Formation of Soluble Fibrin Polymers. Fibrinogen Degradation Fragments D and E Fail to Form Soluble Complexes with Fibrin Monomer†

Gerald F. Smith* and Nils U. Bang

ABSTRACT: Two new findings are presented in this report: (1) it was discovered that a low thrombin activity can convert fibrinogen (I-2 and I-8) essentially completely to soluble fibrin polymers (dimers, trimers, tetramers, and perhaps, higher *x*-mers of fibrin monomers) before gelation or clotting occurs; and (2) it was demonstrated that plasmin-induced fibrinogenolysis fragments D and E (and other fragments formed from extensively digested fibrinogen) do not form soluble complexes or copolymers with fibrin monomer *in vitro*. This study was undertaken to examine the modern hypothesis that fibrin monomer can be complexed and held noncoagulable by lysis products from the plasmin degradation of fibrinogen. The fragments studied in this report were purified fragments D and E and the fragments present in a mixture of plasmin-digested fibrinogen (FLP) containing fragments D and E, and any other smaller nondialyzable fragments. Experiments were designed in which it was demonstrated by agarose gel chromatography that mixtures of fibrinogen (I-2 and I-8) and FLP (or purified D and E) did produce high yields of soluble polymers when the mixtures were exposed to thrombin. However, it was shown that the fibrinogenolysis fragments did not participate in the soluble polymer formation. This led to further experimentation, which demonstrated the heretofore unknown feature of fibrinogen chemistry, whereby thrombin can cause almost complete conversion to soluble polymeric fibrin before clot formation. The soluble fibrin polymers are formed and are stable (remain unclotted) under nearly physiological conditions.

The concept that soluble polymers of fibrinogen-derived species might exist in blood has been emphasized in recent years. Currently, this concept occupies a place of primary importance in the field of blood coagulation and thrombosis.

The term generally used in current literature to refer to these polymeric species is soluble fibrin monomer complexes. The soluble fibrin monomer complexes (SFMC) are considered to be subdivided into two distinct types of compounds, each type utilizing a fibrin monomer as part of a copolymer. The first type of complex (hereafter designated SFMC I) is considered to be a soluble copolymer of fibrin monomer and fibrinogen and is supposedly clottable by thrombin (Niewiarowski and Gurewich, 1971. The existence of this species was postulated by Shainoff and Page (1960, 1962), who demonstrated that a cold-insoluble species containing fibrinogen can be precipitated from solutions containing fibrin monomer and fibrinogen or from plasmas of endotoxin-treated rabbits. The fibrin monomer in the Shainoff and Page complex is an incompletely thrombin-activated monomer devoid in the A fibrinopeptides but retaining the B fibrinopeptides. These authors inferred from their data that fibrinogen was involved in a complex with this special fibrin monomer (possessing the B fibrinopeptides) and in this manner kept the monomer soluble. Thus far, no other experimental data have been reported which confirm (or disavow) the existence of fibrinogen–fibrin monomer soluble complexes (SFMC-I). Sasaki *et al.* (1966) have succeeded in demonstrating that a fibrinogen–fibrin monomer complex can be produced *in vitro* by the action of factor XIII. The relevance of this type of complex to *in vivo* situations has yet to be shown.

The second type of soluble complex is considered to be a copolymer of fibrin monomer and one or more of the various plasmin–lysis products of fibrinogen and/or fibrin. This type of soluble complex (hereafter designated SFMC-II) is considered to be unclottable by thrombin (Niewiarowski and Gurewich, 1971; Lipinski *et al.*, 1967). The degradation products of fibrinogen thought to be able to participate in this complex formation are fragments X, Y, and D, as designated by Marder *et al.* (1969). According to Fletcher's nomenclature these fragments correspond to first derivative, intermediates and 5.27S final product, respectively (Fletcher, 1970). The evidence for the existence *in vitro* of SFMC-II-type complexes is found in two published works. Initially, Lipinski *et al.* (1967) studied the clotting of blood or plasma containing ^{131}I-labeled fibrinogen in the presence of increasing amounts of fibrinogen degradation products. After centrifugation the liquors were found to contain increasing amounts of ^{131}I with increasing quantities of fibrinogenolysis products. The same behavior was observed using purified fibrinogen and fibrin rather than blood or plasma. The authors inferred that complexes had formed between fibrin monomer and the fibrinogenolysis products. The liquors from the incompletely clotted blood or plasma experiments did not clot when exposed to high concentrations of thrombin (33 units/ml). Therefore, the soluble complexes were assumed to be resistant to thrombin action, and they were termed "unclottable" complexes.

In later work, Marder and Shulman (1969) performed experiments in which fibrinogen was mixed with the purified fragments X, Y, and D. Thrombin was added to the mixtures and after clotting (about 50% of the protein was reported to have clotted) the liquors were subjected to analytical ultracentrifugation. The sedimentation patterns showed peaks corresponding to the respective fragments (X, Y, or D) plus some material sedimenting with high *s* values (13, 18, and 23 S). The rapidly sedimenting materials were assumed to be complexes of fibrin monomer and the fibrinogenolysis frag-

† Lilly Laboratory for Clinical Research, Eli Lilly and Co., Indianapolis, Indiana 46206, and from the Indiana University School of Medicine, Department of Medicine, Marion County General Hospital, Indianapolis, Indiana 46202. *Received March 6, 1972.*

* Senior Research Fellow, Eli Lilly and Co., 1971, 1972.

ments; however, no direct evidence for the participation of the degradation products in polymer formation was presented.

Soluble complexes between fibrin monomer and fibrinogen degradation products have been implicated in the mechanism of hermorrhagic diathesis. This pathologic process was assumed to be the result of the rendering of fibrin monomer "unclottable" by formation of SFMC-II-type-soluble complexes (Lipinski *et al.*, 1967; Marder *et al.*, 1967). The concept of a coagulation defect involving SFMC-II complexes originated in Fletcher's laboratory. Defective fibrin polymerization was suggested to occur as a result of fibrin monomer binding with fragment D (5.27S final product) and the incorporation of this abnormal unit into the fibrin network (Alkjaersig *et al.*, 1962; Bang *et al.*, 1962).

Recently Fletcher and Alkjaersig have demonstrated by agarose gel chromatography that soluble, fibrinogen related polymeric species do exist in plasma under certain conditions (Fletcher *et al.*, 1970; Alkjaersig *et al.*, 1970). The exact nature of these polymers was not elucidated, but the authors suggest that both types of soluble fibrin monomer complexes (SFMC-I and SFMC-II) are present, depending upon whether the fibrinolytic system had been activated.

Confirmation of the existence of soluble polymers or complexes of fibrinogen-derived species and elucidation of their structures and mechanisms of their formation were the goals of this study. Experiments were designed to demonstrate the ability of the fibrinogen degradation fragments D and E to form soluble complexes with fibrin monomer (produced from both fibrinogens I-8 and I-2). The interactions of the proteins were studied by separating the various molecular-sized species on columns of agarose (exclusion limit of about 5 million molecular weight).

