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Formation of soluble fibrin oligomers in purified systems and in plasma

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The kinetic parameters for release of fibrinopeptide A (FPA) from human fibrinogen by thrombin are: $K_m = 2.3 \times 10^{-6}$ M and $V_{max.} = 1.1 \times 10^{-10}$ mol of FPA/s per unit of thrombin; for fibrin formation, K_m is similar to that for FPA release, but, under the conditions of the present study, $V_{max.}$ was approximately half of that for FPA release. The formation of fibrin polymer before the sol–gel transition was studied by gel-permeation chromatography combined with effluent analysis for fibrinogen antigen and residual FPA. Polymer formation in purified fibrinogen incubated with thrombin proceeded as a bimolecular association of exposed sites in a manner predicted by probability calculations and assuming random FPA cleavage. Each oligomer consisted of *n* molecules of fibrin monomer and two fibrinogen molecules, each of the latter lacking one FPA molecule, i.e. each oligomer, regardless of molecular size, retains two FPA molecules. The addition of 5 mM-$CaCl_2$ to the reaction mixture changed the rate of polymer formation, so that dimer was no longer the prevalent oligomer; in the presence of Ca^{2+}, the trimer was the oligomer in highest concentration. The polymers formed in the presence of calcium were similar in composition to those without, i.e. 2 mol of FPA/mol of oligomer. EDTA-treated plasma samples incubated for short periods of time, 30 s or less, with thrombin ranging in concentration up to 1 N.I.H. unit/ml did not form clots during the 10–15 min period of observation until they were applied to the column, though a large proportion of the available FPA was cleaved (maximum 45%). The soluble polymers in plasma were mostly of the high-M_r variety (tetramer and greater); these high-M_r polymers contained less than 2 mol of FPA/mol of polymer, whereas dimer and trimer in plasma were similar to those in the purified systems, i.e. 2 mol of FPA/mol.

The formation of fibrin from fibrinogen activated by thrombin is a stepwise reaction that is generally understood in qualitative terms, but less is known of quantitative relationships and kinetics. The release of FPA and FPB was described by Blomback & Vestermark (1957), who estimated a K_m for thrombin release of FPA to be approx. 5 μM (Hogg & Blomback, 1974); Nossel *et al.* (1976) found a roughly similar value, namely 2.99 μM. Others have found higher values (Hermans & McDonagh, 1982). Release of FPB is initially very much slower, but the rate increases substantially with fibrin polymerization (Hurlet-Jensen *et al.*, 1982).

The product of proteolysis is fibrin monomer (fibrinogen lacking both FPA molecules) or des-A-fibrinogen (fibrinogen lacking one FPA molecule); these products polymerize, forming first the dimer and then higher oligomers, by forming end-to-end aggregates with an overlap of half a molecule (Hermans & McDonagh, 1982). The kinetics of fibrin assembly have been studied by Hantgan & Hermans (1979) by means of light-scattering; they concluded that end-to-end association occurred as a bimolecular association of bifunctional monomers, and that the rate constants for end-to-end association was the same for two monomers, monomer–oligomer or oligomer–oligomer. Their data also showed no difference whether fibrinogen was activated by thrombin or reptilase, which only cleaves FPA.

Smith (1979), in a recent *N*-terminal study of oligomers formed during fibrinogen–fibrin conversion, found that the dimer consisted of two

Abbreviations used: SDS, sodium dodecyl sulphate; FPA, fibrinopeptide A; FPB, fibrinopeptide B.

associated des-A-fibrinogen molecules and that each oligomer molecule consisted of n molecules of monomer and two molecules of des-A-fibrinogen. Both Hantgan & Hermans (1979) and Smith (1979) found little if any difference when Ca^{2+} was added to the reaction mixtures.

Plasma from normal subjects contain polymeric forms of fibrin(ogen) and their concentrations are greatly increased in patients with thromboembolic vascular disease. Four mechanisms have been proposed as an explanation for this phenomenon: (1) thrombin action on fibrinogen; (2) plasmin action on fibrin; (3) complexing of fibrin breakdown products with fibrinogen; (4) complexing of fibrinogen or fibrinogen-derived moieties with other plasma proteins. In the present study we examine the first of these possibilities in purified systems in the presence and absence of Ca^{2+} and in plasma.

Materials and methods

Human fibrinogen was obtained from Kabi (grade L) or from IMCO, both of Stockholm, Sweden. Human thrombin [minimum specific activity 3000 N.I.H. (National Institutes of Health) units/mg of protein], soya-bean trypsin inhibitor and the leech anticoagulant hirudin were obtained from Sigma, St. Louis, MO, U.S.A. Absorbed goat antiserum to human fibrinogen was from the Serum Gateway Co., St. Louis, MO, U.S.A. Bio-Gel 5M and reagents for SDS/polyacrylamide-gel electrophoresis were from Bio-Rad, Richmond, CA. U.S.A.

