x the required concentration, passed through a column of chelex-100 and diluted with similarly treated distilled water immediately prior to use. Routine analysis of buffer prepared in this way showed no measurable calcium contamination.

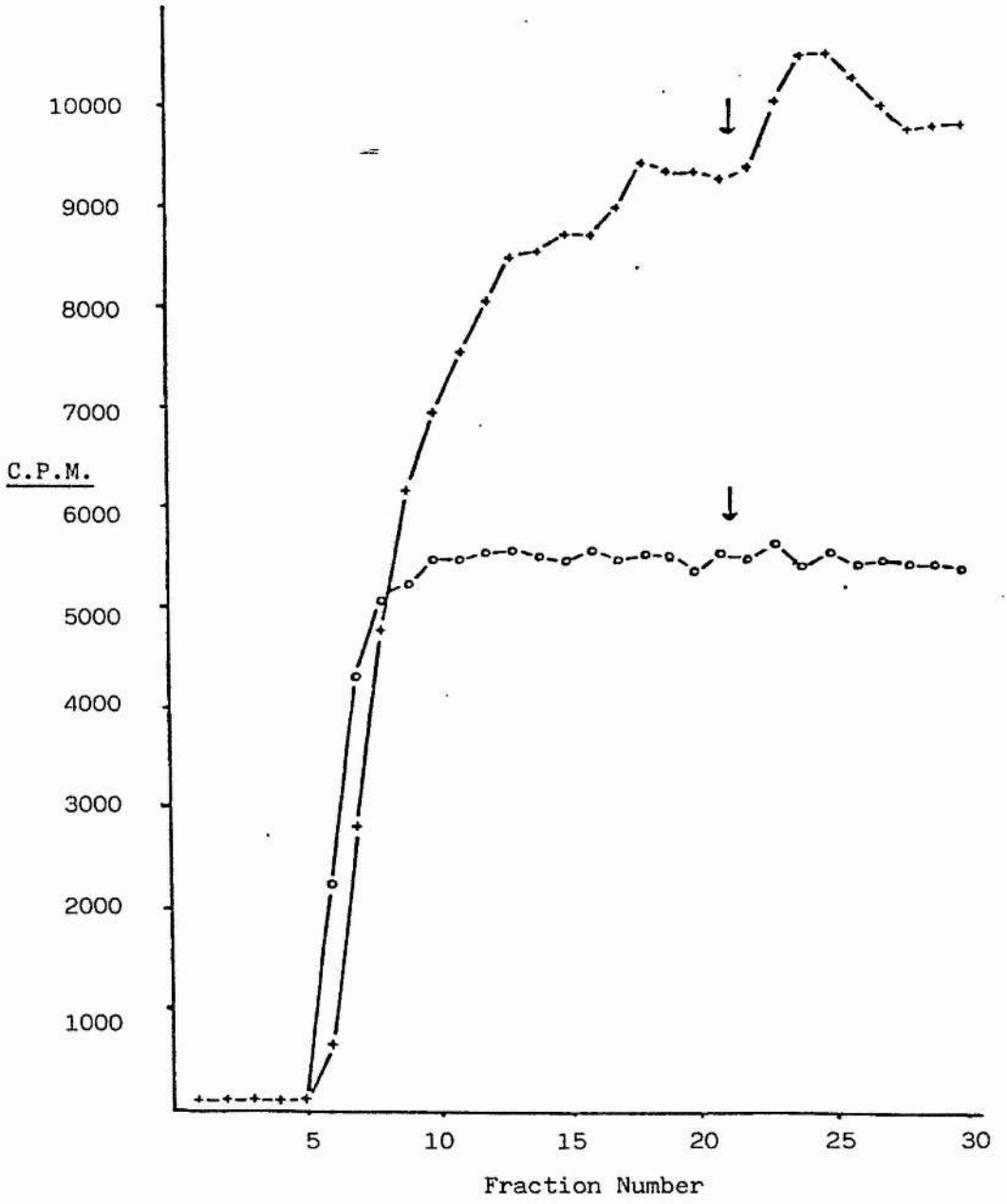


Figure 5.4 Test of Dialysis of $^{45}Ca^{++}$ through Dialysis Membrane

+ - + untreated membrane

O - o acetylated membrane

addition of unlabelled calcium

2) Acetylation of Membrane

Early experiments showed that when Spectropore dialysis membrane was acetylated for a 16 hour period as described by Craig and Konigsberg (1961) it adopted a very rigid texture. It was felt that this could lead to problems in keeping the membrane tightly in position in the dialysis cell. Therefore the period of acetylation was sequentially reduced to the point where the membrane was found to retain its initial plasticity. The time finally adopted was 75 minutes and Figure 5.4 shows the pattern of dialysis of $^{45}Ca^{++}$ through a membrane acetylated for this length of time compared with untreated membrane.

These results clearly show that acetylation reduces the permeability of the membrane for calcium by around 50%. This is important since Colowick and Womack (1969) stressed the importance of minimising the total loss of labelled ligand from the upper chamber during a rate of dialysis experiment.

Figure 5.4 also shows that addition of unlabelled calcium to a dialysis system at equilibrium produces an increase in the steady state level of radioactivity in the lower chamber. effluent if an untreated membrane is used, this effect is abolished by the use of acetylated membrane. This finding supports the work of Reed (1973) who claimed that untreated cellulose membrane could bind calcium.

Therefore acetylation appears to be very valuable for two reasons, firstly it reduces the rate of dialysis of labelled calcium and secondly it eliminates calcium binding by membrane.