In the course of the investigation, it was found that soluble polymeric fibrin species were produced in the experiments, but the fibrinogenolysis fragments were not incorporated into the polymers. Subsequent experimentation was directed to the study of low thrombin activity on solutions of fibrinogen, in attempting to explain the nature of these soluble polymeric materials. Agarose gel chromatography and N-terminal analysis were used to characterize the thrombin-generated soluble polymeric products.

Materials and Methods

Preparation of Human Fibrinogen I-4. This material was prepared by the method of Blombäck and Blombäck (1956). Clottable protein was greater than 96%. The preparation used contained trace procoagulants, as it showed a recalcification time of about 70 hr. The prothrombin time was 9 hr. Plasmin was absent, as no fibrinogenolysis was seen by gel chromatography (see below) after incubating solutions for 48 hr at room temperature. The product was dialyzed exhaustively against 0.3 M NaCl.

Preparation of Human Fibrinogen I-2. This was prepared from frozen human Cohn fraction 1 (a gift from the Michigan Department of Health) by a combination of the methods of Kazal *et al.* (1963, 1964), Mosesson and Sherry (1966), and Mosesson (1962). After two adsorptions with barium sulfate powder (Baker), fractionation was carried out through fractions I-G, I-G_2, I-1, and I-2. The fraction I-2 was dissolved in 0.05 M phosphate buffer (pH 6.4) to a protein concentration of about 1%. An equal volume of 0.2 M ϵ aminocaproic acid–0.3 M NaCl was added. The fibrinogen was precipitated at −4° by 8% ethanol (v/v). The product was extensively dialyzed against 0.3 M NaCl.

Fraction I-2 had a clottable protein content of at least 99%. The product was free of prothrombin (infinite prothrombin time) and free of factor XIII. The I-2 preparation used in the subsequent experiments had a trace of proteolytic activity, as judged by gel elution chromatography (upon sitting 48 hr at room temperature a small "late-eluting" shoulder could be seen in the Bio-Gel A-5m elution pattern, which would indicate lysis). Consequently, as a safeguard, lima bean trypsin inhibitor was added to these solutions (0.005% final concentration). Such solutions were stable at room temperature for at least 48 hr.

Preparation of Human Fibrinogen I-8. This material was prepared from outdated human plasma according to Mosesson and Sherry (1966). The glycine precipitation scheme was performed through the I-8 precipitation (0°, 2 M glycine). In our hands this step left most of the I-8 fraction still in solution (95% of the I-7 fraction was still dissolved). Rather than alter the conditions we precipitated I-8 along with the I-9 fraction (the solution was made 33% saturated with ammonium sulfate). The supernatant was free of ultraviolet-absorbing materials (at 280 nm) after precipitation and 95% of the I-7 weight was recovered. The product was exhaustively dialyzed against 0.3 M NaCl. This product, fraction I-8 (with I-9 included), was 99+% clottable by thrombin. It was free of prothrombin (prothrombin time negative after 48 hr) and other procoagulants (recalcification time greater than 3 days) and free of factor XIII. The I-8 was also free of plasmin activity as, upon incubating at pH 7.6 at room temperature for 48 hr, no lysis could be detected by gel chromatography (nor was polymer formation observed by the gel elution patterns).

Preparation of Fibrinogen Lysis Products (FLP). Fibrinogen was extensively hydrolyzed by plasmin. The substrate solution was 32 ml of human fibrinogen I-4 at a protein concentration of 1.6% in 0.025 M sodium phosphate (pH 7.8). Human plasmin (fibrinolysin—a gift from the Michigan State Department of Health) was 10.1 caseinolytic unit/ml in 50% glycerol. Plasmin (2 ml) was added to the I-4 solutions. The enzymatic digestion was let proceed at 37°. At intervals, 0.2 ml of the reaction mixture was removed and added to tubes containing 0.5 mg of lima bean trypsin inhibitor in 0.1 ml of saline. These samples were assayed for thrombin times and for inhibition of plasma thrombin times (as described below in Figure 1). Benzene was layered over the digest and the mixture left at 37° overnight. After 17 hr the benzene layer was removed, and the aqueous mixture of products was dialyzed exhaustively against a 0.01 M $NaHCO_3$–Na_2CO_3 buffer (pH 8.9) in the cold. This final digest contained 1.1% protein, comprised of fragments D, E, A, B, C, and any other smaller nondialyzable fragments, and is designated FLP.

For use in subsequent experiments the above mixture was diluted to a protein concentration of 0.29% with Tris (0.06 M)–NaCl (0.3 M) (pH 7.6) buffer. The trypsin inhibitor was added to a final concentration of 0.03% in order to inhibit residual plasmin activity in the medium. The FLP solution contained trace amounts of thrombin activity endogenous to the I-4 fibrinogen preparation.

The characteristics of the FLP mixture are presented in Figure 1. The data show the typical features of a "complete" plasmin digest of fibrinogen, sometimes referred to as a "stage-three digest" (Marder *et al.*, 1969). The plasma clotting inhibition curve proceeds through a maximum or "peak anticoagulant" activity and then levels off (Triantaphyllopoulos, 1958). The clotting time of the digest mixture rapidly became infinite. The immunoelectrophoretic pattern of the FLP mixture shows the two distinct arcs coincident with purified frag-

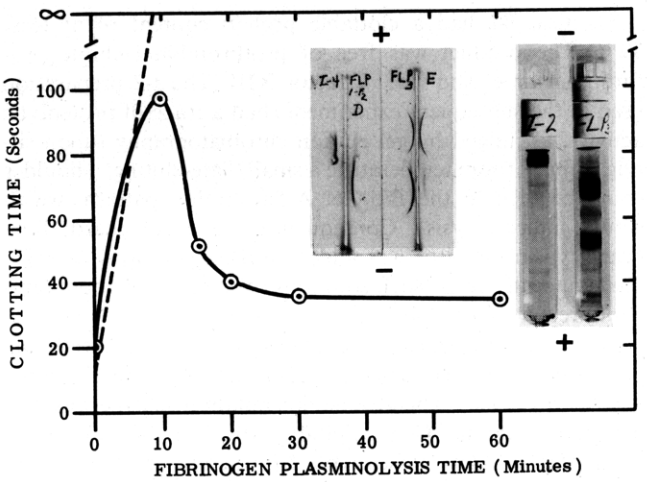

FIGURE 1: Characteristics of FLP preparations. FLP was prepared (20-hr digest) as described in the Experimental Section. The solid curve represents the plasma clotting inhibitory properties of the digest at various times. The broken line shows that the thrombin clotting time of the digest itself quickly became infinite. Immunoelectrophoresis (left to right: I-4, D, FLP, E) was performed on agar slabs with 0.01 mg of protein. Polyacrylamide (7%) gel electrophoresis was done with 0.1 mg of I-2 and 0.15 mg of FLP. The anodic and cathodic directions are indicated in the figure.

ments D and E prepared as below (Nussenzweig *et al.*, 1961). The gel electrophoretic pattern (in 7% polyacrylamide gel) of the FLP mixture shows two thick protein bands—one very anodic (E) and the other heterogeneous band (D) at a smaller distance from the origin, in agreement with Fisher *et al.* (1967).

