The Effect of Plasmin on the Subunit Structure of Human Fibrinogen*

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SUMMARY

The changes in the subunit structure of human fibrinogen during plasmin digestion have been followed sequentially by examining the degraded products electrophoretically in sodium dodecyl sulfate on polyacrylamide gels in the absence or presence of mercaptoethanol. On the basis of the molecular weights of the products, their carbohydrate content, and the relative amounts of each product present during digestion, it has been possible to deduce the sequence of structural changes in fibrinogen during digestion and the polypeptide chain composition of the major transient and terminal digestion products.

The α chains, which contain no detectable carbohydrate, were the first subunits in fibrinogen to be degraded by plasmin. The β chains, which contain carbohydrate, were degraded more slowly than α chains. The initial, but transient, degradation product, Fragment X, was structurally heterogeneous. Forms appearing early in the digestion contained extensively degraded α chains and intact β and γ chains. The next transient fragment formed contained extensively degraded α chains and partially degraded β chains. The γ chains of fibrinogen, which also contain carbohydrate, were more resistant than α and β chains, but on prolonged digestion, were cleaved by plasmin. As γ chains begin to be degraded, and the α and β chains are further degraded, other forms of Fragment X appear along with Fragments D and Y. On the basis of this sequence of structural changes it has been possible to deduce the general subunit structure of the major transient and terminal digestion products. The ability of partially degraded fibrinogen to form visible clots on treatment with thrombin- and fibrin-stabilizing factor has also been examined. Digestion products with extensively degraded α chains retain the ability to form fibrin-like clots, although as these species are degraded further by digestion of their β and γ chains, the ability to clot is lost.

Human fibrinogen has a molecular weight of 340,000 and consists of three pairs of subunit polypeptides, called the α, β, and γ chains (1–5). These chains are held together by several disulfide bonds (3–5), many of which are believed to be located near the amino-terminal end of the molecule (6). The proteolysis of fibrinogen by plasmin has been studied in a number of laboratories (7–23). The final digestion products, which have been designated as Fragments A, B, C, D, and E, have molecular weights of 85,000 or less (8–10, 17) although fragments of large molecular weight have been shown to be formed transient (11–18). Early in the course of fibrinogen digestion by plasmin Fragments A, B, and C appear (13) in addition to a large fragment with a molecular weight of about 240,000 to 265,000 (12, 17). This species, called Fragment X (17), has been shown to form a fibrin-like clot, although the rate of clotting is slow than for undigested fibrinogen (12, 17). Further degradation of the fibrinogen molecule yields a nonclottable fragment with molecular weight of about 155,000 called Fragment Y (17).

The subunit structure of degraded fibrinogen has been examined recently in several laboratories (19–21, 23) by means of electrophoresis on acrylamide gels in sodium dodecyl sulfate (24, 25), a technique of particular value in the study of the subunit structure of fibrinogen and fibrin (26). We wish to report here further studies on the plasminolysis of human fibrinogen by means of this electrophoretic method. On the basis of the results it has been possible to determine where plasmin acts on the subunits of fibrinogen and to ascertain the polypeptide chain composition of the major plasmin digestion products. A reaction sequence for plasminolysis is proposed which takes into account the established structural features of the fibrinogen molecule and its degradation products and the known order of degradation of the fibrinogen polypeptide chains (19, 20, 23).