Preparation of complexes

Fibrinogen was dissolved in 60mM-Tris/80mM-NaCl (pH 7.4)/soya-bean trypsin inhibitor (0.2mg/ml); except where noted, the fibrinogen concentration was 20mg/ml. If Ca^{2+} was required, 0.5M-$CaCl_2$ was added in appropriate amount. Thrombin (100 units/ml) was added, except where noted, to a final concentration of 0.1 unit/ml, and the mixture incubated at 25°C. Samples were removed at timed intervals and added to hirudin, such that hirudin was in 5-fold excess over thrombin. Samples were chromatographed immediately or kept frozen at −20°C until chromatography. A single freeze–thaw cycle did not alter chromatographic findings.

Complexes in plasma were prepared by adding thrombin (100 units/ml) to plasma [anticoagulated with EDTA (1.5mg/ml of blood)] to final concentrations of 0.25, 0.5, 0.75 and 1 unit/ml. After 30s, hirudin was added in 5-fold excess. Thrombin-treated plasma samples were chromatographed 10–15min after the addition of thrombin; no clots were observed under these conditions.

Gel-permeation chromatography was performed on columns (80cm × 2.5cm) of Bio-Gel 5M equilibrated and eluted with 60mM-Tris/0.3M-NaCl buffer, pH 7.4, with 10mg of soya-bean trypsin inhibitor per litre. Elution was by gravity feed at a flow rate of 15–30ml/h. Fractions (5–7ml) were collected and the effluent volume was determined by weighing every fourth tube before and after fraction collection. A portion (3ml) of fibrinogen/thrombin reaction mixture was applied to the column in a closed system, and effluent fractions were assayed: (1) by measurement of A_{280}, the readings being converted into μg of fibrinogen equivalents/ml by the value $A^{1\%}_{1cm} = 16.7$ (Mosesson *et al.*, 1967); (2) by immunoassay in the Technicon immunoprecipitator with the use of goat antiserum to human fibrinogen. The data were expressed as μg of fibrinogen equivalents/ml of effluent, extrapolated from a dilution series of purified fibrinogen of known concentration run simultaneously; and (3) by radioimmunoassay of residual FPA, released by treating a portion with thrombin (10 units/ml) for 10min. The assay was performed with the use of kits donated by Mallinckrodt, St. Louis, MO, U.S.A.

The effluent profiles obtained by all three measurements were resolved into individual components by a computer program developed on the basis of chromatographic-plate theory (Alkjaersig *et al.*, 1973). The column was calibrated by chromatography of purified proteins of known M_r and diffusion coefficient ($D_{20,w}$). Fig. 1(*a*) shows the linear relationship between log M_r of globular proteins and K_D (partition coefficient) where V_0 was 145ml and V_t was 405ml (determined by chromatography of a freeze-dried preparation of *Escherichia coli* and sucrose respectively). Fig. 1(*b*) shows the linear relationship between log $D_{20,w}$ and K_D for globular proteins and for fibrinogen and fibrinogen-derived moieties. Fig. 1(*c*) shows the linear relationship from Fig. 1(*a*) as a broken line, and that for fibrinogen and fibrinogen-derived moieties, which could be independently verified (closed circles). SDS/polyacrylamide-gel electrophoresis was also used to examine all polymers isolated by chromatography; in no instance, under the experimental conditions employed, was γ-dimerization detected. Assuming that fibrinogen-derived moieties are eluted, so that log M_r is linearly related to K_D, V_e values for the various n-mer shown in Fig. 1(*c*) (○) could be extrapolated. Curve-peeling of a variety of elution profiles confirmed protein peaks at these elution volumes. A protein peak was seen at 226ml (K_D 0.3), which corresponds to M_r 530000, compatible with a dimer of fragment X (a fibrinogen breakdown product, M_r 270000).

The elution profile of a pure substance on a gel-permeation column is a Gaussian curve described by the formula:

