High Molecular Weight Derivatives of Human Fibrinogen Produced by Plasmin

I. PHYSICOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION

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SUMMARY

Two high molecular weight derivatives of fibrinogen (Fragments X and Y) produced by plasmin digestion have been identified by comparative analyses of the digests in agar, agarose, and acrylamide electrophoresis, immunoelectrophoresis, Sephadex G-200 gel filtration, and analytical ultracentrifugation. These fragments were purified by salt precipitation and Sephadex G-200 gel filtration and their physicochemical and immunological properties were defined. Fragment X has a molecular weight of 240,000, as compared with 300,000 for fibrinogen, and is slowly but almost completely (85%) clotted by thrombin. Fragment Y has a molecular weight of 155,000 and cannot be clotted by thrombin. Fragments X and Y can be further digested by plasmin to form the known “end stage” products, Fragments D and E. The molecular weight of Fragment D was found to be 83,000, in agreement with previous reports, while the molecular weight of Fragment E was 50,000, somewhat higher than reported values. These various findings clarify the sequence and stoichiometry of reactions involved in the fragmentation of fibrinogen by plasmin.

Following the early observations that identifiable fragments were formed after the addition of a streptococcal extract to human fibrinogen (1) or after the spontaneous lysis of bovine fibrin (2), the physicochemical (3, 4) and immunological (5) characteristics of these degradation products were defined by Nusenzweig et al. Using DEAE-cellulose column chromatography they separated five “final” fibrinogen degradation products after prolonged lysis by plasmin. These products were labeled Fragments A to E, and the molecular weight of the largest fragment was no greater than 85,000. Derivatives of fibrinogen with molecular weights greater than the final products have been reported (6-8), but the number of these fragments that formed and their relationship to the final fragments have not been clarified.

The present report concerns the characterization of the products formed early in the course of fibrinogen digestion by plasmin. Two intermediate degradation products which are themselves digested by continued plasmin action (9-12) have been studied by electrophoresis in agar, agarose, and acrylamide gels and by immunoelectrophoresis, Sephadex gel elution, and analytical ultracentrifugation. These fragments were purified, and in the accompanying article the mechanism of their anticoagulant action was elucidated (13).

METHODS

Human Fibrinogen—Fibrinogen, KABI, Grade L Chuman, lyophilized, Lots 83119, P4084, and 84059 9E (AB KABI, Stockholm, Sweden) was used in these experiments. It was prepared by the ethanol precipitation method of Blomback and Blomback (14).

The plasminogen content of the fibrinogen preparation was measured by the method of Remmert and Cohen (15) with casein (Lot CASA, Worthington) as the substrate for plasmin (16). One unit of plasmin activity was the amount that released 0.1 μg of tyrosine per min from the casein substrate (16). When 10 mg per ml of fibrinogen were used as substrate instead of casein, 0.65 as much tyrosine was released by a standard plasmin preparation. The plasminogen content of a 1% solution of fibrinogen, measured after activation by streptokinase

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(Varidase, Lederle Laboratorie; 200 units per ml final concentration), was 0.38 unit per ml. The fibrinogen was 95% clottable had an E73% of 15.1 at 280 nm (methods described below). **Fibrinogenolysis**—This was performed at 37° by activating plasminogen present in the fibrinogen preparation (10 mg per ml) with streptokinase (Varidase, 100 units per ml final concentration). The pH of the digestion mixture was 7.0 and did not change during the course of fibrinogenolysis. The reaction was stopped with soybean trypsin inhibitor (Mann and Worthington) at a final concentration of 0.1 mg per ml or e-amino-N-caproic acid (K and K Laboratories) at 0.2 mg final concentration, and samples were quick frozen in an ethanol-Dry Ice mixture and stored at −20° for later testing.