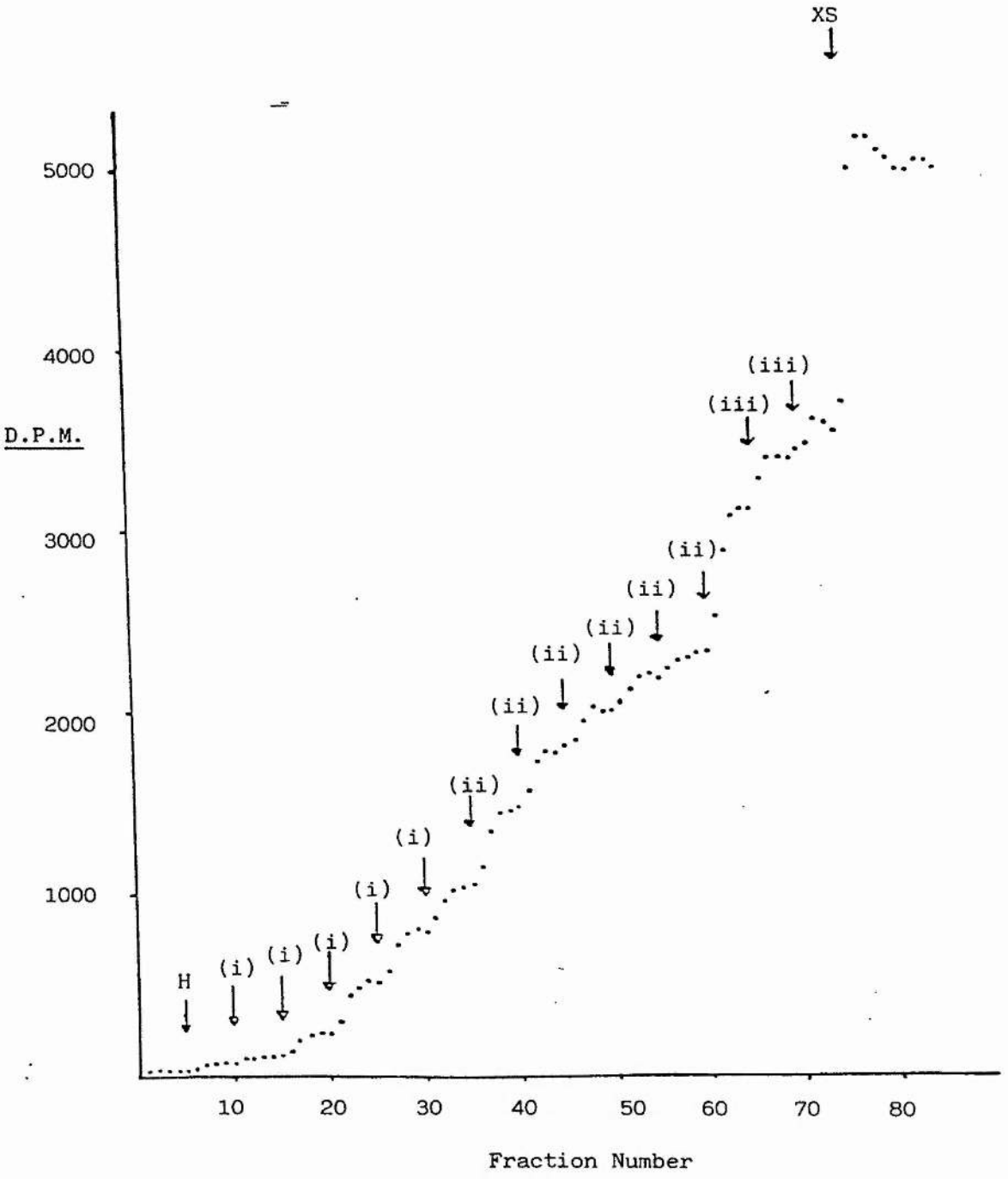


Figure 5.5 Results from a Typical Rate of Dialysis Experiment

H	=	Addition of Labelled Calcium
(i)	=	Addition of 5 ul 0.5 mM $CaCl_2$
(ii)	=	Addition of 10 ul 0.5 mM $CaCl_2$
(iii)	=	Addition of 5 ul 10 mM $CaCl_2$
XS	=	Addition of Excess Calcium

3) Measurement of Calcium Binding by Fibrinogen

Calcium binding properties of Peak I fibrinogen which had been exposed to EDTA before extensive dialysis against calcium-free imidazole buffer were examined using rate of dialysis as outlined earlier. The results of a typical experiment are shown in Figure 5.5.

Using the computer programme "ligand" attempts were made to fit various models to the Scatchard Plot derived from the experimental data. It was found that the model which best fitted the experimental data contained two classes of high affinity calcium binding site. The fit of such a model to the experimental data was improved by the incorporation of non-specific binding into the model, this parameter being used to accommodate for the calcium binding sites of lower affinity ($Kd \simeq 10^{-3}M$) which Marguerie et al (1977) showed to exist within the fibrinogen molecule.

Figure 5.6 shows the Scatchard Plot obtained by analysis of experimental data (open circles), it also shows the straight lines defining the derived parameters of the best fitting model and the computer generated curve from which these parameters are derived. It may be seen that the experimental and computer generated curves are very similar, indicating the closeness of the fit. The values of the parameters of the high affinity calcium binding sites derived from this model are given in Table 5(ii).