$$C = C_{max.} \exp\left(\frac{(V - V_e)^2 N_p}{2 V_e^2}\right)$$

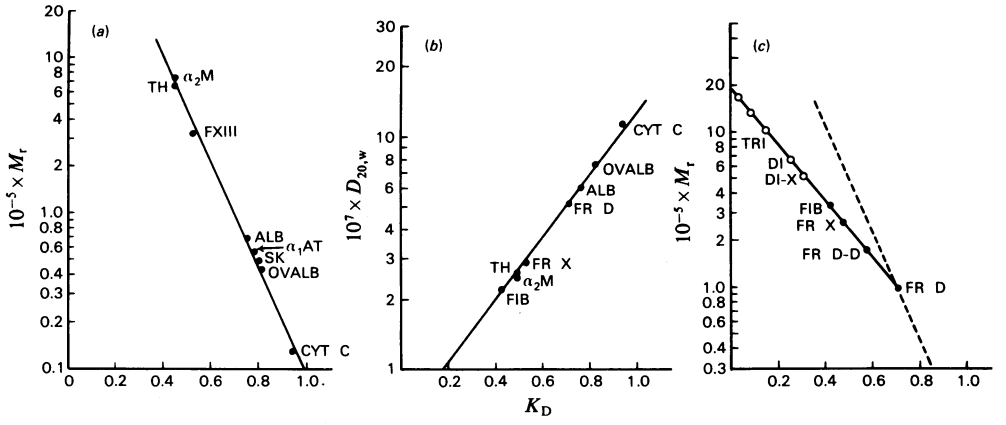

Fig. 1. *Gel-permeation chromatography of marker proteins and fibrinogen-derived moieties*

The eluent was 0.06M-Tris/0.3M-NaCl, pH7.4, with 10mg of soya-bean trypsin inhibitor/litre. Effluents were monitored by A_{280}. Marker proteins were thyroglobulin, albumin, α_1-antitrypsin, streptokinase, ovalbumin, and cytochrome *c*. Plasma was applied to the column and other markers were determined by specific assay: factor XIII by activity and α_2-macroglobulin and α_1-antitrypsin by immunoassay. Fibrinogen-derived proteins were prepared by plasmin proteolysis; assay was by immunoprecipitation; M_r was determined by SDS/polyacrylamide-gel electrophoresis (●) or extrapolated (○). Abbreviations used: α_2M, α_2-macroglobulin; TH, thyroglobulin; FXIII, factor XIII; ALB, albumin; α_1AT, α_1-antitrypsin; SK, streptokinase; OVALB, ovalbumin; CYT C, cytochrome *c*; FR D, fragment D; FR X, fragment X; FIB, fibrinogen; TRI, trimer of fibrin(ogen); DI, dimer of fibrin(ogen); DI-X, dimer of fragment X; FR D-D, cross-linked fragment D dimer.

where C is the concentration at elution volume V, $C_{max.}$ is the peak concentration at elution volume V_e, and N_p is a constant for a given column, the theoretical number of plates calculated from the elution profiles of purified biophysically defined proteins (Winzor, 1969).

Results

The kinetic parameters for FPA release from fibrinogen and for fibrin formation were determined. K_m was 2.3μM for both reactions and $V_{max.}$ was 1.1×10^{-10}mol/s per unit of thrombin for FPA release, and 5.6×10^{-11} half-mol/s per unit of thrombin for fibrin formation. The fibrinogen concentration ranged from 0.21 to 9.44mg/ml, the thrombin concentration was 0.05 unit/ml, the pH was 7.4 and the ionic strength was 0.15.

The polymerization that occurred as a consequence of thrombin action on fibrinogen and before sol–gel transformation was demonstrated by analytical gel-permeation chromatography. Human fibrinogen was exposed to human thrombin; portions removed at timed intervals were added to hirudin and analysed on the agarose column (Fig. 2, left-hand panels). A similar series of reaction mixtures containing 5mM-Ca^{2+} is illustrated in Fig. 2 (right-hand panels). The control fibrinogen showed a peak at 256ml; this peak was asymmetric and computer analysis showed that 25% of the protein was in the form of early fibrinogen-breakdown products. This was confirmed by SDS/polyacrylamide-gel electrophoresis of the reduced fibrinogen; 35% of the α-chains were degraded to fragments of M_r 32000–36000. After 6min in the absence of Ca^{2+}, a dimer was clearly discernible, being eluted just before the fibrinogen peak, and further incubation resulted in other polymer species in addition to the dimer. In the presence of Ca^{2+} only small amounts of dimer were formed, and polymers were eluted closer to the void volume. Thrombin treatment of polymer fractions released FPA from all polymer species (Fig. 2, ○). The late reaction mixtures were viscous, but no gels were seen during the first 30min of incubation.

Analysis of effluent profiles

A computer program developed on the basis of chromatographic-plate theory was used to analyse the elution profiles in Fig. 2. Table 1 shows the analysis of the profiles in Fig. 2 (bottom right-hand panel). The first three columns, V_e, K_D and M_r, are derived as described in the Materials and methods section. The fourth column is the computer analysis of the elution profile of the fully drawn line (the data showing fibrinogen equivalents/ml of fraction), which gives the proportion of the original fibrinogen in the form of pentamer (M_r 1700000), tetramer (M_r 1350000), trimer (M_r 1000000), and dimer (M_r 650000), intact fibrinogen (M_r 340000), fragment X (M_r 270000), and X dimer (M_r 530000).