**Antifibrinogen and Antidegradation Products Antisera**—These were prepared in rabbits (6) by three weekly subcutaneous injections of antigen (5, 10, and 10 mg) in complete Freund's adjuvant (Difco), followed 3 weeks later by 12 intravenous injections over a 3-week period of antigen (2.5 to 10 mg) in 0.75% sodium aluminum sulfate. The total amount of antigen injected per animal was approximately 80 mg. One week after the last injection, osalate or citrate plasma was collected, heated to 56° for 30 min to precipitate fibrinogen, and centrifuged at 35,000 × g for 20 min to remove the precipitate. The antisera was adsorbed for 60 min at 23° and then 4 hours at 4°, with 0.20 volume of normal human serum. The precipitate was removed by centrifugation at 35,000 × g for 20 min. Adsorptions were repeated until a precipitate no longer formed and the double diffusion test against normal human serum was negative.

**Electrophoresis in agar gel (Noble agar, Difco)** was performed in agar and agarose gel (Seakem agarose, Lot 962506, Marine Colloid, Inc., Lawsche Instrument Company, Inc., Bethesda, Maryland) (18, 19), double diffusion studies in agar gel (20), and Pevikon (Pevikon C-870, Mercer Chemical Corporation, New York, New York) block electrophoresis (21) were performed according to previously described techniques.

**Sephadex G-200 Gel (Pharmacia) Filtration**—This was performed at 21° as described by Flodin (22), with an eluting solution of 1.0 M NaCl, 0.025 M Tris-hydrochloric acid buffer at pH 7.4, 0.025 M sodium citrate to prevent clotting, and soybean trypsin inhibitor (0.1 mg per ml) plus 0.2 M e-aminocaproic acid to prevent proteolysis of the digest during elution. For analytical studies, the column size was 2.5 × 80 cm, flow rate was 10 to 15 ml per hour, sample size was 4.0 ml (10 mg per ml), and eluate aliquots were 3.0 ml. The optical density of the eluates was measured in a Beckman DU spectrophotometer at 280 nm.

**Purification**—To purify large amounts of fibrinogen degradation products formed early in the course of digestion (Fragments X and Y as labeled in the text), 1 to 1.5 g of the plasmin digests were passed through Sephadex G-200 gels in a column, 6 × 100 cm, with a flow rate of 25 to 30 ml per hour, other conditions being the same as above. Eluates were concentrated by ultrafiltration or precipitation with ammonium sulfate at 35% saturation. After three elutions Fragments X and Y were >95% pure.

Fragments D and E were prepared by Pevikon block electrophoresis of the final plasmin digestion products, after an initial separation from the small digestion Fragments A, B, and C (4) by precipitation with ammonium sulfate at 50% saturation. Fragment E migrated farther toward the anode than Fragment D. After electrophoresis for 24 hours at 10 volts per cm at 4° each fragment was eluted with the 0.05 M barbital buffer at pH 8.2 and concentrated by ultrafiltration.

**Clotting with Thrombin and Heat Precipitation**—The per cent total protein of preparations of fibrinogen and degradation products that was clotted by thrombin (topical, bovine origin; Parke, Davis) or precipitated by heating at 56° for 30 min was calculated on the basis of optical density at 280 nm before and after clotting or precipitation and removal of insoluble material by centrifugation. The results were corrected for the optical density of soybean trypsin inhibitor which remained in solution after clotting or heating at 56°.

**Extinction Coefficients—**E1% at 280 nm of fibrinogen and the degradation products were determined by micro-Kjeldahl digestion with a factor of 6.25 to convert nitrogen to protein. Degradation products were precipitated with ammonium sulfate at 35% final saturation, dissolved in 0.15 M sodium chloride containing 0.0025 M sodium bicarbonate buffer at pH 10.0, and dialyzed extensively against this buffer, to obtain preparations free of soybean trypsin inhibitor and e-aminocaproic acid.

**Sedimentation Velocity Determinations**—These were performed at 25° at 60,000 rpm in a Spinco model E ultracentrifuge, with a model AN-D rotor with Tetlon center pieces. Unless noted, all samples were dialyzed against a solution containing 1.0 M sodium chloride, 0.025 M Tris-hydrochloric acid buffer at pH 7.4, 0.025 M sodium citrate, and 0.2 M e-aminocaproic acid. The mean viscosity of six dialyzates, as measured in a Ubbelohde viscometer with a water outflow time of 98 sec, was 1.286 with a standard deviation of 0.004.