EXPERIMENTAL PROCEDURE

Reagents—Human fibrinogen, 97% clottable, was prepared by the method of Blombäck and Blombäck (27) from fresh froz...
plasma. This fibrinogen contained sufficient fibrin-stabilizing factor as a contaminant to catalyze complete crosslinking during fibrin formation (26) and even after plasmin digestion. Human urokinase (Calbiochem) was dissolved in 0.2 M sodium phosphate buffer, pH 7.2, to give a final concentration of 400 Ploug units per ml and stored at \(-20^\circ\). This solution was used for the activation of plasminogen preparations. Bovine thrombin (The Upjohn Company) was dissolved in 0.075 M sodium chloride containing 50% glycerol to give a concentration of 500 NIH units per ml and stored at \(-20^\circ\). This solution was used to determine thrombin clotting times on aliquots removed from the fibrinogen-plasmin digestion mixture. Hammarsten quality casein (Nutritional Biochemicals) was heat treated and precipitated by perchloric acid (28) and stored at \(-20^\circ\) as a 4% solution for use in caseinolytic assays. Streptokinase (Lederle Laboratories) was dissolved in distilled water to a final concentration of 1000 Ploug units per ml and stored at \(-20^\circ\). This solution was used for the activation of plasminogen in the caseinolytic assays. Carrier ampholytes, ranging from pH 3 to 10, were obtained from LKB. All other reagents used in this study were analytical reagent grade unless stated otherwise.

**Plasminogen Purification**—Partially purified plasminogen was prepared from 100 g of Cohn Fraction III with the use of DEAE-cellulose chromatography according to the method of Wallen and Bergstrom (29). It was assayed by a modification (30) of the method of Remmert and Cohen (31). The activities of chromatographic samples were expressed in caseinolytic units. The fractions which contained the major portion of caseinolytic activity were pooled and lyophilized.

Further purification of plasminogen was achieved by dissolving the lyophilized sample in 5 ml of 0.04 M ammonium acetate buffer, pH 9.0, centrifuging at 2000 rpm for 15 min at 4\(^\circ\) to remove small amounts of insoluble material and then applying the supernatant solution, which contained about 525 mg of protein (460 CTA units of plasminogen activity) to a Sephadex G-100 column (95 x 2 cm). The column was eluted at 4\(^\circ\) with 0.04 M ammonium acetate buffer, pH 9.0, and 5-ml fractions were collected. Two major peaks were eluted, the second of which contained highly purified plasminogen. The second peak was pooled and dialyzed against four changes of distilled water in a ratio of 1 part sample to 50 parts water. The sample was concentrated by lyophilization, then dissolved in 0.2 M sodium phosphate buffer, pH 7.2, to give a concentration of 8 mg per ml and stored at \(-20^\circ\). This preparation showed a single band on polyacrylamide gel electrophoresis at pH 4.3 (32) and on SDS gel electrophoresis (24, 25). It also behaved as a single species on isoelectric focusing by the method of Hayes and Wellner (33), which was modified by reducing the riboflavin concentration during polymerization from 0.0015% to 0.0005%, since polymerization failed to occur at the higher concentration. This preparation of human plasminogen contained 9.7 CTA units per absorbance unit when assayed against reference plasmin (American National Red Cross), which was comparable to the very high specific activity of the preparation reported by Deutsch and Mertz (34). The molecular weight for reduced plasminogen by the SDS 5% polyacrylamide gel electrophoretic method was 82,000. Plasmin, prepared by activating 10 \(\mu\)g of plasminogen with 18 Ploug units of urokinase, showed two bands with molecular weights of 59,000 and 27,000 after reduction.

1 The abbreviation used is: SDS, sodium dodecyl sulfate.

These values are in good agreement with previously reported molecular weights obtained by ultracentrifugation (35, 36).

**Plasminogen Digestion with Plasmin**—Purified human fibrinogen was dissolved in 0.05 M Tris-HCl, pH 7.4, containing 0.15 M sodium chloride to give a final concentration of 5 mg per ml and cooled to 0\(^\circ\). Human plasminogen (10 \(\mu\)l; 8.0 mg per ml) and urokinase (20 \(\mu\)l; 0.5 mg per ml) were added to give a final concentration of 0.08 mg per ml of plasminogen and 7.4 Ploug units per ml of urokinase. Immediately after the urokinase solution was added and while the incubation mixture remained at 0\(^\circ\), samples were removed for analysis as described below. The digestion mixture was then incubated at 37\(^\circ\), and samples were removed at intervals to determine the thrombin time (0.2 ml) and for analysis by electrophoresis on polyacrylamide gels in SDS (0.5 ml). For the gel analyses, protein was precipitated immediately by adding samples of the digest to an equal volume of 8 M urea containing 2% SDS. One-half of this sample was reduced by the addition of \(\beta\)-mercaptoethanol to give a 1% final concentration. Both the nonreduced and reduced samples were then incubated at 37\(^\circ\) for 20 hours before analysis on the gels.