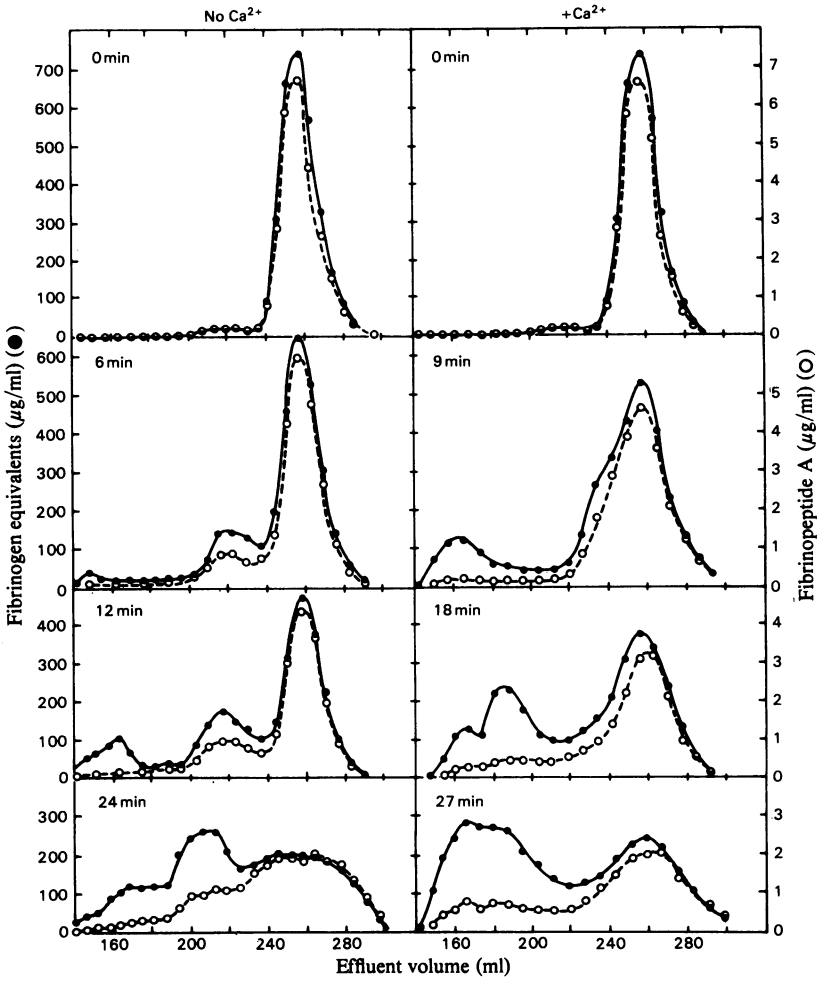

Fig. 2. *Gel-permeation chromatography of fibrinogen/thrombin reaction mixtures*
Column and eluent were the same as in Fig. 1. Reaction mixtures were human fibrinogen (20 of dry wt/ml), human thrombin (0.1 unit/ml) and soya-bean trypsin inhibitor (0.2 mg/ml) (left-hand panels); the reaction mixtures in the right-hand panels also had 5 mM-$CaCl_2$. The reaction was stopped at the indicated times by addition of a 5-fold excess of hirudin. Effluents were assayed immunologically, for fibrinogen in a Technicon immunoprecipitator (●), and for residual fibrinopeptide A (○) after thrombin treatment of the fractions, by a radioimmunoassay.

Table 1. *Computer analysis of the elution profiles of components eluted after a 27 min incubation of fibrinogen with thrombin and Ca^{2+}*

	V_e (ml)	K_D	$10^{-5} \times M_r$	Fibrinogen equivalents (%)	Residual FPA (%)	FPA (mol/mol)
Pentamer	153	0.03	17	9.8	3.4	2.14
Tetramer	165	0.077	13.5	14.2	8.4	2.49
Trimer	182	0.142	10	23.4	10.8	1.87
Dimer	211	0.254	6.5	11	6.9	1.73
X-dimer	226	0.300	5.4	8.4	14.3	2.43
Fibrinogen	256	0.427	3.4	19.6	31.2	1.98
Fragment X	268	0.473	2.7	13.6	24.9	1.74

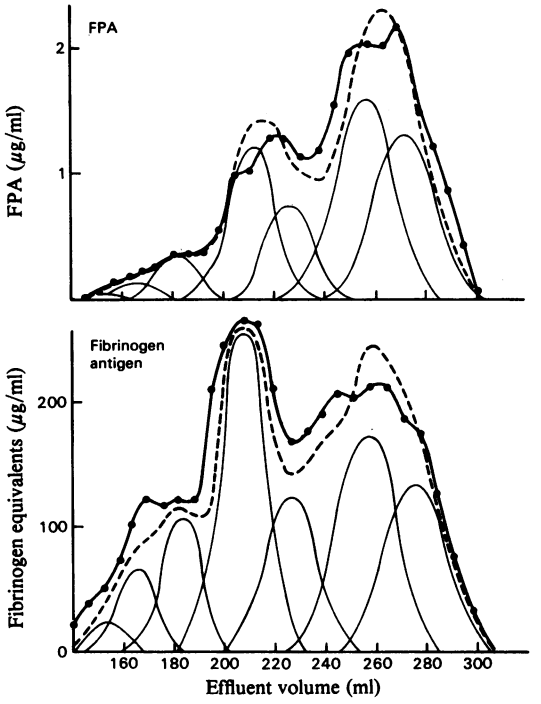

Fig. 3. *Resolution of a 27 min fibrinogen/thrombin mixture into its component parts*