**Diffusion Constants**—These were determined by a method of free diffusion, as followed by Rayleigh interference fringes, and measured according to the technique of Longsworth (25). The diffusion measurements for fibrinogen and Fragment X were carried out in the Amicon model B diffusion apparatus, while those for Fragments Y, D, and E were performed at 4000 rpm in the double sector synthetic boundary cell of the Spinco model E ultracentrifuge. Analysis for concentration dependence was made by the fringe deviation procedure suggested by Creeth (24).

The molecular weight of each fragment was calculated according to Svedberg's equation (25).

\[
\frac{S_{0.2}}{D_{w,s}} = \frac{1}{(1 - \frac{V}{V_w})}
\]

where R is measured in ergs °K⁻¹ mol⁻¹, V was assumed to be 0.718 (26), the diffusion and sedimentation constants were cor-
rected to a solvent with the density and viscosity of water at 25°, and the corrected sedimentation and diffusion constants were extrapolated to null concentration.

**RESULTS**

When fibrinogen was digested by plasmin, a reproducible sequence of molecular changes occurred that could be characterized by the following physicochemical and immunological techniques.

**Agar Gel Electrophoresis**—The pattern of progressive digestion of fibrinogen by plasmin are shown in Fig. 1. Fibrinogen entered the gel poorly, and remained at the origin. At 3 min under conditions of digestion described under "Methods," almost all of the material shifted from the origin toward the cathode, reflecting the conversion of fibrinogen to the first intermediate degradation product, which has been labeled Fragment X (9, 11). At 10 min, Fragment D (4) was seen as a light spot closer to the
cathode than Fragment X, and the reappearance of material at the origin in the same electrophoretic position as fibrinogen was due to a second intermediate degradation product which has been labeled Fragment Y (9, 11). Fragment Y had electrophoretic properties of fibrinogen in agar gel but is actually much smaller, as is shown below. At 15 and 20 min of digestion, Fragment X decreased in amount and Fragments D and Y increased. At 30 min, Fragment X was barely visible and Fragment Y also had decreased; Fragment D was the major degradation product and Fragment E (4) was present near the anode. At 45 min, there was a further decrease of Y and an increase in D and E. After 16 hours of digestion (not shown), Fragment D moved slightly less toward the cathode and Fragments X and Y were absent. Further addition of plasmin did not change the character or position of the degradation products.

The rate of fibrinogen degradation was increased by adding human plasmin (1.4 to 2.1 units per ml final concentration) instead of streptokinase to human fibrinogen and decreased by lowering the pH of the reaction mixture to 6.0 or raising it to 9.0. However, the same sequence of electrophoretic patterns always appeared at a rate proportional to the rate of digestion.

**FIG. 1.** Agar gel electrophoretic patterns of fibrinogen digests. Fibrinogenolysis was carried out as noted under “Methods”; reaction was stopped at times noted by the addition of soybean trypsin inhibitor at a final concentration of 0.1 mg per ml. Origin indicated by arrow at the top of the figure; cathode at the left, anode at the right. The relative electrophoretic positions of Fragments D, X, Y, and E are shown by the arrows at the bottom of the figure.

**FIG. 2.** Sephadex G-200 gel elution of fibrinogen digests. Enzymatic digestion was terminated at the times noted by the addition of soybean trypsin inhibitor (final concentration 0.1 mg per ml) and 2-aminocaproic acid (0.2 mg/ml final concentration) to the digestion mixture. All samples were eluted from the same column, the void volume of which was 115 ml as determined by the elution of a dextran blue marker (mol wt >2,000,000). Elution of normal human serum from this column showed the macroglobulin peak at 125 ml, 7 S globulins at 180 ml, and albumin at 230 ml.