Preparation of ^{125}I-Labeled FLP. The labeled material was prepared according to Hunter (1966) with minor modifications. One milliliter of 1.1% FLP mixture was treated with 2 μl of Na^{125}I (0.1 mCi) in the presence of 0.5 mg of chloramine-T. The reaction was stopped with 0.5 mg of ascorbic acid, and the protein was separated from the nonbound ^{125}I by Sephadex G-25 gel chromatography. The eluant used was Tris (0.06 M)–NaCl (0.3 M) (pH 7.6). The product was 3.5 ml of 0.33% ^{125}I-labeled FLP (containing 18 μCi/ml). Lima bean trypsin inhibitor was added to a final concentration of 0.04% by adding 0.3 ml of the inhibitor (5 mg/ml) in Tris buffer (pH 7.6).

Preparation of Purified Fragments D and E. These fragments were separated from FLP digests by DEAE-cellulose chromatography, as modified from Nussenzweig *et al.* (1961). DEAE-cellulose (Selectacel from Schleicher and Schuell Co.) was equilibrated with 0.01 M $NaHCO_3$–Na_2CO_3 buffer (pH 8.9). Four milliliters of FLP digest (about 44 mg) was applied to a column (0.9 × 29 cm). Elution of the materials was performed in three steps: (a) 200 ml of 0.01 M carbonate buffer (pH 8.9), (b) 1000 ml of a linear gradient produced from 500 ml of 0.01 M carbonate buffer (pH 8.9) plus 500 ml of this same buffer made 0.1 M in NaCl, and (c) 0.01 M carbonate buffer (pH 9.9), 0.1 M in NaCl (at least 200 ml). The elution flow rate was about 2 ml/min. During step a fragments A, B, and C were eluted; no protein eluted during step b; during step c fragment D eluted as a single peak, followed by a peak containing both D and E material, and finally a peak eluted containing only E material (judged by immunoelectrophoresis). The materials were concentrated by freezing and lyophilization. The purified fragments D and E failed to demonstrate procoagulant activity. It has been established that both D and E materials are heterogeneous (Jamieson and Gaffney, 1968; Niewiarowski and Nandi, 1971). Reference in this report to fragments D and E are meant to include any heterogeneous components.

General Assays. Prothrombin was assayed according to Pechet (1964). One-stage prothrombin times were performed according to Quick (1950) using solutions 0.1 M in ϵ-aminocaproic acid.

Clotting inhibitory activity of fibrinogen digests was assayed according to Latallo *et al.* (1962). Thrombin times of the digests were performed with 0.1 ml of digest and 0.1 ml of thrombin (0.5 unit). Alternatively, 0.05 ml of digest was mixed with 0.1 ml of normal plasma and then treated with 0.1 ml of thrombin (0.5 unit).

Recalcification times were measured by incubating 0.1 ml of fibrinogen with 0.1 ml of $CaCl_2$–ϵ-aminocaproic acid (0.08 M $CaCl_2$–0.2 M ϵ-aminocaproic acid). Also, thrombin could be detected by allowing 0.2 ml of fibrinogen (about 0.3% protein) to stand overnight at room temperature (pH 7.6) and subsequently testing for polymer formation by gel chromatography on Bio-Gel A-5m agarose columns (see below).

Clottable protein was measured by a microultraviolet assay. The clotting mixture consisted of 0.1 ml of fibrinogen (0.3–1.0%) in Tris (0.06 M)–0.3 M NaCl (pH 7.6) and 0.1 ml of $CaCl_2$–ϵ-aminocaproic acid (0.08 M $CaCl_2$–0.2 M ϵ-aminocaproic acid). Parke-Davis topical thrombin (0.1 ml of a 20-units/ml solution) was added, and the mixture was allowed to clot overnight at room temperature. Tris buffer (1.0 ml) was added to the glass tube, and the mixture was allowed to stand 1 hr with frequent mixing. The tubes were centrifuged at 5000 rpm for 10 min, and the clot liquor was transferred to a quartz microcell (volume of about 1.0 ml), path length 1 cm. The ultraviolet spectrum was recorded (Cary Model 15) from 320 to 240 nm or a single reading is taken at 280 nm. Appropriate blanks were constructed (for fibrinogen and thrombin) and the clottable protein was calculated by the disappearance of 280-nm-absorbing protein. Alternatively, a macrotechnique was used in which 2.0 ml of fibrinogen and 0.2 ml of 0.4 M $CaCl_2$–1.0 M ϵ-aminocaproic acid were clotted with 1.0 ml of thrombin.

Factor XIII activity was assayed by estimating the 5 M urea solubility of fibrin formed in the presence of 0.04 M $CaCl_2$ overnight at room temperature. The fibrinogen (0.1 ml) was assayed for coagulable protein, as described above, and the fibrin residue after centrifugation was treated with 1.0 ml of 5 M urea. After one hour at room temperature, with frequent gentle shaking, the dissolved protein was estimated by ultraviolet spectroscopy.

Protein concentration of fibrinogen solutions was determined spectrophotometrically by assuming that $E^{1\%}_{1\,cm} = 15$ for fibrinogen at 280 nm (Pollara, 1963).

Immunoelectrophoresis. This technique was performed on agar slabs (7.5 × 2.5 cm) according to Hirschfeld (1960) using rabbit antihuman fibrinogen antibody (Behring Diagnostics). The buffer medium used was sodium diethyl barbiturate–sodium acetate (0.1 M) (pH 8.6).

Polyacrylamide Gel Electrophoresis. Continuous gel columns (0.5 × 7 cm) (Davis, 1964) were prepared (7% final acrylamide concentration, 0.16% bisacrylamide, and 0.03% *N,N,N′,N′*-tetramethylenediamine buffered at pH 8.9 with 0.37 M Tris·HCl). Electrophoresis was performed for 2 hr at 3 mA/tube. The running buffer medium (top and bottom reservoirs) was Tris–glycine (0.005 M Tris–0.04 M glycine), pH 8.3. Staining was done with Coomassie Brilliant Blue (Chambrach *et al.*, 1967).

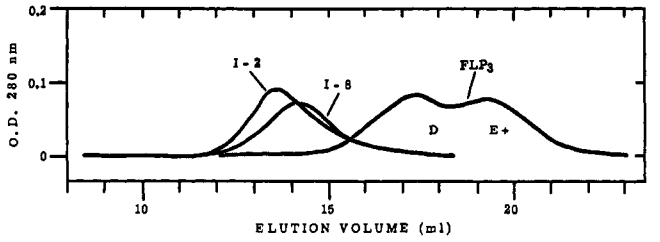

FIGURE 2: Gel chromatographic behavior of fibrinogens I-2 and I-8 and the FLP proteins. All of the proteins were run on the same column (0.9 × 29 cm) of Bio-Gel A-5m agarose. (See Materials and Methods for chromatographic details.) The amounts of the respective proteins applied to the column were: I-2 = 0.6 mg; I-8 = 0.5 mg; FLP = 1.1 mg.