A computer program based on chromatographic-plate theory resolved the complex elution profiles in Fig. 2 (lower left-hand panel) into a series of Gaussian curves representing in order along the abscissa, pentamer, tetramer, trimer, dimer, dimer of fragment X, intact fibrinogen and fragment X (drawn with thin lines). The broken line is the sum of these curves, and the experimental points (●) are shown. The upper panel shows the analysis of the FPA elution profile and the lower panel that of the fibrinogen-antigen profile.

The broken elution curve obtained from the FPA assay (residual FPA content per ml of fraction) yields the data shown in column 5. The resolution into individual components of the elution profiles of one reaction mixture is shown in Fig. 3.

All the Fig. 2 profiles were analysed as described above, and the data for FPA content per mol of each polymer species, fibrinogen and fragment X are shown in Table 2 as means ± S.E.M. for experiments with and without Ca^{2+}. Each molecular species contains approx. 2 mol of FPA/mol. There are no significant differences in relative FPA content in oligomers derived from intact fibrinogen between experiments with and without Ca^{2+}, but less FPA/mol of fragment X ($P < 0.01$) and less FPA/mol of X-dimer ($P < 0.02$) was found in experiments without Ca^{2+} than in those where Ca^{2+} was added.

Table 2. *Residual fibrinopeptide A content of fibrinogen-derived oligomers in reaction mixtures with and without Ca and in plasma*

	FPA (mol/mol of polymer) (mean ± S.E.M.)		
	No Ca^{2+}	5 mM-Ca^{2+}	Plasma
Pentamer	2.18 ± 0.35	2.02 ± 0.13	0.86 ± 0.13
Tetramer	1.91 ± 0.14	2.07 ± 0.15	1.09 ± 0.32
Trimer	2.69 ± 0.38	1.88 ± 0.13	2.10 ± 0.19
Dimer	1.98 ± 0.19	1.77 ± 0.12	2.89 ± 0.26
X-dimer	2.07 ± 0.08	2.54 ± 0.15	1.55 ± 0.53
Fibrinogen	1.96 ± 0.02	2.00 ± 0.03	2.02 ± 0.15
Fragment X	1.51 ± 0.05	1.90 ± 0.09	1.76 ± 0.45
n	10	9	5

A significant linear relationship ($r = 0.972$) was demonstrated to exist, in both the presence and absence of Ca^{2+}, between residual FPA, expressed as FPA/μg of fibrinogen equivalents, and chromatographic elution volumes between 160 and 260 ml (K_D 0.06–0.45) (result not shown).

Fig. 4 summarizes the data from the experiments in Fig. 2. The rates of FPA release and reduction in intact fibrinogen are not influenced by Ca^{2+} in the reaction mixture. The formation rates of individual polymer species, however, differ. In the absence of Ca^{2+} (Fig. 4, ●), the rate of formation of each polymer was close to that expected if bimolecular reactions and random thrombin action at susceptible sites are assumed; the lines in Fig. 4 were calculated as suggested by Hantgan & Hermans (1979). In the presence of Ca^{2+}, dimer formation was much less than predicted by these calculations, and higher oligomers were more abundant.

The X-dimer eluted just before the fibrinogen peak was present in low concentration in the starting fibrinogen (2% of total fibrinogen), but was increased in all samples after thrombin had been added; its concentration did not change with time [mean value 13.7 ± 0.6% (S.E.M.), $n = 19$]. Fragment X, which was a contaminant of the starting fibrinogen (25%), was decreased in all thrombin-treated mixtures, but there was no correlation with time [mean 14.3 ± 0.7% (S.E.M.)].

Other experiments (results not shown) with higher Ca^{2+} concentrations resulted in much earlier clot formation, though FPA was released at the same rate, or slower, than without Ca^{2+}; the rate of FPA release was not increased at any Ca^{2+} concentration (tested over the range 1 μM–100 mM), but was slightly inhibited at Ca^{2+} concentrations of 0.05 M or higher; this was probably a consequence of the higher ionic strength, since an increase in ionic strength brought about by the addition of NaCl resulted in similar inhibition. Whereas clot formation was rapid at high Ca^{2+} concentrations, high