**Gel Filtration**—Filtration of the same digests shown in Fig. 1 through Sephadex G-200 gel gave two early high molecular weight degradation products of fibrinogen, as well as the known lower molecular weight final products (Fig. 2). Fibrinogen eluted as a homogenous peak at 130 ml just after the appearance of the dextran blue marker (Lot 7759, Pharmacia). The 3-min and 10-min digestion products, which no longer had fibrinogen characteristics on agar gel (see Fig. 1), eluted primarily as a major peak at 140 ml, slightly later than fibrinogen. At 15 min, new peaks were distinguished at elution volumes of 160 and 210 ml and, at 20 min, these peaks were further increased at the expense of the peak at 140 ml.

The peaks at 140 and 160 ml, which contained Fragments X and Y, respectively, as shown by agar gel electrophoresis (Fig. 3), disappeared completely by 120 min. At the same time, the peak at 210 ml, which was almost entirely composed of Fragment D at 20 min (Fig. 3), increased. This peak also contained small amounts of Fragments A, B, C, and E, which were visible only after removing Fragment D by precipitation at 56° and concentrating the supernatant solution 10-fold by ultrafiltration. The peak at 270 ml, which appeared distinctly at 15 min and increased slightly by 120 min, contained small polypeptides.

**Immunoelectrophoresis**—The immunoelectrophoretic patterns of the plasmin digests in agar and agarose gels are shown in Fig. 4. Fibrinogen remained near the origin. The single precipitin arc of the 3-min digest, representing the initial degradation of fibrinogen to Fragment X, was closer to the cathode in agar and slightly closer to the anode in agarose. The relative positions of the different fragments formed on further digestion were
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FIG. 3. Agar gel electrophoresis of peaks eluted from Sephadex G-200 gel. Arrow at the top denotes the origin, cathode at left, anode at right. The eluates of the 20-min digest (see Fig. 2) were concentrated by ultrafiltration or precipitation with ammonium sulfate at 50% saturation. Peak 1 is the Sephadex G-200 eluate at 140 ml (Fig. 2), Peak 2 the eluate at 160 ml, and Peak 3 at 210 ml. The electrophoretic positions of Fragments A, B, and C corresponded to those of D, X, and Y, respectively (9). Fragments A, B, C, and E were not detectable in Peak 3 until Fragment D was removed by precipitation at 56° and the supernatant was concentrated approximately 10-fold. The small polypeptide units in the peak at 270 ml were not demonstrable under the conditions of this experiment.

The immunoelectrophoretic positions of the individual degradation fragments, purified as described under "Methods," are in Fig. 5, which also shows the degree of purity obtained. Whereas Fragment Y was indistinguishable from fibrinogen in agar gel, its position in agarose gel was clearly closer to the anode.

Acrylamide Gel Electrophoresis—The patterns of fibrinogen digests in acrylamide (Fig. 6) showed a sequence of changes that was similar to that noted in agar and agarose gels. Undigested fibrinogen penetrated the gel only slightly. The 10-min digest showed two distinct bands; with continued digestion, the band nearer to the cathode disappeared and the distal band intensified. At 60 min, only the distal band persisted, and a new spot was present near the brom phenol blue marker. By comparing patterns of crude digests (shown on the left) with results obtained with partially purified fragments (shown on the right), it was apparent that in the 10-min digest the band nearer the cathode represented Fragment X, while the distal band contained both Fragments Y and D. The anodal spot in the 60-min digest was Fragment E.

Sedimentation Velocity—Fig. 7 shows the extrapolations to null concentration of the corrected sedimentation constants of fibrinogen and its purified digestion products. Individual determinations of sedimentation values of fibrinogen and Fragment X overlapped to some extent at all but the lowest concentrations. The $s_{20,w}$ value obtained for fibrinogen was 8.34, and for Fragment X, 7.90. The observed sedimentation constants of Fragment Y were clearly higher than those of Fragment D at all concentrations. The $s_{41, w}$ of Fragment Y was 6.47, while that of Fragment D was 5.54. Fragment E had an $s_{41, w}$ of 3.3.