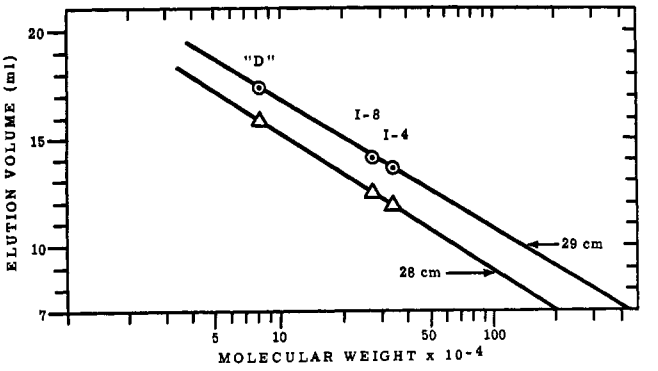

FIGURE 3: Elution volume–molecular weight relationship of fibrinogens I-4 and I-8 and the fragment D. The ordinate represents elution volumes of these proteins on Bio-Gel A-5m columns (0.9-cm diameter). Experimental conditions were described in Materials and Methods. The abscissa indicates the log molecular weight of these materials. The upper curve (represented by ○) is a plot of the data from Figure 2 using a 29-cm column. The lower curve (△) represents averages of multiple runs on a 28-cm column.

Gel Chromatography. Columns (0.9-cm diameter) were packed with Bio-Gel A-5m (Bio-Rad) spherical agarose beads (200–400 mesh) and equilibrated with Tris buffer (0.06 M Tris–0.3 M NaCl), pH 7.6. Column flow was regulated by gravity feeding using a Mariotte flask. The flow was always maintained at values near 11 ml/hr. Fractions were collected automatically (usually at 5.5-min intervals) and elution volumes were measured directly with micrograduate cylinders. The column effluents were monitored continuously at 280 nm with an ISCO Model UA-2 ultraviolet analyzer, using 1-cm path-length flow cells. The chart speed was 2 in./min, and the optical density ranges were either 0–0.25 or 0–1.0. When quantitative comparisons were to be made on a sequence of samples, the same column was used and all conditions would be kept constant.

The Bio-Gel A-5m agarose is reported by Bio-Rad Laboratories to give packed columns with void volumes of about 0.38 of the total bed volume. For a column of 28-cm length the void volume is calculated to be about 6.8 ml.

N-Terminal Amino Acid Analysis. This technique was performed as previously described (Smith and Murray, 1970), using the phenylthiohydantoin method.

Results

Behavior of Fibrinogen Species on Gel Chromatography. The elution patterns of human fibrinogens I-2 and I-8 and the mixture of plasminolysis products designated as FLP (containing fragments D, E, A, B, C, and any other nondialyzable fragments) are shown in Figure 2. These fractions were eluted from the same column of Bio-Gel A-5m. This particular column produced the following peak elution volumes of the respective materials: I-2 at 13.8 ml, I-8 at 14.3 ml, and the FLP mixture at 17.5 ml (fragment D) and 19.5 ml (fragment E plus smaller fragments).

The two elution peaks in the FLP elution pattern of Figure 2 are designated as "D" (containing fragment D) and "E+" (containing fragment E plus smaller nonimmunogenically reactive fragments, *e.g.*, A, B, C). These peak assignments were verified by running purified fragments D and E on the same columns and subjecting the resulting peaks to immunoelectrophoresis.

Figure 3 demonstrates the linear relationship between the gel elution properties of the various fibrinogen species and their molecular weights. The molecular weights were taken to be: I-2 as 340,000 (Caspary and Kekwick, 1957), I-8 as 273,000 (Sherman *et al.*, 1969), and fragment D as 83,000 (Marder *et al.*, 1969). The upper line in Figure 3 represents the data from the column described in Figure 2 (the column length was about 29 cm). The lower line in Figure 3 represents data obtained by multiple runs on a different column (length of 28 cm) and averaging the elution volume data. The average elution volumes from this 28-cm column were: I-4 = 12.0 ml (8 runs), I-8 = 12.6 ml (5 runs), and the D peak of FLP = 16.0 ml (3 runs).

The average deviation of these data was less than 2 percent for each of the three proteins. The 0.5-ml difference in elution volume between fibrinogen I-4 and fibrinogen I-8 was constant. The linear relationship between elution volumes and the molecular size of these species (Figure 3) implies that fragment D might retain much of the three-dimensional structure of the parent molecule. This is in agreement with recent observations that bovine fragment D possesses near-ultraviolet circular dichroic spectral properties identical with bovine fibrinogen (Budzynski, 1971).

Polymerization of Fibrinogen I-8 by Thrombin in the Presence of FLP. The ability of any of the fragments in the FLP mixtures to copolymerize or complex with fibrin monomer from I-8 fibrinogen was studied by generating fibrin monomer in the presence of the FLP proteins.

Figure 4 demonstrates the conversion of fibrinogen I-8 to

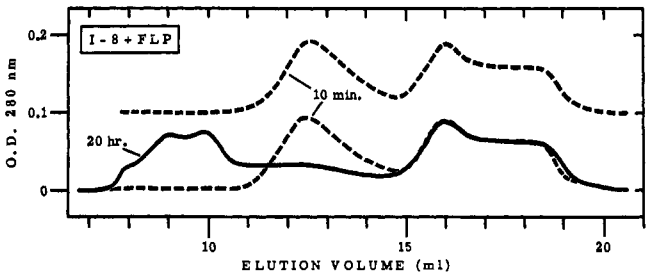

FIGURE 4: The polymerization of fibrinogen I-8 in the presence of the FLP proteins. The experimental clotting mixture consisted of I-8 and FLP (1:1 ratio by weight) with the thrombin activity endogenous to the FLP mixture as the source of clotting enzyme. The dashed curve shows the Bio-Gel A-5m (28 cm) elution pattern 10 min after mixing the reactants. The solid curve represents the elution profile after 20-hr reaction time. Because these two curves are superimposable near the FLP elution area, the dashed curve is redrawn in the upper part of the figure. (See Materials and Methods for chromatographic details.)