**Determination of Thrombin Time and Preparation of Fibrin from Plasmin-degraded Fibrinogen**—Thrombin times were determined throughout plasmin digestion of fibrinogen by adding 0.2 ml of the digestion mixture to a tube containing 5 \(\mu\)l of thrombin solution (25 NIH units) and 20 \(\mu\)l of a 0.22 M calcium chloride solution. After the onset of fibrin formation, each sample was kept at 37\(^\circ\) for 3 min and then suspended for 1 to 2 min in 3 ml of 0.05 M Tris-HCl, pH 7.4, containing 0.15 M sodium chloride to remove excess calcium. If calcium is not removed from the samples, it precipitates the SDS. The fibrin was then removed, blotted with filter paper, and incubated for 20 hours in 0.25 ml of 5 M urea containing 1% SDS and 1% \(\beta\)-mercaptoethanol. The fibrin, which was completely soluble in this solvent, was then analyzed by SDS polyacrylamide gel electrophoresis.

**Gel Electrophoresis**—Polyacrylamide gel electrophoresis in SDS was performed according to methods previously described (24, 25). Throughout these studies, 5% polyacrylamide gels in the presence of 0.1% SDS were prepared in glass columns (15 x 0.6 cm). Approximately 10 to 20 \(\mu\)g of protein were applied to each gel. The electrophoresis was performed at 20-25\(^\circ\) at a constant current of 9 ma per gel for approximately 6 hours, by which time the bromphenol blue dye marker (0.05%) had migrated to within about 2 cm of the bottom of the gel. The gels were stained with Coomassie brilliant blue (Colab Labs, Inc.) for 6 hours and then destained 18 to 24 hours in a solution containing 14% acetic acid and 7% methanol. The molecular weights of the component polypeptide chains or the degraded chains of fibrinogen were calculated from their mobilities and the experimentally determined mobilities of proteins of known molecular weight. The following proteins were used as standards (the assumed molecular weights are given in parentheses): phosphorylase a (92,900), human transferrin (77,000), bovine serum albumin (69,000), catalase (60,000), ovalbumin (43,000), pepain (35,000), and chymotrypsinogen (36,000). Each gel was stained for carbohydrate-containing proteins with the periodic acid-Schiff base reagent according to the method of Zaborski et al. (37), except that after staining the gels were washed overnight in distilled water and then destained in 50% methanol for 8 hours. The gels were allowed to reswell in 7% acetic acid.
Fig. 1. The digestion of human fibrinogen by plasmin as monitored by electrophoresis on SDS-gels. A, electrophoretic patterns of digestion mixtures without treatment with mercaptoethanol; B, electrophoretic patterns after treatment of the same samples shown in A with mercaptoethanol. Gels stained with Coomassie blue are designated by the letter P, and those stained with the periodic acid-Schiff base for carbohydrate-containing proteins are indicated by the letter C. The numbers at the bottom indicate the time of digestion.
and stored in that solution. The stained bands tend to fade after several days.

EXPERIMENTAL RESULTS

Electrophoretic Analysis of Fibrinogen Degraded by Plasmin

Unreduced Digestion Products—Fig. 1A shows the course of digestion of fibrinogen by plasmin as observed by electrophoretic analysis on polyacrylamide gels in SDS. The gels were stained with Coomassie blue which detects only protein, or with the periodic acid-Schiff reagent which detects only carbohydrate-containing species. As expected on the basis of earlier studies (17, 38), the unreduced gel patterns show that lower molecular weight species are formed as digestion proceeds. Because the fragments contain disulfide bonds, however, the molecular weight of each unique species observed on the gels can only be estimated approximately (39). Nevertheless, from these weight estimates and from knowledge of the pattern of digestion and the molecular weights of the products reported by others (9, 12, 17), it is possible to identify each species on the gel with one of the degradation products observed earlier. Fig. 2A shows the relative amounts of fibrinogen and each of the unique fragments produced by plasmin action throughout the course of digestion. The relative amounts for each species were estimated on the basis of the intensity of staining with Coomassie blue.