Ultracentrifugal patterns of crude digests shown in Fig. 8 could be interpreted in light of the sedimentation constants of
FIG. 5. Immunoelectrophoresis of fibrinogen and Fragments X, Y, D, and E in agar and agarose gels. Experimental conditions as noted in Fig. 4. One microliter of antigen was applied to the wells at the following concentrations: fibrinogen, 10 mg per ml; Fragment X, 7.5 mg per ml; Fragment Y, 4 mg per ml; Fragment D, 4 mg per ml; Fragment E, 55 mg per ml. The troughs contained 75 μl of rabbit anti-human fibrinogen antiserum (NIH No. 60).

FIG. 6. Acrylamide gel electrophoresis of fibrinogen (FIBR.) digests and purified degradation fragments. Cathode at top, anode at bottom; migration time 30 min at 5 ma per tube. Acrylamide gel at 7.5%; buffer, 0.0125 M Tris-glycine at pH 8.3 in the gels, and 0.025 M in the reservoirs. The concentration of fibrinogen and the digests was 5 mg per ml; Fragment X, 2.1 mg per ml; Fragment Y, 4 mg per ml; Fragment D, 4 mg per ml; and Fragment E, 2.1 mg per ml. Arrow denotes brom phenol blue marker.

FIG. 7. Sedimentation constants of fibrinogen and purified fragments. The data presented were obtained from three preparations or more of fibrinogen (■—■), and Fragments X (○—○) and Y (□—□) and from two different samples of Fragments D (□—□) and E (▲—▲). The regression lines were calculated by a least squares analysis (28) of the observed data and drawn between theoretical points of null and 1% concentrations (circled symbols). The slopes of the regression lines were -1.4 for fibrinogen, -1.09 for Fragment X, -0.79 for Fragment Y, -0.62 for Fragment D, and -0.02 for Fragment E. The respective coefficients of correlation for these lines were -0.97, -0.91, -0.68, -0.02, and -0.12, with values closer to -1.0 reflecting better agreement of the observed points to the calculated slope (28). The value for Fragment E was of little significance, as the slope of this regression line was virtually zero.

FIG. 8. Ultracentrifugal analysis of fibrinogen and the fibrinogen digests. Sample concentration was 10 mg per ml, pictures were taken after the indicated times (t) at 60,000 rpm, and peaks moved from left to right in each cell. The crude digests used were the same as those shown in Figs. 1 and 2.

the purified fragments shown in Fig. 7. Fibrinogen descended as a homogeneous peak contaminated with a small amount of heavier material which was seen in all subsequent digests (see arrow, left upper panel). During digestion, four major peaks were distinguished. A slow peak, representing the minor degradation Fragments A, B, and C, remained essentially constant in all digests. The most rapidly descending peak, representing Fragment X, decreased as digestion continued, and was not seen.
after the 30-min digest. A peak containing both Fragments D and Y was first visible as a small shoulder on the trailing end of the Fragment X peak in the 10-min digest. The D and Y components were individually distinguished only after prolonged centrifugation (t = 121 min, 20-min digest). With continued digestion, Fragment Y in the “D, Y” peak progressively decreased and, in the 45-min digest, this peak was almost entirely undetectable in the 20-min digest (t = 121 min), after which it increased in prominence.

**Molecular Weight and Other Properties**—Table I summarizes results of the diffusion studies on fibrinogen and the various fragments at 25°C. The value for the diffusion coefficient of human fibrinogen of 2.43 × 10⁻⁹ cm² sec⁻¹ was calculated from the data of Caspary and Kekwick (29) from determinations at 20°C. There was no evidence for a markedly increased diffusion at concentrations below 0.1%. Fringe deviations were present only in Fragment X diffusion measurements, indicating slight heterogeneity of this fragment, but there was no evidence of heterogeneity for fibrinogen or Fragments Y, D, and E. The K₁ and the diffusion coefficient for Fragment X represent average values for the heterogeneous mixture; hence the molecular weight value in Table II may be low for pure Fragment X.