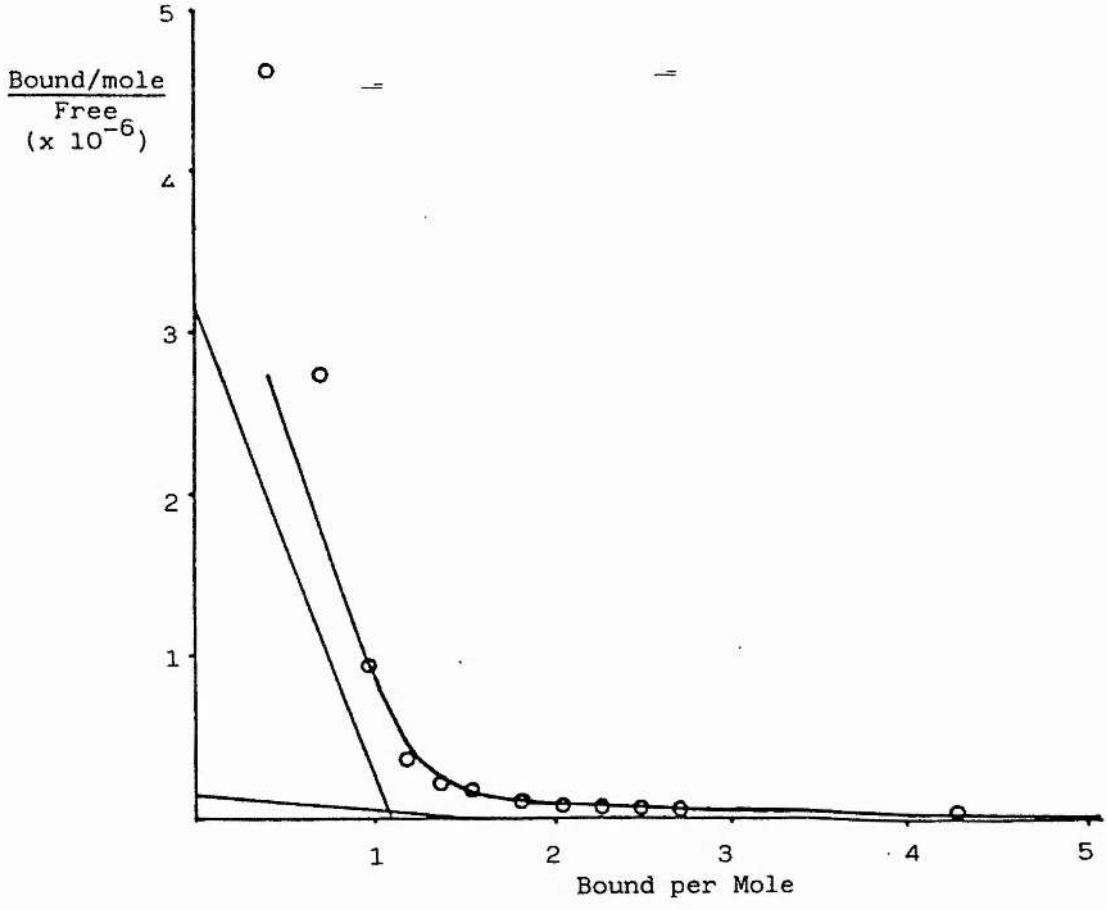


Figure 5.6 Scatchard Plot showing Experimental Points from Rate of Dialysis Experiment, Computer Generated Curve and the Lines Defining the Parameters from which the Curve is Synthesised

SITE	DOSSOCATION CONSTANT	NO.OF SITES/ MOLECULE
1	2.8×10^{-7}M	1.1
2	6×10^{-5}M	1.4

Table 5(ii) Computer Derived Parameters of the High Affinity Calcium Binding Sites of Fibrinogen

These results suggest that fibrinogen contains one calcium binding site of very high affinity in addition to two sites of affinity comparable to that reported by other workers (Marguerie et al,1977; Nieuwenhuizen et al 1979, 1981; Lindsey et al,1978).