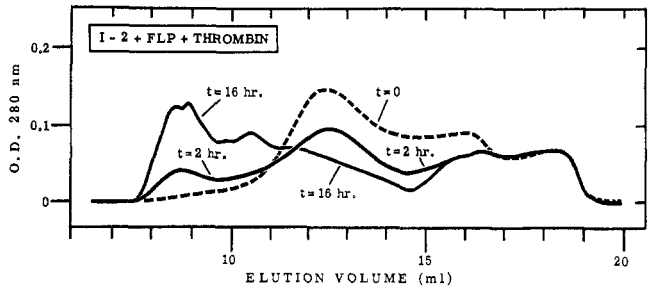

FIGURE 5: The polymerization of fibrinogen I-2 by thrombin in the presence of FLP. The reaction mixture consisted of I-2 (0.18%) and FLP (0.10%) at room temperature. Thrombin was added and at various timed intervals 0.5 ml of the mixture run on the same 28-cm column of Bio-Gel A-5m. The dashed curve marked $t = 0$ represents the elution profile before thrombin addition. The other two curves shown represent the elution profiles after 2 hr and after 16 hr. (See Materials and Methods for chromatographic details.)

soluble fibrin polymers in the presence of FLP. The reaction mixture contained 0.18% I-8 and 0.19% FLP (see the Materials and Methods section for preparation of the FLP solution). The thrombin endogenous to the FLP mixture was used as the source of clotting enzyme in this experiment. Ten minutes after mixing the FLP and the I-8 solutions a 0.3-ml aliquot was applied to a 28-cm Bio-Gel A-5m column and the gel elution pattern recorded. The elution profile of this 10-min reaction time is shown by the broken line in Figure 4. This curve coincides exactly with the two control curves (0.3 ml of 0.18% I-8 and 0.3 ml of 0.19% FLP, respectively) obtained on the same column. Also, controls were run in which 0.3 ml of 0.18% I-8 were let sit 48 hr at room temperature and then run on the gel column. No changes resulted in the observed elution patterns. Similarly, 0.3 ml of 0.19% FLP solution was held for 48 hr and no change was produced in the subsequent gel elution curves. As a final control, the FLP mixture was incubated with thrombin (0.04 unit/ml) at room temperature overnight. Again, no change in this mixture was apparent by gel chromatography.

An aliquot taken from the I-8-FLP reaction mixture 20 hr after mixing the solutions produced the dramatically altered gel elution pattern shown by the solid curve in Figure 4. Essentially all of the fibrinogen had been converted to high molecular weight species which remained soluble. The elution patterns of these polymeric species show peaks at about 8-, 9-, and 10-ml elution volumes. These data correspond to molecular weights of about 1,400,000, 990,000, and 690,000, according to the graph in Figure 3. The latter figures are representative of tetramers, trimers, and dimers of fibrin monomer. The curves in Figure 4 show that the material which is present in the FLP mixture (fragments D, E, A, B, and C) is not being utilized in the formation of the polymeric species. The tracing of the 20-hour reaction time curve shows no diminution of the area under the FLP portion of the curve. When these same experiments were performed in the presence of hirudin, an irreversible, highly specific thrombin inhibitor (Markwardt and Wolsman, 1958), no polymeric species were formed. Rather, the gel elution pattern of the reaction mixture remained the same as the dashed control curve in Figure 4. This confirmed that the trace of clotting activity in the FLP mixture was, indeed, thrombin.

Purified fragment D was subjected to experimentation similar to the above. Mixtures consisting of fibrinogen I-8 (0.18%) and purified fragment D (0.06%) were let sit overnight at room temperature. Upon gel elution chromatography no changes were observed relative to control curves (obtained on each component separately). However, when these mixtures were treated with thrombin (0.06 unit/ml) overnight the bulk of the fibrinogen was converted to soluble polymers (gelation would occur a few days later). The gel elution patterns showed that the fragment D peak was not diminished, suggesting that no complexing was occurring between the polymerizing fibrin monomer and fragment D. Similar results were obtained with fragment E.

The inability of fibrin monomer from fibrinogen I-8 to bind or complex with any of the FLP proteins was further demonstrated by using thrombin concentrations high enough to cause clotting within a few hours. Reaction mixtures containing equal concentrations of fibrinogen I-8 and FLP (0.2%) were treated with thrombin at a final concentration of 0.6 unit/ml. After 10 min, gel chromatography showed extensive conversion to soluble polymers. After 20 hr firm clotting had occurred. The liquor was squeezed from the clots and subjected to gel chromatography. The gel elution patterns showed that the FLP peaks remained unchanged relative to controls, even though all of the fibrinogen had been clotted and removed from the reaction mixture.

Studies of Fibrinogen I-2 Polymerization by Thrombin in the Presence of the FLP Fragments. In order to determine if fibrinogen I-2 behaved similarly to its more soluble counterpart (I-8) the experiments described above were repeated using low solubility fibrinogen. The fibrinogen was allowed to interact with the FLP mixture in the presence of added thrombin activity. The resulting mixtures of products were analyzed by gel chromatography. The experiments described in Figure 5 utilized reaction mixtures containing 0.18% fibrinogen I-2 and 0.10% FLP. The final thrombin added was 0.002 unit/ml. In Figure 5 the curve labeled $t = 0$ (dashed) represents the gel elution pattern (0.5-ml aliquot) obtained before adding thrombin to the protein mixture. Here, the fibrinogen curve overlaps with the elution of the FLP proteins. The control experiments were similar to those described earlier; solutions of I-2 left at room temperature for 24 hr did not give altered elution curves. The elution curve (0.5-ml aliquot) labeled $t = 2$ hr in Figure 5 shows that by this time the production of soluble fibrin is occurring, with subsequent decrease in the fibrinogen elution peak. The curve marked $t = 16$ hr shows the elution pattern (0.5-ml aliquot) of the reaction mixture at this time. Most of the fibrinogen has been converted to soluble polymers. It appears that the FLP proteins were not involved in the polymerization process, as the areas under the respective peaks seem unchanged.

Conclusive evidence for the lack of participation of fragments D, E, A, B, and C in the observed soluble polymer formation was provided by experiments using ^{125}I-labeled FLP. The radioactive FLP mixture was composed of 0.05 ml of [^{125}I]FLP (as prepared in Materials and Methods) and 1.0 ml of nonradioactive FLP (0.27% protein–0.03% lima bean trypsin inhibitor in Tris (pH 7.6)). The resulting mixture was 0.26% FLP–0.03% lima bean trypsin inhibitor and 0.82 μCi/ml of radioactivity. This solution (0.2 ml) was mixed with 0.2 ml of fibrinogen I-2 (0.3%). Thrombin was added to a final concentration of 0.0025 unit/ml and the reaction let proceed for 20 hr at room temperature. The total reaction mixture (0.4 ml) was then chromatographed on a Bio-Gel A-5m column and the eluted fractions were assayed for Gamma counts along with continuous ultraviolet monitoring. Figure 6 demonstrates the results of these experiments. The ultraviolet elution curve (dashed curve) shows the same phe-