**Table II**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>D₂</th>
<th>K₁</th>
<th>D₁ · e⁻²</th>
<th>Amount clotted by thrombin</th>
<th>Precipitated at 56°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>5200 µg/ml</td>
<td>2.24</td>
<td>-0.22</td>
<td>2.43</td>
<td>95</td>
<td>+</td>
</tr>
<tr>
<td>Fragment X</td>
<td>7.90</td>
<td>240,000</td>
<td>15.1</td>
<td>95</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Fragment Y</td>
<td>6.47</td>
<td>155,000</td>
<td>17.6</td>
<td>95</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Fragment D</td>
<td>5.54</td>
<td>85,000</td>
<td>20.8</td>
<td>95</td>
<td>+</td>
<td></td>
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<tr>
<td>Fragment E</td>
<td>3.30</td>
<td>50,000</td>
<td>10.2</td>
<td>95</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

a Diffusion constant at infinite dilution calculated from

\[ D₀ = D₁ [1 + K₁(e⁻²)] \]

where K₁ is determined according to Creeth (24) from the Rayleigh fringe deviation patterns.

**Table II**

<table>
<thead>
<tr>
<th>Component</th>
<th>Molecular weight</th>
<th>E₁% cm⁻¹</th>
<th>Amount clotted by thrombin</th>
<th>Precipitated at 56°C</th>
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<td>Fibrinogen</td>
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<td>Fragment E</td>
<td>50,000</td>
<td>10.2</td>
<td>95</td>
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</tbody>
</table>

**Immunological Analysis**—The antigenic relationship between fibrinogen and Fragments X, Y, D, and E is illustrated in the double diffusion study with rabbit anti-human fibrinogen antiserum shown in Fig. 9. Fibrinogen spurred over both Fragments X and Y (upper arrows), more so with Y, and both Fragments X and Y spurred over the line containing Fragments D plus E (bottom arrows). The precipitin arcs of Fragments X and Y were continuous, with this and 15 other rabbit anti-human fibrinogen antisera.

These differences in antigenic content were corroborated by double diffusion studies with adsorbed antisera (Fig. 10). After adsorption with an excess of Fragments D plus E (upper left panel), the antiserum still formed precipitin lines with fibrinogen and Fragments X and Y. The fibrinogen line spurred over (see arrow) the fainter line of Fragment X, which was heavier than but continuous with that of Fragment Y. Antiserum adsorbed with an excess of Fragment Y (upper right panel) did not precipitate with Fragments X, Y, D, or E, but did form a precipitin line with fibrinogen (see arrow). This line was lighter than that formed between fibrinogen and unadsorbed antiserum (top well). Antiserum adsorbed with Fragment X (lower left panel) reacted even less with fibrinogen (see arrow) than did the antiserum adsorbed with Fragment Y (see arrow, upper right panel), and antiserum adsorbed with native fibrinogen (lower right panel) did not react with fibrinogen or any of the degradation products. Fifteen other rabbit anti-fibrinogen antisera also failed to react with Fragment X.
with any of the degradation products after prior adsorption with fibrinogen.

**DISCUSSION**

Although the final plasmin digestion products of fibrinogen were defined and characterized 7 years ago (3-5), the intermediate degradation products have only recently been appreciated as distinct molecular entities (7-12). This is primarily due to the transient presence of the intermediate products during digestion and to an overlap of certain physical, electrophoretic, and immunological properties of intermediate products with those of fibrinogen and final products. Specifically, the first intermediate product, Fragment X, has properties that are similar to fibrinogen in the ultracentrifuge (Fig. 7) and in Sephadex G-200 gel (Fig. 2), but it is distinguished from fibrinogen by its characteristic mobility in agar gel (Fig. 1) and acrylamide gel (Fig. 6). The second intermediate product, Fragment Y, has the same electrophoretic position as fibrinogen in agar gel (Fig. 1) but resembles Fragment D in agarose (Fig. 5) and acrylamide gels (Fig. 6). Fragment Y can best be distinguished by its characteristic elution from Sephadex G-200 gel (Fig. 2).