The initial fragment formed was visible at 3 min of digestion and corresponds to Fragment X which has a molecular weight of about 240,000 to 265,000 (12, 17). Its intensity increased as the fibrinogen was hydrolyzed, and at 15 min it was the major species present in the digest. Three species, in minor amounts, could be seen after 3 min of digestion and persisted in constant amounts throughout the digestion. These products correspond to species similar in size to Fragments A, B, and C noted earlier (9). After 7 min of digestion, two unique species first appeared and increased in amounts during digestion. One of these species which corresponds to Fragment Y of Marder et al. (17) and has a molecular weight of about 155,000, reached a maximum concentration between 25 and 30 min and then began to disappear. The second species, with a molecular weight of about 83,000, corresponds to Fragment D of Marder et al. (17). It persisted beyond 30 min and remained as the major product of fibrinogen digestion after 24 hours. After 20 min of digestion another species appeared, Fragment E, with a molecular weight of about 50,000 (9, 17). This species and Fragment D were the products in major amounts after 60 min of digestion and persisted as the major products after 24 hours. Two species in minor amounts, immediately below Fragment D, became visible 30 min after digestion and remained as terminal fragments at 24 hours.

Reduced Digestion Products—Fig. 1B shows the electrophoretic patterns of the same digest shown in Fig. 1A, except that each sample was reduced with mercaptoethanol before electrophoresis. Under these conditions of analysis the subunit peptides of fibrinogen and its degradation products are observed on the gels. In addition, the molecular weights of the peptides can be calculated accurately from their electrophoretic mobilities in the gels (24, 25). Fig. 2B graphically depicts the relative amounts of the subunits of fibrinogen and the subunits derived from the degradation products.

The gel at zero time, which was stained with Coomassie blue, shows the α, β, and γ chains. This gel also shows that the α chain is heterogeneous, as noted earlier (26), and contains at least three different species with slightly different molecular weights. Carbohydrate could not be detected in the α chains in gels stained with periodic acid-Schiff reagent, but the β and γ chains, which stained heavily, contained significant amounts of carbohydrate. After 5 min of digestion the α chains were present in much smaller amounts and appear to have been degraded extensively. At this time the β chains appear to have been degraded but to a much lesser extent than α chains. Two new bands appear after 5 min of digestion which have molecular weights of 52,000 and 25,000. The species of 52,000 molecular weight must have originated from the β chains since it contains carbohydrate and has a larger molecular weight than the γ chain. This species will be designated as β' chain throughout the following discussion. The species of 25,000 molecular weight, which does not contain carbohydrate, is derived from α chains, since the γ chains remain intact at this time and the β and β' chains account well for the total amount of β chain on the gels. This species will be designated as α' chain. By 9 min the α chain has been completely digested, and the decrease in the
The amount of β chain appears to be roughly proportional to the increased amounts of the species with a molecular weight of 52,000. At 5 to 9 min another new species, with a molecular weight of 44,500 and containing carbohydrate, first appears and increases in amount throughout digestion as the β chain and the β' chain decrease in amount. The γ chain appears to remain essentially undigested at 9 min as judged by protein and carbohydrate stains. Because the γ chain is not degraded at 9 min, the chain with a molecular weight of 44,500 must be derived from β' chain and will be designated here as β'' chain. After 15 min the β chain appears to be degraded completely, and the β' chain (mol wt 52,000) is found in greatest amount and the β' chain (mol wt 44,500) increases in amount. In addition, the β' chain increased in amount, as judged with both stains, as the β' chain disappeared (Fig. 2A). After 15 min the amounts of the γ chain have decreased only slightly; however, by 30 min of digestion, the γ chain is completely degraded. As the γ chain disappears between 15 and 30 min of digestion, two new species appear with molecular weights of 42,000 and less than 15,000. The 42,000 molecular weight species was devoid of carbohydrate but because it appears to be derived from γ chains, it will be designated as the γ' chain. Also, by 30 min, the β' chain and the α' chain, which first appeared after 5 min of hydrolysis, have essentially disappeared, and a new species with a molecular weight of 37,000 began to appear. Between 30 and 60 min of digestion this species increased in amount as the 42,000 molecular weight species (γ' chain) decreased, suggesting that the 37,000 molecular weight species is derived from the γ' chain. For this reason the 37,000 molecular weight species is designated as the γ'' chain. Between 60 and 120 min of digestion, the β'' chain reached maximal intensity while the γ'' chain band increased slightly in intensity as the last of the γ' chain disappeared. The product of less than 15,000 molecular weight remained unchanged in intensity after 30 min of digestion. After 120 min of digestion the electrophoretic patterns of the products showed no further changes. The terminal reduced digestion products were the 44,500 molecular weight species containing carbohydrate (β'' chain), the 37,000 molecular weight species, which is devoid of carbohydrate (γ'' chain), and a mixture of species containing carbohydrate with molecular weights of less than 15,000.