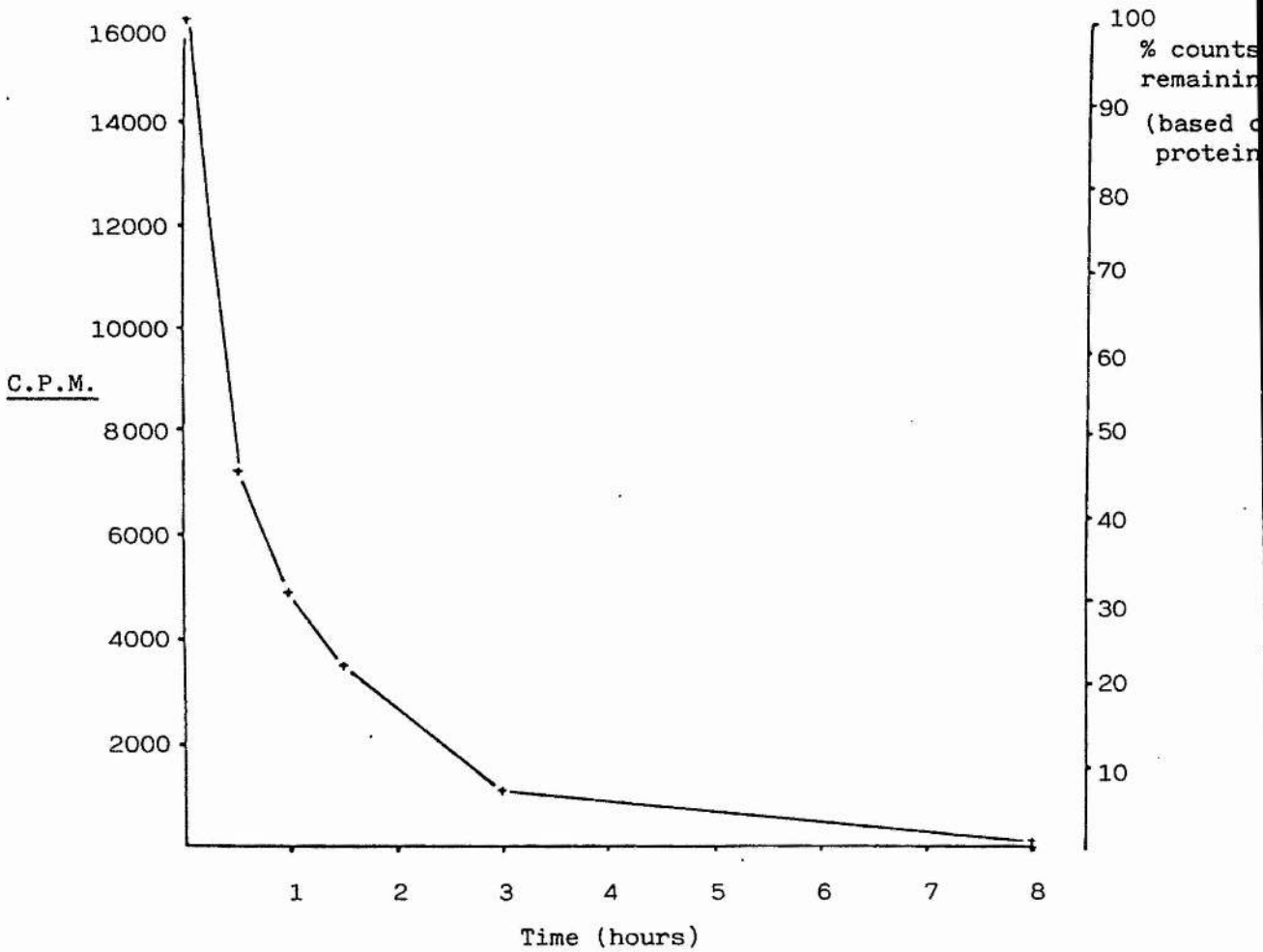


Figure 5.7 Dialysis of ^{14}C-EDTA from Fibrinogen Solution

4) Examination of the Removal of EDTA from Fibrinogen Solutions

One possible explanation of the results obtained in the previous experiments could be that the protein samples contained EDTA and it was this that was responsible for the observed very high (Kd $\simeq 10^{-7}$M) affinity calcium binding site. To test this idea the dialysis of labelled EDTA from solutions of fibrinogen was measured. Figure 5.7 shows the pattern of dialysis of ^{14}C-EDTA from a fibrinogen solution and from this, it can be seen that after 8 hours of dialysis less than 2% of the EDTA remains in the protein solution.

Therefore, using the dialysis regime outlined earlier the maximum concentration of EDTA which could remain in fibrinogen solutions used for rate of dialysis experiments would be at a level three orders of magnitude lower than the molarity of the fibrinogen. This of course only accounts for EDTA in free solution, if, as suggested by Nieuwenhuizen et al (1981) fibrinogen does bind EDTA then this assumption may not be valid.

5) Effect of Sample Preparation on the Calcium Binding Properties of Fibrinogen

The previous experiment showed that the calcium binding site of very high affinity found in earlier experiments could not be accounted for by the presence of free EDTA in fibrinogen solutions, it did not however preclude the possibility of protein bound EDTA being responsible for the observed very high affinity site. Therefore it was decided to carry out rate of dialysis experiments on fibrinogen preparations which had never been exposed to EDTA.

Peak I fibrinogen was extensively dialysed against calcium free buffer and some was then used for rate of dialysis experiments. The remainder was additionally treated with Calex resin (immobilized parvalbumen) before rate of dialysis.

Computer analysis of experimental data showed that,in both cases the model which best fitted the observed results was one which contained two classes of high affinity calcium binding site and non-specific binding. The computer derived parameters of the high affinity calcium binding sites for each preparation are given in Table 5(iii), results from the previous experiments on EDTA treated fibrinogen are included for comparison.

PREPARATION	Kd(1)	Kd(2)	N(1)	N(2)
Chelex dialysed	7×10^{-7}M	5×10^{-5}M	0.43	2.0
Calex treated	3.3×10^{-7}M	7.6×10^{-5}M	0.82	1.5
EDTA treated	2.8×10^{-7}M	6×10^{-5}M	1.1	1.4

Table 5(iii) Parameters of the High Affinity Calcium Binding Sites of Fibrinogen Subjected to Differing Treatments

These results clearly show that the existence of the site of very high affinity is not dependent upon the exposure of fibrinogen to EDTA and therefore it does not appear to be artefactual. It is also interesting to note that the number of sites of very high ($Kd \simeq 10^{-7}M$) affinity is greatly influenced by sample preparation with fewer sites per firbinogen molecule being found when the sample is subjected to less rigorous regimes for calcium removal.

6) Measurement of Calcium Binding by Fibrinogen Fragment D (Ca^{++})

Fragment D (Ca^{++}) prepared as outlined earlier was freed from calcium by exposure to EDTA followed by extensive dialysis against calcium-free imidazole buffer. Rate of dialysis experiments were then carried out and it was found that the experimental data was best fitted to a model containing approximately one high affinity calcium binding site and non-specific binding. Typical derived parameters for the high affinity site in such a model are given in Table 5(iv).

	Kd	No.of Sites/Molecule
Fragment D (Ca^{++})	5.8×10^{-5}M	0.89

Table 5(iv) Parameters Derived for the High Affinity Calcium Binding Site of Fibrinogen Fragment D (Ca^{++})

It can be seen that the affinity of this site is very close to that found for the high affinity sites of similarly treated fibrinogen (Kd = 6×10^{-5}M) and this would appear to confirm the findings of Lindsay et al (1978) and Nieuwenhuizen et al(1979) who ascribed two of the calcium binding sites of fibrinogen to the fragment D domains of the molecule.

DISCUSSION

The stated aim of this series of studies was to utilise the technique of rate of dialysis coupled with computerised data analysis to assess the calcium binding properties of fibrinogen and its degradation product fragment D (Ca^{++}) in the hope that information so derived would be of use in formulating a model of fibrinogen shape based on other findings in this work. However, before looking at the calcium binding properties of these proteins certain points revealed in experiments during the development of the system for rate of dialysis should be considered.

The increase in the rate of dialysis of labelled calcium caused by the addition of unlabelled calcium to a steady state system observed whilst using untreated membrane in membrane tests would appear to back the suggestion made by Reed (1973) that calcium can bind to unmodified cellulose membranes. To date, none of the studies of the calcium binding properties of fibrinogen have been carried out using acetylated membranes. Standard pre-treatment of membranes has involved washing with EGTA, a regime which could conceivably increase the affinity of the membrane for calcium. Thus it would appear that the studies of calcium binding by fibrinogen carried out to date have included an inherent unknown factor which could affect the distribution of calcium thereby affecting results. It would therefore be useful to carry out more calcium binding studies using acetylated membranes since acetylation appears to abolish this possible source of error.