On the basis of the early changes observed in plasmin digests (Figs. 1 and 4), it appears that fibrinogen is first digested to Fragment X. The appearance of this large molecular weight intermediate product probably was noted first by Schwick et al. (6) in 1963, when they separated fibrinogen digests by Sephadex G-100 gel filtration and found a product which had an $s_{20,w}$ of 7.1, compared to 7.6 for undigested fibrinogen. Larrieu, Marder, and Inceman (9) in 1965 noted two previously unidentified fibrinogen digestion products in agar gel electrophoretic and immunoelectrophoretic patterns and labeled them Fragments X and Y. They showed that both were precipitable by heating at 56°C and possessed potent anticoagulant effects. Studies preliminary to the present report (10, 11) showed that purified Fragment X had a sedimentation constant that was slightly less than that of fibrinogen (30). In 1966, Fletcher et al. (7) identified a “fibrinogen first derivative” in early plasmin digests of fibrinogen which had a molecular weight of 265,000 and was slowly but completely clottable by thrombin. In 1968, Mosesson et al. (8) isolated a “high solubility fibrinogen” (Fraction I-8) from outdated human ACD plasma which had a molecular weight of 265,000 and was slowly but completely clottable by thrombin. This was similar to a “high solubility” fragment (I-8D) isolated from early plasmin digests of fibrinogen by Sherman, Mosesson, and Sherry (31). The calculated molecular weights of Fragment X, fibrinogen first derivative, and Fractions I-8 and I-8D are in close agreement, suggesting that they describe the same intermediate degradation product. The slightly lower molecular weights noted for Fragment X and for human fibrinogen in the present study may reflect the correction of observed diffusion coefficients to null concentration (see Table 1). All four high molecular weight derivatives clot more slowly with thrombin than does fibrinogen, and the small differences in
the amount of material that can be clotted may reflect simply the degree of purity of the original fibrinogen preparation.

The second intermediate product, Fragment Y, was first identified by agar gel electrophoresis (9), and certain of its physicochemical and immunological characteristics have been noted in preliminary reports (10, 11). Clear-cut purification from mixtures of digestion fragments is best obtained by Sephadex G-300 gel elution (Fig. 2). The molecular weight of 155,000 is intermediate between that of Fragments X and D (Table II). Fragment Y is not clottable by thrombin but can be precipitated by heating at 56° for 30 min.

On the basis of acrylamide gel electrophoresis and ultracentrifuge studies, Fletcher et al. (7) and Fletcher and Alkjaersig (32) concluded that a variety of closely related fibrinogen intermediates were derived from the first fibrinogen derivative. These secondary derivatives were described as a "distinct ultracentrifugal entity (s20,w approximately 5.7)" and were found to be partially clottable by thrombin. Fisher et al. (33) have since postulated on the basis of electrophoresis in 15% acrylamide gel that intermediate products could be divided into "early intermediates" of approximately 250,000 to 150,000 molecular weight and "late intermediates" of less than 150,000 molecular weight. In contrast, the present work shows that only one nonclottable degradation product, Fragment Y, is intermediate in size between the first fibrinogen derivative, Fragment X (mol wt 240,000), and the final digestion products (mol wt 83,000 or less).