Determination of Thrombin Times and Analysis of Fibrin Formed from Plasmin-degraded Fibrinogen—Fig. 3 shows that the thrombin-clotting times of plasmin-degraded fibrinogen increased as the length of digestion increased. The fibrin formed with each sample of degraded fibrinogen was reduced and anal-

Fig. 3. The thrombin clotting times of plasmin-degraded fibrinogen during the course of the digestion. The clotting time at point A refers to the thrombin time for fibrinogen prior to addition of urokinase.

Fig. 4. The electrophoretic patterns on SDS gels of the clots formed during plasmin digestion. These patterns were obtained with the digestion mixtures shown in Fig. 3. All samples were treated with mercaptoethanol prior to analysis. The gel indicated by the letter A refers to the digestion mixture prior to addition of urokinase.
lyzed by SDS polyacrylamide gel electrophoresis as shown in Fig. 4. It is important to note that once the end point in the thrombin times was determined, fibrin formation was allowed to proceed for only 3 min at 37°C, conditions which allow crosslinking of γ chains and lead to formation of γ dimers, but not α polymers (26). Of course, the α chains were extensively degraded even at very short times of digestion and completely absent after 9 min of digestion.

The patterns in Fig. 4 show that the fibrin formed from fibrinogen degraded with plasmin for short intervals is completely devoid of γ chain and that these chains have been crosslinked to give γ dimers with a molecular weight of 90,000. Clearly γ dimers are noted in the fibrin formed from fibrinogen degraded with plasmin for periods of up to 15 min. Samples of fibrinogen degraded for 30 min with plasmin did not produce visible fibrin clots on treatment with thrombin. These results, when correlated with the gel patterns shown in Fig. 1B, suggest that the ability of plasmin-degraded fibrinogen to form fibrin clots disappears with the loss of Fragment X and that the degradation of α and β chains is sufficiently extensive after 15 min of digestion such that fibrin cannot be formed even though γ chains are only partially digested.

**DISCUSSION**

**Pattern of Plasmin Digestion of Fibrinogen**—From the studies described here and those reported earlier, it is possible to determine where plasmin acts on the three different polypeptide chains of fibrinogen and also to ascertain the polypeptide chain composition of the major plasmin digestion products. Examination of the action of plasmin on fibrinogen by means of electrophoresis on acrylamide gels in SDS has revealed the following major points. (a) The three subunit chains of fibrinogen differ in their susceptibility to plasmin. The α chains are more susceptible to plasmin than the β chains, which, in turn, are more susceptible than the γ chains. This order of susceptibility agrees with that given in our earlier report (20) and those of others (19, 23). (b) Plasmin digestion leads initially to the formation of Fragments X and Y and low molecular weight peptides. These fragments are then digested by plasmin to give Fragments D and E and additional low molecular weight peptides. (c) The α chains of fibrinogen contain no detectable carbohydrate as judged by staining gels with a periodic acid-Schiff reagent. Although this observation differs from an earlier report which indicates that all three chains contain carbohydrate (40), it has been particularly helpful in judging which digestion products contain fragments of the α, β, and γ chains. On the basis of these general observations and the ability to correlate the disappearance of fibrinogen and its subunits with the appearance of new fibrinogen fragments and new subunits, it has been possible to construct the details for digestion of fibrinogen by plasmin as shown in Fig. 5. Before discussing the details of this figure, it should be emphasized that the structures shown for fibrinogen and its degradation products have been drawn so that the action of plasmin and the subunit structures of the fragments can be understood conveniently. Further studies are clearly required to establish the orientation of the subunits and the positions of the interchain disulfide bonds.