Another very important point raised during the development of this system was the finding that Tris-HCl buffer

appears to have an affinity for calcium and that,even treatment of the buffer with Chelex-100 chelating resin cannot remove all traces of contaminating calcium. Marguerie et al (1977) reported using NaCl solutions which had calcium contamination of less than 10^{-7}M, findings which are entirely consistent with those of this work. However,before experiments were carried out in NaCl solution the protein was exposed to Tris-HCl which was assumed to be calcium-free,an assumption which, in view of the findings presented in this work may not be valid. These workers also claim that the protein used for equilibrium dialysis experiments could contain contaminating calcium at a level below 3×10^{-6}M, it must be pointed out that,in the study of very high affinity calcium binding sites this represents a significant level of contamination (approximately one calcium for every ten fibrinogen molecules).

In an effort to alleviate such problems, all studies in this work were carried out using an imidazole buffer system which was shown to have no affinity for,or contamination with calcium.

Turning to the results of the studies on calcium binding by fibrinogen,this work suggests that fibrinogen possesses more than one calcium binding site of high affinity (Kd approx. 3×10^{-7}M) Subsequent work on fragment D suggested that the sites of Kd 10^{-5}M resided within the D domains of fibrinogen. This would imply that the actual number of such sites in fibrinogen would be two and,when this value was inserted and tested in models these gave a statistically acceptable fit to the experimental data obtained in rate of dialysis studies of fibrinogen. The fact that the two

sites of fibrinogen and the site found in fragment D have very similar affinities for calcium backs the suggestions made earlier in this work that the fragment D domains of fibrinogen undergo little or no conformational changes during the digestion of fibrinogen to core fragments.

It is however in the finding of one calcium binding site of very high affinity that this work differs from others, although the suggestion that two classes of high affinity calcium binding site exist in fibrinogen is not new and dates back to the original report of calcium binding by fibrinogen by Marguerie et al in 1977. In view of the generally accepted symmetry of fibrinogen and the fact that two of the calcium binding sites reside within the fragment D domains of the molecule any third calcium binding site would have to bridge the two "halves" of the molecule. Therefore it seems extremely unlikely that any third site could be chemically identical to the other two sites, thus it could be expected to exhibit different binding parameters to those of the site within fragment D.

Nieuwenhuizen et al (1979) reported the existence of two classes of calcium binding site in fibrinogen having dissocation constants of 9×10^{-6}M and 3.2×10^{-5}M respectively, values which are very similar to those reported in this work. However, Niewenhuizen et al obtained these values by taking tangents to curved Scatchard plots and, as Norby et al (1980) pointed out this is not a valid approach. When Niewenhuizen et al (1981a) reassessed their experimental data using a curve fitting

computer programme they concluded that fibrinogen actually contained three calcium binding sites of equal affinity. However the model could be made to fit the experimental data much more closely if 0.08 of a site with a dissocation constant almost identical to that of the single very high affinity site found in this study was included in the model. This is particularly interesting in view of the findings in this work that less stringent conditions for calcium removal result in the observed number of very high affinity sites being reduced and that Tris -HCl buffer appears to have an affinity for calcium. Since these workers employ a Tris-HCl buffer system in the early stages of calcium removal from fibrinogen it is possible that not all of the calcium is removed from the "calcium-free" fibrinogen used in the equilibrium dialysis experiments of this study. This could result in under-estimation of the number of very high affinity sites. The over-estimation of the number of sites of slightly lower affinity ($Kd \simeq 10^{-5}M$) could then result from a combination of this calcium contamination coupled with a failure to take into account the influence of the low affinity ($Kd \simeq 10^{-5}M$) calcium binding sites in the calculation of the binding parameters of the higher affinity sites.

This touches upon one of the major criticisms of this current work; the grouping together of the lower affinity calcium binding sites and utilising the inclusion of non-specific binding in models to account for them. Obviously it would be preferable to carry out experiments in the presence of magnesium which would block the low affinity sites whilst not affecting the

high affinity calcium binding sites. However,this would involve carrying out experiments in the presence of levels of magnesium around 0.01M and to date it has proved impossible to find a magnesium chloride preparation from which such a solution could be prepared and used without the concomitant introduction of an unacceptably high level of calcium contamination. Thus the approach used in this work, although not entirely satisfactory appears to be the best currently available and is certainly more valid than that of ignoring the influence of the low affinity sites completely.

In conclusion, this study has emphasised the difficulties in examining the calcium binding properties of fibrinogen, however all the data obtained are consistent with the idea that fibrinogen contains three binding sites with a high affinity for calcium. Two of these sites are identical and reside within the fragment D domains of the molecule whilst the location of the third site, which exhibits a significantly higher affinity for calcium has not yet been identified. It has also been noted that the sites within the fragment D domains of the molecule appear to be unaffected by digestion of fibrinogen to core fragments.

GENERAL DISCUSSION

The major aim of this work was to develop and assess various methods for the study of fibrinogen with a view to obtaining information about the shape and calcium binding properties of the molecule. Obviously before such studies could be carried out it was necessary to obtain a high quality fibrinogen preparation. This was particularly important in view of the results of Tooney and Cohen (1972) who showed that,whilst intact fibrinogen is not crystallizable, the protein could be crystallized after degradation of the Aα chain C-terminus. This suggests that an important conformational change takes place on cleavage of this labile portion of the molecule. Therefore a rapid fibrinogen purification scheme was developed which produced very pure and highly intact fibrinogen although this was achieved at the expense of a low yield. However the yield was improved by the collection of a second pool of fibrinogen which, although of poorer quality than the first, proved suitable for digestion to core fragments. The fragment D species produced by such digestions in the presence of calcium was shown to be homogenous and,in view of the higher stability of this species in the presence of calcium (Haverkate & Timan, 1977) it was decided to use fragment D (Ca^{++}) in the pilot studies of the various analytical techniques.