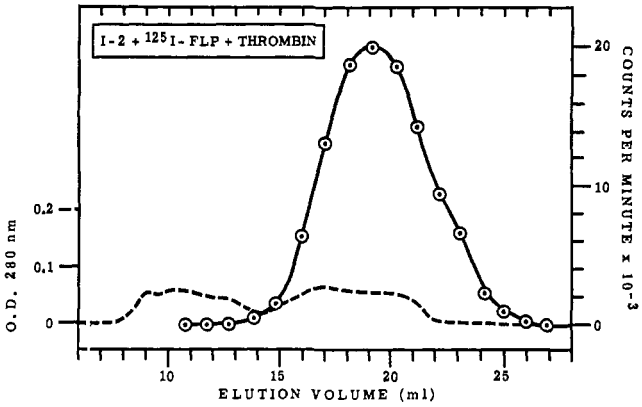

FIGURE 6: The polymerization of fibrinogen I-2 by thrombin in the presence of ^{125}I-labeled FLP. The reaction mixture consisted of I-2 and FLP in a 1:1 ratio. The FLP contained ^{125}I-labeled FLP to a final concentration of 0.4 μCi/ml in the reaction mixture. Thrombin was added to the mixture to a concentration of 0.0025 unit/ml and the reaction let proceed for 20 hr at room temperature. Four-tenths milliliter of the reaction mixture was chromatographed on a 28-cm Bio-Gel A-5m. The solid curve (⊙) shows the elution of radioactivity from the column. The dashed curve represents the optical density at 280 nm elution profile. Fibrinogen I-2, untreated, eluted at 12.5 ml on this column.

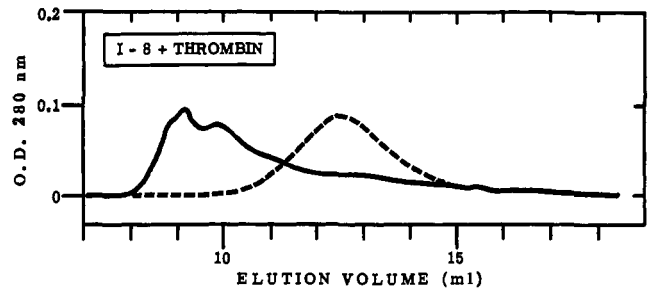

FIGURE 7: Polymerization of fibrinogen I-8 by thrombin alone. The dashed curve shows the control gel elution pattern (28-cm column of Bio-Gel A-5m). Solid line resulted after 20-hr thrombin reaction at room temperature (see text for details).

nomena observed in the earlier experiments. Most of the fibrinogen has been converted to soluble polymers, while the FLP proteins were not changed relative to the controls. However, all of the eluted radioactivity is associated with the FLP proteins, while none of the radioactivity was eluted with the soluble polymers or with any unpolymerized fibrinogen, as shown by the solid curve of Figure 6.

Polymerization of Fibrinogens I-8 and I-2 by Thrombin Alone. Experiments were designed to examine the ability of fibrinogen to form soluble polymers in the absence of the FLP proteins. A typical experiment with fibrinogen I-8 is shown in Figure 7. A solution of I-8 (0.18%) was reacted with thrombin (at a final concentration of 0.06 unit/ml) for 20 hr at room temperature. The solid elution curve in Figure 7 (0.3-ml aliquot) shows that, indeed, essentially all of the fibrinogen has been converted to high molecular weight soluble polymers. The polymeric species produced seem to be mainly dimers and trimers of the parent molecule, as judged by the elution peaks (about 9 and 10 ml) and the graph in Figure 3. The broken line in Figure 7 shows the control curve of fibrinogen I-8 (0.3-ml aliquot) that sat at room temperature for the same amount of time.

Low solubility fibrinogen was similar to fibrinogen I-8, in that soluble polymer formation by thrombin action did not require the presence of the FLP proteins. A typical experiment is illustrated in Figure 8. A solution of fibrinogen I-2 (0.3%) was reacted with thrombin (0.01 unit/ml) at room temperature. At various timed intervals 0.2-ml aliquots were analyzed on a Bio-Gel A-5m column (29 cm). In Figure 8 the gel elution curves of these aliquots are labeled according to their reaction time. The sequential changes show that fibrinogen is depleted while various species of soluble polymers are being produced.

To demonstrate that the soluble polymeric species, which we produced in the above experiments, were soluble fibrin and not the result of some other type of fibrinogen aggregation, an experiment was designed to monitor the N-terminal amino acid changes under these "low thrombin" conditions. Fibrinogen I-2 (0.3%) was allowed to react with thrombin (0.02 unit/ml) at room temperature. At timed intervals (0, 1, 2.5, and 4 hr) 0.2-ml aliquots were taken for gel chromatography and additional 10-ml aliquots were removed for N-terminal amino acid analysis. The protein was precipitated with 10 ml of 70% saturated ammonium sulfate, and the product was washed well with 50% acetone and with water. The resulting products were lyophilized and subjected to quantitative N-terminal amino acid analysis. Table I includes the results from this experiment. At $t = 0$ (before thrombin addition) the only N-terminal groups found were alanine (with aspartic acid from the A-chain variant) and tyrosine in a 1:1 ratio. The gel elution pattern of this material is shown by the dashed curve in Figure 9 and is the same as the control curve of fibrinogen I-2. At $t = 1$ hr, the N-terminal content (from Table I) of the product has changed to include much glycine (alanine:glycine:tyrosine = 1:2.2:2.7). The gel elution pattern of this material, shown in Figure 9 by the solid curve, depicts a high degree of conversion of the fibrinogen to soluble polymers. Planimetry of the gel elution curve indicated the polymer:unpolymerized fibrinogen ratio was about 2:1.

At $t = 2.5$ hr, the clotting mixture was still clear and fluid. However, when this clear liquid was applied to the top of a Bio-Gel A-5m column the solution clotted. Therefore, no elution pattern of this material could be obtained. The N-terminal contents of the products at this stage (Table I) showed that no alanine remained, only glycine and tyrosine, indicating that all of the fibrinogen had been converted to some type of fibrin monomer. The glycine-to-tyrosine ratio of the protein was 1.6:1. At $t = 4$ hr the reaction mixture clotted. The products in this mixture had a glycine-to-tyrosine ratio of 1.7:1, showing a progressive conversion toward completely proteolized fibrin monomer.

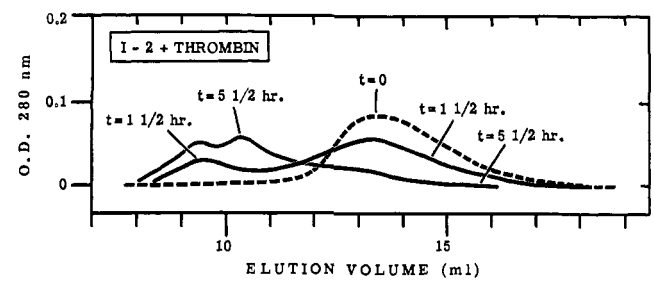

FIGURE 8: The polymerization of fibrinogen I-2 by thrombin alone. The dashed curve shows the control Bio-Gel A-5m gel elution pattern (29 cm column). Solid curves show elution profiles at indicated thrombin reaction times. (See text for details.)