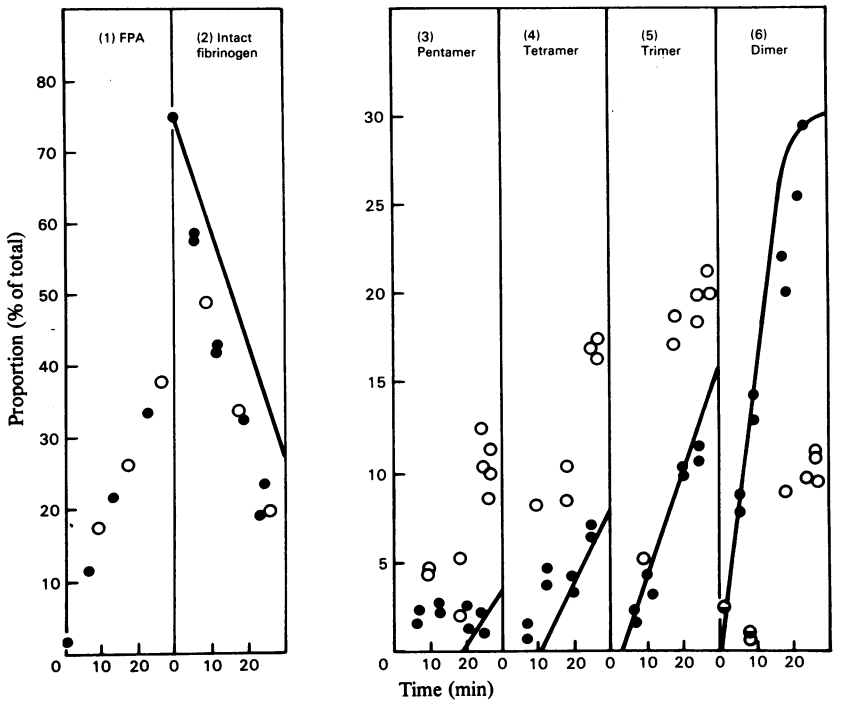

Fig. 4. *Rates of FPA release, decrease in intact fibrinogen and appearance of each oligomer in fibrinogen/thrombin reaction mixtures*

Panel (1) shows FBA release as a function of incubation time with thrombin. Effluent profiles of fibrinogen antigen (Fig. 2) were analysed as shown in Fig. 3 and Table 1, the various moieties being quantified as percentages of the total protein. Panel (2) shows the decrease in intact fibrinogen with incubation time, whereas subsequent panels (3–6) show the appearance rate of pentamer, tetramer, trimer and dimer. The experiments performed in the absence of Ca^{2+} are shown as closed circles (●) and those in its presence as open circles (○). The lines in panels (2)–(6) were calculated on the basis of bimolecular reaction between exposed sites and random thrombin action at susceptible sites.

Na^+ concentrations induced a prolonged lag phase, but, when clotting started, it proceeded at a more rapid rate than in its absence (result not shown).

Fibrin polymer formation in plasma

Thrombin added to plasma is rapidly inhibited by the plasma inhibitors, with inhibition nearly complete after 4 min. These observations suggested that fibrin polymerization in plasma should be studied by use of increasing thrombin concentrations, rather than as a function of time. EDTA/plasma was treated with thrombin in concentrations ranging from 0.25 to 1 unit/ml for 30 s, and the reaction mixtures chromatographed in the same manner as illustrated in Fig. 2. The effluent profiles (not shown) differed from those in Fig. 2 in that mostly high-M_r polymers eluted close to the void volume. None of the reaction mixtures clotted, but, at the two highest thrombin concentrations, part of the fibrinogen-derived moieties precipitated on the column; there was an 18% loss at 0.75 unit of thrombin/ml and a 38% loss at 1 unit/ml.

The data obtained by analysis of the elution profiles of thrombin-treated plasma are summarized in Fig. 5. FPA release was comparable with that of the experiments illustrated in Fig. 3, but decrease in intact fibrinogen was less for a given degree of FPA release. The addition of thrombin in smaller concentrations for longer periods of time to plasma, anticoagulated with EDTA or citrate, induced late clot formation (~10 min after stopping thrombin action), even though FPA release was less than 20%. The relative concentrations of polymers resulting from thrombin action on fibrinogen in plasma also differed from those in the purified system; dimer and trimer were found in low concentrations and pentamer and tetramer in high concentrations.