The first technique to be studied was photosensitized labelling. This was developed by Brandt (1974) and Hemmendorf et al (1981). The technique involves labelling the surface oriented

regions of a protein with a small radioactive marker in a sensitized photochemical reaction and has the major advantages that the labelling procedure takes place rapidly under mild conditions. Hemmendorf et.al. (1981) actually studied fibrinogen using this technique. Since however this is the only reported study using this technique to date, it was decided to examine the system rigourously before attempting to study fibrinogen. Using fragment D for these early experiments it was shown that the technique did induce labelling, that this labelling was by covalent attachment and that the degree of labelling was light dependent. Furthermore the degree and distribution of label were shown to be conformation dependent, both being significantly altered in protein samples labelled under denaturing conditions. The distribution of label within native fragment D labelled in these studies suggested that the γ chain was highly exposed whilst the β chain was protected. These suggestions are completely consistent with the observed susceptibilities of each of these chains to proteolytic attack in the absence of calcium (Furlan et.al. 1975).

The technique was then applied to fibrinogen and label distributions which were almost identical to those of Hemmendorf et.al. (1981) were obtained suggesting that the technique is highly reproducible which makes it even more useful for analysis of protein. Analysis of the distribution of label showed that the Aα chain was heavily exposed, a suggestion which has been made by a number of workers using different

techniques (Gaffney & Dobos, 1971; Blomback, M. 1976; Furlan & Beck, 1975). It was further shown that photo-sensitized labelled fibrinogen could be digested by plasmin and studies of digestions of fibrinogen to core fragments led to the conclusion that the fragment D domains of fibrinogen and fragment D (Ca^{++}) in solution have a very similar shape. This finding was particularly important because fragment D (Ca^{++}) had been used for early studies on photosensitized labelling and therefore much information about its label distribution had been obtained which could be directly applied to fibrinogen.

The technique of chemical cross-linking has been used to study ribosomal proteins (Nomura, 1973) and structure-function relationships in a number of proteins (Peters, 1977). However, in the context of this work it has the major disadvantage in that it generally yields a product which cannot be subjected to plasmin degradation, in this study this point was of particular importance since much of the information about fibrinogen has been obtained from such studies and the pattern of digestion has been well characterised (Marder et al, 1969: Latallo, 1973).

In spite of this, chemical cross-linking was used in this work to study fragment D (Ca^{++}) and fragment D (EDTA) yielding results which were in good agreement with those about the shape of fragment D revealed by photo-sensitized labelling.

The use of chemical cross-linking reagents revealed a major conformational difference between fragment D (Ca^{++}) and fragment D (EDTA). It was further shown that this difference was

a direct result of digestion rather than calcium removal.

In an effort to alleviate the problem of not being able to digest cross-linked protein the system for photo-sensitized labelling developed in earlier work was modified to induce cross-linking in the protein sample. This proved very successful and yielded a product which could be digested by plasmin. Digestion of such cross-linked fibrinogen samples suggested that the fragment D domains of fibrinogen were shielded within the molecule since normal fragment D was produced and there was no evidence of any abnormal high molecular weight material in these digests. This is in agreement with the results from the photo-sensitized labelling of intact fibrinogen which showed that the Aα chain bound most of the label whilst the fragment D domains bound less label than free fragment D (Ca^{++}) in solution. This suggests that the Aα chains play some role in shielding the fragment D domains of the molecule. The increased cross-linking of the N-terminal portions of fibrinogen observed when partially degraded fibrinogen was subjected to photo-sensitized cross-linking suggests that the Aα chains of fibrinogen also play a role in protecting the N-terminus of the molecule.

The technique of rate of dialysis exhibited attributes which make it a very useful system for the analysis of ligand binding by macromolecules although in this study it also emphasised the difficulties in the analysis of high affinity calcium binding.

The major advantages of the system lie in the speed of analysis, the small amounts of protein which it uses and the fact that all the measurements for a full Scatchard plot are derived from the same sample. However,the system does require that the sample under test is freed from the ligand under study and this presented one of the major problems of this work as the removal of calcium from protein and buffers and the determination of very low residual levels of calcium proved difficult. Other problems encountered in this study were the elimination of artefactual binding of calcium by membrane and the interpretation of curved Scatchard plots.

However these problems were overcome and analysis of the calcium binding properties of fibrinogen and fragment D (Ca^{++}) were carried out. All the data obtained were consistent with fibrinogen possessing three binding sites having a high affinity for calcium. Two of these sites would appear to be identical,one residing in each of the fragment D domains of fibrinogen. The characteristics of the third site found in this study differ slightly from those found by others (Marguerie et al, 1977; Niewenhuizen et al, 1979) although Marguerie's work indicated that the third site was different from the others, an idea which seems more likely than sites which are not chemically identical exhibiting the same binding affinity.

Combining data obtained using each of the techniques in this study it becomes possible to make a number of suggestions about the shape of the fibrinogen molecule in solution.

From the photo-sensitized labelling and calcium binding studies it would appear that the D domains of fibrinogen and fragment D (Ca^{++}) in solution are conformationally very similar. Cross-linking studies of fragment D (Ca^{++}) suggest that this is a compact globular conformation, thus it would appear that 58% of the mass of the fibrinogen molecule is contained in two compact domains.

The cross-linking pattern of partially degraded fibrinogen showed, as expected, that at the N-terminus of the molecule the constitutent chains of fibrinogen are in close association. Thus the N-terminal region of fibrinogen may be considered as an area of dense protein mass and when this is considered in conjunction with the evidence from this study that the fragment D regions of fibrinogen represent two discrete domains the evidence strongly supports the suggestion made by many workers (Hall & Slayter, 1959; Johnson & Mihalyi, 1965, Schrager et al 1976; Doolittle et al 1977) that fibrinogen is a trinodular structure.