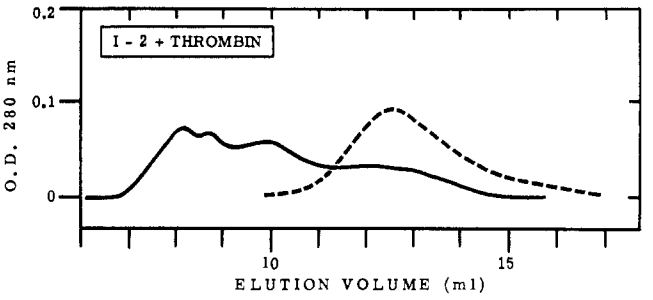

FIGURE 9: The polymerization of fibrinogen I-2 by thrombin. These gel elution patterns (28-cm column of Bio-Gel A-5m) correspond to N-terminal amino acid changes in Table I. The dashed curve represents the elution pattern at $t = 0$ (before thrombin). The solid curve is the elution profile of the aliquot from $t = 1$ hr.

TABLE I: Thrombin Action on Fibrinogen I-2. Gel Chromatography and N-Terminal Group Changes.[f]

Reaction Time (hr)	Gel Elution Pattern	N-Terminal Groups (Molar Ratios)	Fibrin Glycine: Tyrosine
0	Shows the normal[a] fibrinogen single peak	Alanine[b] / Tyrosine = 1/1	
1	Shows about 60–70%[c] soluble polymers	Alanine[d] 1; Glycine 2.2; Tyrosine 2.7	1.3[e]
2.5	Not obtainable; solution clotted when applied to surface of gel column	Glycine / Tyrosine = 1.6/1	1.6
4	Not obtainable; reaction mixture had clotted	Glycine / Tyrosine = 1.7/1	1.7

[a] This pattern is shown by the dashed curve in Figure 9. [b] The alanine content is inclusive of the alanine and aspartic acid (a-chain variant) values. [c] This pattern is shown by the solid curve in Figure 9. [d] No measurable aspartic acid included in the alanine value. [e] Calculated by correcting total tyrosine by subtacting the amount due to unaltered fibrinogen. [f] These data correspond to the experiments described in Figure 9. At the indicated time intervals the 10-ml aliquots of the 0.3% fibrinogen substrate solution yielded about 30 mg of protein upon purification, as described in text. N-Terminal amino acid analysis was performed in duplicate on these products. The data are listed below in terms of the ratios of the groups present. No other end groups were found, even in trace amounts, in the samples. This includes the $t = 0$ aliquot (before thrombin addition) which serves as a control for the N-terminal group assays and for the gel elution behavior.

Discussion

In the experiments presented in this report model systems were constructed containing potentially interacting proteins. The validity of the experimental results are dependent upon the sensitivity with which the reaction mixtures can be analyzed for polymeric (or degradation) products. Equally important to the validity of the results is the extent to which the purity and characteristics of the protein preparations are understood.

The FLP mixtures used in the experiments described above were thoroughly characterized (Figures 1 and 2). The fragments present in the FLP mixture were D, E, A, B, C, and any unknown small molecular weight nondialyzable species. There were no species larger than fragment D in the mixtures. When solutions of FLP were held overnight at room temperature, with or without thrombin, no changes were observed in the gel elution patterns. The purified fibrinogen fractions used (I-2 and I-8) were free from polymeric contaminants and degradation products (Figures 1 and 2). The fibrinogens were at least 99% coagulable by thrombin. Upon standing overnight at room temperature neither polymeric species nor degradation products were observed by gel chromatography. Therefore, the results presented above are not due to artifactual contaminants or from artifacts produced in the protein preparations.

The system of analysis of fibrinogen mixtures for polymeric content by Bio-Gel A-5m gel chromatography is extremely sensitive. For example, a fibrinogen dimer (mol wt 680,000) will elute from a 0.9 × 28 cm column about 2 ml earlier than fibrinogen I-4, as shown in Figure 3. Polymeric species (dimers, trimers, etc.) are easily separable from fibrinogen with this technique in a reproducible fashion.

When fibrinogen I-8 was allowed to interact with the FLP mixture (fragments D, E, A, B, and C) in a 1-to-1 weight ratio, the slight endogeneous thrombin activity of the FLP mixture was sufficient to convert the fibrinogen almost completely to soluble polymers in 20 hr (Figure 4). The areas under the gel elution curves indicated that the FLP proteins had not been diminished. This suggested that the fragments in the FLP mixture were not complexing with fibrin monomer or with fibrinogen in the production of this soluble polymeric material. Similarly, the FLP proteins remained undiminished even when I-8–FLP mixtures were treated with enough exogenous thrombin to result in clotting of the reaction mixture.

When purified fragments D and E were allowed to interact with fibrinogen I-8 for lengthy periods, no polymeric products were formed. We concluded that no soluble complexes were formed between I-8 fibrinogen and fragment D (or fragment E). However, thrombin converts the fibrinogen in these mixtures (fibrinogen to D weight ratio = 3:1) to soluble high molecular weight polymers, provided that the thrombin concentration is sufficiently low. Gel chromatography data indicated that fragment D was not included in the soluble polymeric material. The results of these experiments suggested that the fragments present in the FLP mixtures (fragments D, E, A, B, and C) do not form soluble complexes with I-8 fibrin monomer. Moreover, their presence is not even required for the conversion of I-8 fibrinogen into soluble polymers. It was unexpectedly discovered that fibrinogen I-8 could be completely converted by thrombin (at low enzyme levels) into soluble polymers of fibrin monomers long before gelation occurred (Figure 7).

The polymerization behavior of fibrinogen I-2 is essentially the same as that of I-8. Mixtures of fibrinogen I-2 and FLP will develop soluble polymers, in high yields, upon the action of thrombin. However, the polymers did not appear to include any of the fibrinogenolysis fragments (D, E, A, B, and C) from the FLP mixture (Figure 5). This was confirmed by utilizing ^{125}I-labeled FLP in the same experiments. When this labeled material was present in fibrinogen–thrombin reactions,

no radioactivity was eluted from the gel chromatographic columns in the region of the soluble polymers; rather, all of the radioactivity eluted with the FLP proteins (Figure 6). Therefore, none of the FLP proteins were complexed with the soluble polymers.

Fibrinogen I-2 also proved capable of being converted by thrombin to polymeric forms of fibrin monomer which were soluble under nearly physiological conditions in the absence of fibrinogenolysis fragments and other "inhibitors of fibrin formation" (Figure 8). This heretofore unrecognized feature of fibrinogen–fibrin chemistry must be considered in the evaluation of hypothetical schemes which depict fibrin monomer to be complexing or copolymerizing with some other species.