Table 2 shows the residual FPA content of the fibrin polymers in plasma expressed, as mol of FPA/mol. These values are similar to those found

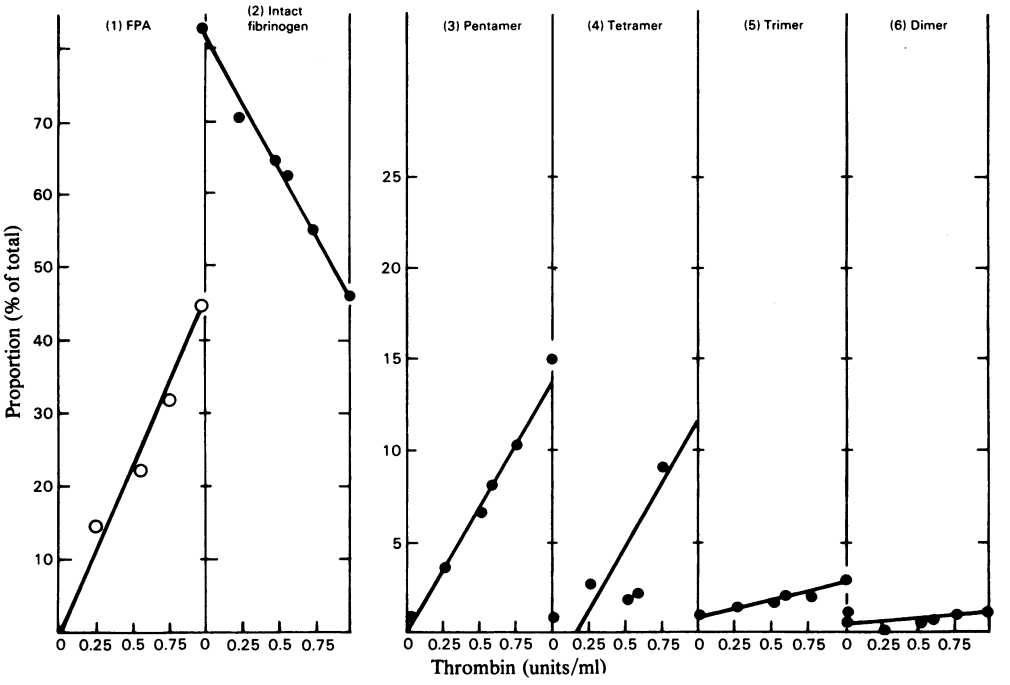

Fig. 5. *Rates of FPA release, decrease in intact fibrinogen and appearance of oligomers of fibrin(ogen) in plasma incubated with thrombin*

Normal human plasma (collected in EDTA) was incubated for 30 s with human thrombin ranging in concentration from 0.25 to 1 unit/ml. The reaction mixtures were chromatographed under conditions similar to those in Fig. 2, and the elution profiles analysed in the same manner as shown in Fig. 3 and Table 1. Panel (1) shows release of FPA as a function of thrombin concentration; panel (2) shows the decrease in intact fibrinogen, and subsequent panels (3–6) the proportions of pentamer, tetramer, trimer and dimer at each thrombin concentration.

for similar-sized polymers in purified systems for dimer and trimer, but differ for the more abundant higher-M_r polymers.

Discussion

Fibrinogen–fibrin conversion is classically viewed as a three-step reaction (Scheraga & Laskowski, 1957):

Fibrinogen + thrombin → fibrin monomer + fibrinopeptides A and B

Fibrin monomer → fibrin polymer

Fibrin polymer → fibrin gel

We have measured the first step of the reaction sequence by quantification of FPA release, since release of FPA causes polymerization, and FPB is not released to any appreciable extent until fibrin I (des-A-A fibrin, i.e. fibrin lacking both FPA molecules, but retaining FPB) is formed. Hurlet-Jensen *et al.* (1982) demonstrated a slow FPB release from thrombin-treated fibrinogen, but a 10-fold increase in FPB release rate after fibrin I was formed; moreover, light-scattering studies (Hantgan & Hermans, 1979) showed no difference between thrombin- and reptilase-mediated reactions, though reptilase only released FPA.

During the lag phase, before fibrin gel is formed, activated fibrinogen, lacking one or two FPA molecules, reacts with other similarly activated molecules to form polymer. Hitherto, polymerization has mostly been studied by light-scattering (Hantgan & Hermans, 1979; Ferry *et al.*, 1954; Blomback & Laurent, 1958; Wiltzius *et al.*, 1982), and most of these studies have employed substrate/enzyme concentrations where the physical self-association reaction is rate-limiting (Hermans & McDonagh, 1982). In the present study, thrombin was used in low concentration and FPA release was the rate-limiting step, permitting determination of rate of formation of each polymer species. Smith (1979), who used gel-permeation chromatography for polymer separation before *N*-terminal analysis, concluded that initially two fibrinogen molecules, each lacking one FPA molecule, associate to form a dimer which retains two FPA molecules; additional thrombin action and FPA release lead to further association into larger polymers. This concept is

supported by the present study; from *N*-terminal analysis, Smith (1979) found the dimer to be composed of two $\alpha(A\alpha)(B\beta)_2(\gamma)_2$ components. (The three pairs of polypeptide chains of fibrinogen are designated Aα, Bβ and γ; when the fibrinopeptides have been removed from Aα- and Bβ-chains, the chains become α and β respectively.) The only alternative that could fit the data would be a dimer of fibrin monomer and fibrinogen, which, for a number of reasons discussed by Smith (1979), appears untenable. We failed to detect fibrin-monomer–fibrinogen dimer when purified fibrin monomer dissolved in 5 M-urea and free of thrombin was added to purified fibrinogen; in each study, fibrin monomer formed fibrin gel and the chromatographic elution pattern of the mixture was identical with that of the original fibrinogen solution.