Most of the observations of Fletcher et al. (7) noted above can be explained by findings of the present study. The heterogeneous ultracentrifugal peak of 5.7 s20,w seen in partially digested fibrinogen probably reflects the dual contributions of both Fragments Y and D, as shown in Fig. 8 (20-min digest, t = 121 min). With continued digestion of Fragment Y, this peak becomes more homogeneous, and the S value approaches that of pure Fragment D (Fig. 8, 45 min). The partial clottability of intermediate products purified by Sephadex G-150 gel filtration of incompletely digested fibrinogen (7) can be explained by contamination of the sample with clottable Fragment X (Figs. 1 and 2, and Reference 13), which is not adequately separated from Fragment Y by elution from this gel. The broad range of molecular weights reported for the intermediates by Fisher et al. (33) was based on the electrophoretic mobilities in acrylamide gels and not on direct physical measurements of purified degradation products. In addition, others have shown that the multiple bands seen after electrophoresis of digest in starch or acrylamide gels and not on direct physical measurements of purified degradation products. In addition, others have shown that the multiple bands seen after electrophoresis of digest in starch or acrylamide (34-36) represent heterogeneity of the Fragment D molecule, and do not reflect a multiplicity of intermediate products. There is at present no evidence for even minor heterogeneity of Fragment Y.

The antigenic determinants of Fragment Y closely resemble those present on Fragment X and may be only quantitatively different (Figs. 9 and 10). Both X and Y contain determinants in excess of those present on Fragments D plus E (Figs. 9 and 10), and could be termed the X-Y determinants. Barring the detection of "hidden" determinants (37), fibrinogen contains all of the antigenic material present on Fragments D, E, and X-Y. In addition, fibrinogen appears to possess a fourth antigenic group which is missing from X-Y, and is shown in the panels on the left in Fig. 10 (arrows).

Since the rate of digestion of fibrinogen varied according to experimental conditions, digests of identical incubation times may differ considerably in their content of digestion products and in their consequent physiological properties. For example, Triantaphyllopoulos (38) noted the maximal anticoagulant effect in fibrinogen digests at 48 hours, whereas maximal anticoagulant effect was found by Niewiarowski and Kowalski after 10-min incubation (39), and in the present study after 20-min incubation (13). Regardless of the rate of digestion, however, the same intermediate and final degradation products always appear in exactly the same sequence. Thus the degree of digestion can be sensitively by the presence or absence of specific intermediate products, and the course of fibrinogen degradation can be divided into three arbitrary stages (11). During the first stage, Fragment X but not Y is present; during the second, both X and Y are present; and during the third or final stage, X and Y are absent. This guide to degree of digestion can be used to compare digests formed under different conditions, both in vivo and in vitro, with regard to their anticoagulant activity (9, 11, 13) or other physiological effects.

Schemes describing the molecular fragmentation of fibrinogen by plasmin have been proposed, based primarily upon calculations of molecular weight and final concentrations of Fragments D and E (40, 41). In view of the present study, a fragmentation scheme must account for complete conversion of fibrinogen to Fragment X before Fragments Y, D, or E appear (Figs. 1 and 4), the presence of antigenic determinants of Fragments D and E in both Fragments X and Y; the appearance of Fragment F, concomitant with decreases in Fragment Y concentration (Fig. 1); the final yield of Fragment D amounting to 50 to 55% and of E to 15 to 20% of the amount of fibrinogen from which they were derived; and the molecular weights of Fragments X, Y, D, and E as listed in Table I. The molecular weight of Fragment X excludes the possibility of 4 Fragment D molecules (40) derived from fibrinogen, and the existence of Fragment Y further limits the number of D molecules to 2. The initial conversion of fibrinogen to Fragment X precludes the possibility that fibrinogen is initially split in half by plasmin (41), and makes it unlikely that Fragment Y represents the units of a fibrinogen dimer that have been proposed (42). Thus, fibrinogen (mol wt 300,000) appears to lose approximately 20% of its mass as the minor Fragments A, B, and C (4) when it is converted to Fragment X (mol wt 240,000). Fragment X may then be split unevenly into 1 Fragment Y (mol wt 155,000) and 1 Fragment D molecule (mol wt 83,000), following which Fragment Y appears to split into a 2nd Fragment D molecule and a single Fragment E molecule (mol wt 50,000). The molecular weight of Fragment E limits the number of Fragment E molecules derived from each Y molecule to 1. The heterogeneity of Fragment D described by Jamieson and Gaffney (34, 35) and by Nilén (36) is consistent with the scheme (11) which suggests that the Fragment D molecules are derived from different portions of the fibrinogen molecule.

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