The production of soluble fibrin polymers from fibrinogen I-2 by thrombin action was accompanied by N-terminal group changes, which showed that the polymeric material is indeed fibrin and not the result of some other type of fibrinogen aggregation phenomenon. Also, the N-terminal changes agree with existing theories of thrombin activity. The release of fibrinopeptide A from fibrinogen by thrombin is much faster than fibrinopeptide B release; consequently, small amounts of thrombin initially release the A peptides preferentially (Copley and Luchini, 1964; Stocker and Straub, 1970). The slow kinetics operative in the experiments described in Figure 9 and Table I favor the production of at least two fibrin monomers: (1) monomer which has lost only the A peptides and (2) monomer which has lost both the A and B peptides.

The N-terminal group changes that accompany the formation of these fibrin monomers are shown in Chart I.

CHART I

$(\text{alanine})_2\text{–}(\text{tyrosine})_2\text{–}(\text{pyroglutamic acid})_2$
Fibrinogen
↓ loss of A fibrinopeptides
$(\text{glycine})_2\text{–}(\text{tyrosine})_2\text{–}(\text{pyroglutamic acid})_2$
Fibrin monomer I
↓ loss of B fibrinopeptides
$(\text{glycine})_2\text{–}(\text{tyrosine})_2\text{–}(\text{glycine})_2$
Fibrin monomer II

The results presented in Table I show that the clotting reaction mixture at t = 1 hr shows an N-terminal group pattern of alanine:glycine:tyrosine = 1:2.2:2.7. This ratio indicates that 37% of the protein is still unaltered fibrinogen.[1] The remaining 63% would then be fibrin molecules, having a glycine:tyrosine ratio of 2.2:1.7 = 1.3. This interpretation is confirmed by the gel elution pattern of this clotting mixture (t = 1 hr) shown by Figure 9. The areas under the elution peaks indicate that about one-third of the protein elutes as unaltered fibrinogen (peak near 12.5 ml); thus, the soluble polymers comprised about 67% of the reaction products at t = 1 hr. The glycine:tyrosine ratios of the clotting mixture at the time intervals shown in Table I demonstrate the presence of fibrin monomer I. At t = 1 hr the glycine:tyrosine ratio was 1.3 (see above). This indicates that 70% of the soluble fibrin molecules still retained their B fibrinopeptides. At t = 2.5 hr the glycine:tyrosine ratio was 1.6 (see Table I), or the percentage of fibrin monomers that possess B peptides was only 40%. By t = 4 hr, at which point the reaction mixture had clotted, 30% of the fibrin monomers were type I (glycine:tyrosine = 1.7). Thus, the clotting mixture progressively develops fibrin monomer II but still contains significant amounts of fibrin monomer I at the physical gelation point. This is in agreement with findings by Abildgaard (1965).

The experimental data presented in this work warrant the following conclusions. (1) The fibrinogenolysis fragments designated as fragments D, E, A, B, and C, present in complete fibrinogen digests, do not form soluble complexes with fibrin monomer or with fibrinogen (either high solubility or low solubility fibrinogen). The purified fragments D and E, likewise, do not form soluble complexes with fibrin or fibrinogen. (2) Soluble fibrin polymers can be produced in high yield, under nearly physiological conditions, simply by the action of thrombin. The presence of other materials, including fibrinogenolysis products, is not necessary. Almost all of the fibrinogen in a clotting reaction mixture can be converted to soluble polymers before gelation occurs, if the system is not overloaded with thrombin.

The apparent composition of these "soluble fibrin polymers" represents a mixture of fibrin monomer I (devoid in only the A fibrinopeptides) and fibrin monomer II (devoid in both the A and B fibrinopeptides) spontaneously aggregated into dimers, trimers, tetramers, and perhaps higher x-mers of the monomer units. The ratio fibrin monomer II:fibrin monomer I becomes larger as thrombin activity continues. The quantitative data presented suggest that fibrinogen is not incorporated into the soluble fibrin polymers, but definitive experiments are necessary to resolve this issue. The important feature of these soluble fibrin polymers is that they can exist for long periods of time and at high concentration under nearly physiological conditions. This presents the possibility of the production and clinical importance of these soluble fibrin polymers *in vivo*. Their production (and hence, existence) requires only a low level of thrombin activity. Recently, Kierulf and Abildgaard (1971) have demonstrated that treatment of human plasma with small amounts of thrombin resulted in the production of a small yield of a soluble fibrinogen-related material bearing N-terminal glycine. The authors could not positively identify the species, but their material and the soluble fibrin polymers produced in our present report have similar properties.

The gradual production *in vivo* of soluble fibrin polymers could play a role in the development of hypercoagulable or prethrombotic states. A sufficiently high blood concentration of these polymers might represent a labile state in which clotting could be initiated by certain provocations (*e.g.*, sudden additional thrombin liberation, platelet aggregation, or stasis). It is also possible that soluble fibrin polymers may appear in the circulation as a result of a local ongoing thrombosis (Fletcher *et al.*, 1970).

The findings in this report have relevance to existing reports and theories in this field.

The defective fibrin polymerization theory (Fletcher, 1970; Alkjaersig *et al.*, 1962; Bang *et al.*, 1962; Fletcher *et al.*, 1966), which involves the binding of fragment D with fibrin monomer, must be reconsidered. Also the data reported by Marder *et al.* (1967) and Marder and Shulman (1969) can be reinterpreted in view of the present findings. These authors observed small amounts of soluble polymers in ultracentrifugal patterns

[1] It can be assumed that the alanine content is due to unaltered fibrinogen and the tyrosine content is proportional to the total amount of all three species. Remember that pyroglutamic acid is not reactive to phenyl isothiocyanate and cannot be removed from the peptide chain for analysis.

obtained with liquors from mixtures of fibrinogen, fragment D (or Y) and thrombin which had partially clotted. As a control the authors reported that fibrinogen with only thrombin, at a concentration of 43 units/ml (Marder and Shulman, 1969) did not yield the high *s* value peaks. They inferred that the soluble high *s* value species observed in their mixtures containing fibrinogenolysis fragments could not have been fibrin polymers, but must have been complexes of fibrin monomer with the respective fragments. These authors did not have the luxury of knowing that a much lower thrombin level would have produced soluble fibrin polymers in their control fibrinogen solution and would not have completely clotted out all of the fibrin. Our data suggests that the polymers these authors observed were soluble fibrin polymers and not complexes of fragment D with fibrin monomer. Apparently, the anti-coagulant property of fragment D resides in its ability to competitively bind thrombin. Whether the same conclusion can be reached about fragment Y (and other nonclottable fibrinogenolysis fragments) will await direct experimental verification. Fragment X (first derivative), however, is clottable itself, and would be expected to participate, in the presence of thrombin, as a type of fibrin monomer in the formation of intermediate soluble polymers.

The findings presented here strongly support the work of Fletcher and Alkjaersig (1971; Fletcher *et al.*, 1970; Alkjaersig *et al.*, 1970), who demonstrated by gel chromatography the presence of high molecular weight polymers in the plasma of patients with hypercoagulability and thrombotic symptoms. The existence of soluble polymers, such as they observed, have been confirmed herewith, *in vitro*, and shown to be easily separable from fibrinogen by Bio-Gel A-5m agarose chromatography.

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