The results of photosensitized labelling however, suggested that the Aα chain of fibrinogen is heavily exposed at the surface of the molecule and digestion of labelled fibrinogen suggested that the fragment D domains are protected within the interior of the molecule. It therefore seems likely that the Aα chain C-terminii fold round the outer part of the molecule shielding the interior. This idea is similar to that proposed by Cierniewski et al (1977) who, using immunochemical studies suggested that the N-terminal of the γ chain of fibrinogen was exposed by digestion of the Aα chain C-terminus. Further

evidence for this is provided by cross-linking patterns where, with intact fibrinogen Aα dimers were observed whilst a different pattern of cross-linking was observed with partially degraded fibrinogen. The difference in the two patterns of cross-linking was not so large as to suggest a major conformational change, only an increased accessability of the N-terminal portions of the molecule. Of course a major conformational change would not be expected in view of the previous finding that a large proportion of the mass of fibrinogen is contained in two compact globular domains.

A number of workers have suggested that the C-terminal portions of the Aα chain are surface oriented (Gaffney & Dobos, 1971; Blomback.M., 1976; Furlan & Beck, 1975) and more recently Mosssesson et al (1982) suggested that the terminal portions are folded back towards the N-terminal region of the fibrinogen molecule. This idea is supported by this work and the folding back of the Aα chain C-terminii could account for some of the controversy about the shape of fibrinogen.

Taking account of the evidence which shows the existence of discrete domains within the fibrinogen molecule and coupling that with the accumulating evidence (Cierniewski et al 1977; Mossesson et al 1982) about the folding back of the Aα chains it would appear that fibrinogen could best be described as a protected trindoular structure. This being the case it could be envisaged that, under certain conditions of preparation it could appear as a globular structure. The model proposed here could also account for the differences in the reported results of Hemmendorf et al (1981) and Takagi and Kawai (1978). Hemmendorf et al reported that the early peptide

cleaved from the Aα chain C-terminal had a molecular weight of 44,000 daltons whilst Takagi & Kawai (1977) reported that this peptide weighed 20,000 daltons, both groups finding very similar amino acid compositions for the peptide. If, as suggested here the C-terminii of the Aα chains are in close proximity it is possible that Hemmendorf was actually examining a dimeric product formed by photo-sensitized cross-linking of Aα chain C-terminii prior to cleavage.

Analysis of fragment D (Ca^{++}) and fragment D (EDTA) using chemical cross-linking reagents revealed a gross conformational difference between these species and indicated that Fragment D (EDTA) has a much more open conformation. Furthermore it was shown that the conformational change was only induced by cleavage of the C-terminal portion of the γ chain of fragment D (Ca^{++}) and could not be produced solely by removal of calcium, however the cleavage could only occur after removal of calcium. This is particularly interesting in view of the finding from photo-sensitized labelling that the γ chain and more specifically the C-terminal portion of the γ chain is exposed at the surface of fragment D (Ca^{++}). The picture of fragment D (Ca^{++}) which therefore emerges is one of a compact molecule held in its compact conformation by the calcium binding C-terminal portion of its γ chain. This is particularly relevant in view of other results from photo-sensitized labelling showing the conformational similarly between fragment D (Ca^{++}) in solution and the fragment D domains of fibrinogen. However they take on an

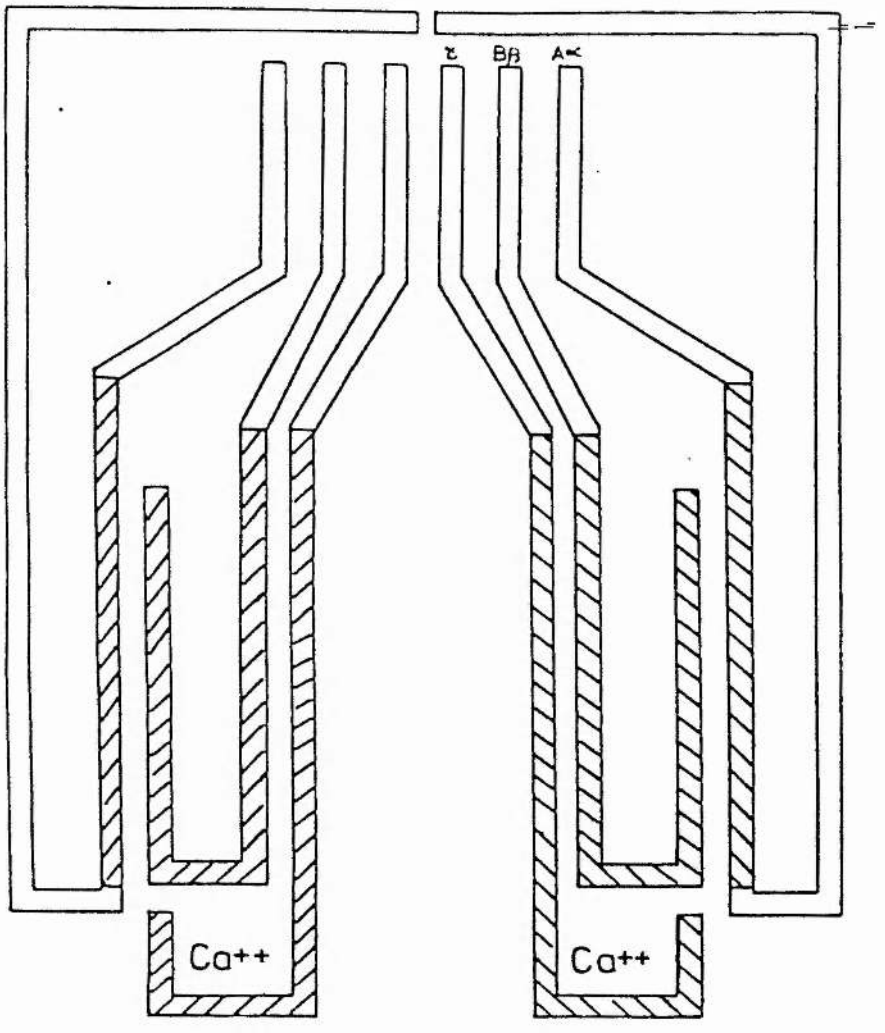


Figure 6.1 Schematic model of fibrinogen shape

(Shaded regions = fragments D)

even greater significance in view of the finding from photo-sensitized labelling that almost all of the label bound by the γ chain of fibrinogen is bound by that portion which constitutes the γ chain remnant of fragment D (Ca^{++}).(See tables 3(vi) and (vii)).This provides direct evidence that the portion of fibrinogen responsible for calcium binding (Lawrie & Kemp 1979). Factor XIII induced cross-linking of fibrin (Haverkate et al.,1979) and interaction of fibrinogen with platelet receptors (Marguerie et al,1982) is exposed at the surface of native fibrinogen, an idea which is of course suggested by its physiological role.One interesting point is that these regions which are on the surface of fibrinogen and prone to photo-sensitized labelling are not subject to chemical or photo-sensitized cross-linking,this could suggest that there is some repulsion between these regions of native fibrinogen in solution.