On the other hand, Olexa & Budzynski (1980) have presented evidence for the existence of binding sites additional to the two *N*-terminal sites exposed by thrombin. These authors suggest that linear polymer are formed by the binding of one of these additional sites to a thrombin-exposed binding site on another molecule, permitting formation of a fibrin-monomer–fibrinogen dimer, and others (Heene & Matthias, 1973; Kudryk *et al.*, 1974) have shown that fibrinogen binds to an agarose–fibrin-monomer column and fibrin monomer to an agarose–fibrinogen column; however, steric relationships in free solution and under affinity-column conditions may differ.

Assuming end-to-end aggregation and formation of protofibrils as described by Hermans & McDonagh (1982), a protofibril can be visualized as a series of fibrin-monomer units linked through the sites exposed by FPA release and with a half-activated fibrinogen molecule at each end; thus each polymer molecule, regardless of size, should retain two FPA molecules; such a composition is shown in Table 2.

The rate of formation of each oligomer in solutions of fibrinogen and thrombin was predictable, and could be calculated on the basis of probability, assuming random thrombin action at susceptible sites and bimolecular reactions (Hantgan & Hermans, 1979). In the presence of Ca^{2+}, additional binding sites appeared to play a role, for dimer formation was much less than in its absence, and higher oligomers were more abundant.

It has been assumed that fibrin polymerization in plasma was qualitatively similar to that encountered in purified systems; however, the present study shows both quantitative and qualitative differences.

The soluble polymers that appeared in the column effluents differed from those in the purified system, where, in the absence of Ca^{2+}, dimer was the predominant species and, in the presence of Ca^{2+}, trimer was predominant; in plasma, dimer and trimer only appeared in low concentrations, whereas larger polymers (greater than tetramer) predominated (Fig. 6). These large-molecular-size polymers were unstable; at least they tended to precipitate on the column, where they were separated from fibrinogen. The size of these large molecules cannot be estimated accurately, since they are essentially eluted in the void volume. If each polymer is similar in composition to polymers in purified systems, i.e. 2 mol of FPA/mol, the mean M_r might be of the order of 3×10^6.

The data suggest that thrombin action at susceptible sites is not random in plasma, and that thrombin has greater affinity for oligomers; as a consequence, more fibrinogen should remain intact than in the purified system. Comparison of Figs. 4 and 5 shows that at the time when approx. 50% of the available FPA had been released in the purified system, only 20% of the fibrinogen remained in intact form, whereas in plasma with the same degree of FPA release, 50% of the fibrinogen was intact. Thus, in the complex milieu of plasma, some mechanism appears to exist that protects the circulating fibrinogen from proteolytic action by thrombin; a possible explanation for this phenomenon is binding of thrombin to oligomer. Seegers *et al.* (1945) first demonstrated adsorption of thrombin to fibrin; Liu *et al.* (1979) confirmed the observation and found (i) that the active site is not required for binding, (ii) that the absorbed thrombin is to some extent protected from inactivation by plasma inhibitors, thereby increasing thrombin half-life 8-fold over that of free thrombin in plasma, and (iii) that maximal binding is 1 mol of thrombin/2 mol of fibrin(ogen). Thus thrombin bound to oligomer would still be able to release FPA from fibrinogen but only in close proximity to an existing oligomer. Liu *et al.* (1980) also found subtle differences in thrombin binding between reaction mixtures with and without Ca^{2+}; perhaps these differences might explain the differences in rate of formation of individual oligomers (Fig. 4).

In previous clinical studies, on analysis of patient plasma samples by gel-permeation chromatography, we found a correlation between the concentration of fibrinogen-derived high-M_r complexes of M_r higher than that of fibrinogen itself and thromboembolic disease (Fletcher *et al.*, 1977, 1979; Alkjaersig *et al.*, 1976, 1980). Several possible mechanisms can be invoked to explain the appearance of these high-M_r derivatives: thrombin action on fibrinogen, plasmin action on fibrin, complexing between fibrin proteolysis products and fibrinogen or complexing of either of these moieties with other plasma proteins. The complexes found in patient plasma samples probably arise through several mechanisms, since it is generally assumed that activation of the blood-coagulation system *in vivo* is accompanied by activation of the fibrinolytic

enzyme system. However, thrombin action on fibrinogen plays a significant role in the genesis of the high-M_r fibrin(ogen) complexes found in human plasma. Oligomers formed by thrombin action contain FPA (two molecules per molecule of oligomer), whereas those formed by plasmin action on fibrin do not contain FPA.

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