These points also suggest that the study of fibrinogen Bern I, where an abnormal γ chain has been shown to exist in the D domain (Rupp et al 1981) could yield useful information about the calcium binding sites of the D domains of fibrinogen and their role in polymerisation and in the interaction of fibrinogen with other species.

The major points about the shape of fibrinogen raised by this work are illustrated in Figure 6.1. This diagram of the fibrinogen molecule shows the Aα chains folding round to protect the N-terminal of the molecule and clearly shows how the Aα chain represents the major proportion of the solvent exposed surface of fibrinogen.It also shows the fragment D domains (shaded) which are in a similar conformation to that of fragment D (Ca^{++}) in solution. The calcium binding γ chain C-terminii are exposed at the surface of the molecule however the mid sections of the

γ chains are in close proximity and thereby shield each other, a point which is consistent with the recently published data of Fair et al, (1982) who examined fibrinogen using antibodies and showed that the portion of the γ chain between residues 95 and 264 was protected in fibrinogen but was progressively exposed on digestion to fragment D.

The position of the Aα chain in this model could also account for the pattern of digestion of fibrinogen by plasmin where Aα chain cleavage takes place followed by subsequent cleavages leading to core fragments D and E.

The shielding effect of the Aα chain on the compact domains may also in part explain difficulties in assessing the shape of the fibrinogen molecule.

A very interesting point about this model is that, whilst it possesses three distinct domains, its overall shape is very similar to that proposed by a number of workers (Hudry-Clergeon et al, 1975; Marguerie and Stuhrmann, 1976; Marguerie, 1979). Therefore the data supplied in this study could, in part, reconcile the two most extreme models of fibrinogen shape. The coiled-coil connecting regions proposed by Doolittle et al, 1978 depend on the formation of a hydrophobic core with polar side chains pointing out from this core. Thus when dehydration takes place, as is the case for many electron microscopic studies these coiled coils could break down and this breakdown of secondary structure could result in connecting regions becoming much narrower on electron micrographs. Furthermore, there is

nothing in this study which is in disagreement with the findings of Lederer (1979) or Hantgen (1982) who proposed that fibrinogen was a flexible molecule. Thus, linear arrangement of the domains of fibrinogen could be seen as a distinct possibility, particularly if the coiled-coil connecting regions were wholly or partially destroyed.

The model of fibrinogen proposed in Figure 6.1 shows only two calcium binding sites, one in each of the D domains of the molecule. The position of the third site has not yet been identified however there are really only two possible sites for it. The third site could be located at the C-terminal ends of the Aα chains of fibrinogen as suggested by Marguerie and Ardaillou (1982) or it could be in the region between the fragment E and fragment D domains. Van Ruijen-Vermeer, (1978) and Lindsay et al,(1978) showed that late fragment E does not bind calcium, they did not however preclude the possibility that early fragment E does so. If early fragment E and the Aα chain C-terminii were shown not to bind calcium then the conclusion could be drawn that the third calcium binding site of fibrinogen was dependent upon some interaction between either the two inter-domainal coiled-coil or the fragment E and fragment D domains.

As stated earlier,the main aim of this work was to develop and assess techniques for the examination of fibrinogen. In this respect the work has been successful however the information about fibrinogen which has been obtained is not completely conclusive and a number of suggestions about further work can be made.

One of the major points still to be resolved concerns the third high affinity calcium binding site. On the basis of this work it would appear that this site does exist and is not artefactual and so the main question remaining concerns the position of this site. The two positions mentioned earlier seem most likely and it would appear to be a simple matter to resolve the problem by carrying out calcium binding studies on partially degraded fibrinogen which has lost its Aα chain C-terminii. However, this has been attempted by a number of workers (Niewenhuizen & Gravesen, 1981; Marguerie & Ardaillou, 1982) and has yielded equivocal results,this may be attributable to problems in assessing the degree of digestion which has taken place coupled with the inherrent difficulties in measuring calcium binding sites of such high affinity highlighted in this study. Nevertheless this would seem to be a very useful area for future examination.

In this work the technique of photo-sensitized labelling has been shown to be very useful in assessing protein shape and more information about fibrinogen could be obtained if this technique was applied to the study of the fragment E region of the molecule,on the basis of the model presented here it would be predicted that fragment E from partially degraded fibrinogen would bind more label than fragment D from intact material.

If the technique of photo-sensitized labelling were combined with HPLC it could represent an even more powerful tool allowing complete characterisisation of the surface oriented regions of proteins.

As mentioned earlier, the usefulness of the techniques of cross-linking as a tool in protein chemistry has long been established however,this study has indicated that the technique may be even more useful if photo-sensitized cross-linking is used since it yields similar patterns to chemical cross-linking reagents whilst yielding a product suitable for enzymic degradation.

In conclusion,the main aim of this project which was to examine techniques for the analysis of fibrinogen has been achieved and these techniques have revealed a number of points about the fibrinogen molecule. On the basis of these studies it would appear that fibrinogen is a protected trinodular structure which possesses two calcium binding sites of equal affinity in its D domains and a third site of significantly higher affinity at some other part of the molecule.

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