The proposed subunit structures of the fragments produced by plasmin digestion of fibrinogen are listed in Table I. This tabulation will be helpful in the following discussion of Fig. 5.

The α chains are the first subunits to be hydrolyzed by plasmin (Figs. 1B and 2B). The rapid destruction of these chains may coincide with the initial cleavage of two to three bonds, which have been reported to be split at a much faster rate than other bonds on degradation of bovine fibrinogen with plasmin (41). Concomitant with the destruction of α chains is the appearance of the first degradation product of fibrinogen, Fragment X (Fig. 1A), which was noted earlier by Marder et al. (17) (Re-action 1 in Fig. 5). Coincident with the loss of α chains and the appearance of Fragment X is the appearance of three peptides, whose molecular weights appear to be about 15,000 each. These peptides correspond to Fragments A, B, and C described earlier (9, 17). They have no detectable carbohydrate, just as α chains, and because they appear concurrently with degradation of α chains, they must represent fragments of α chains. With the loss of Fragments A, B, and C, which together would have a total molecular weight of about 90,000, Fragment X should have a molecular weight of about 250,000 and contain degraded α chains with a molecular weight near 25,000. Fragment X has a reported molecular weight of 240,000 to 260,000 (12, 17). In addition, analysis of digest (Fig. 1B) reveals that when Fragment X is the major digestion product, a subunit chain of about 25,000 molecular weight is formed, which is designated here as α' chain.

In Fig. 5, the formation of Fragment X occurs on symmetrical cleavage of the α chains in fibrinogen. Clearly, the initial events of plasmin degradation are unlikely to be this simple. Probably some species of Fragment X are formed transiently by degradation of only one of the two α chains and species could be formed by the release of only one or two of the three peptides corresponding to Fragments A, B, and C. For this reason, it is likely that Fragment X represents a population of degradation products with different molecular weights and contains α chains degraded to differing degrees. The broad bands corresponding to Fragment X in Fig. 1A probably represent such intermediate species although they are not resolved in the electrophoretic system used here. Unpublished studies indicate that Fragment X can be resolved into several species by electrophoresis on 3.4% crosslinked SDS gels (42), buffered at pH 7.2. These intermediates have not been designated in Fig. 5 and for simplicity, only one of the possible fragments, called X1, is shown. It is believed that plasmin digestion results in release of peptides from the COOH-terminal parts of the α chains. This conclusion is based on the earlier studies of Mossesson who showed that the NH2-terminal groups of fibrinogen are unchanged during the initial stages of plasmin action and that the fragments initially formed release fibrinopeptides A and B on treatment with thrombin (43). The proposal by Blomback et al. (6) that disulfide bonds of fibrinogen are clustered in the NH2-terminal region of the molecule would account for the retention of α' chain in Fragment X, despite extensive proteolysis of the α chains.

It is clear from the time course of degradation (Figs. 1B and 2B) that Fragment X may also contain partially degraded β chains. When Fragment X is present in maximum amounts in digests, about half of the β chains are absent and appear to be degraded to a species with a molecular weight of 52,000. This species, designated as β' chain, is formed on cleavage of the β chain to release a peptide with a molecular weight of about 4,000 which cannot be detected by the methods used in this study. In view of the observations of Mossesson concerning the NH2-terminal groups and the release of fibrinopeptide B from the

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3 S. V. Pizzo and P. A. McKee, unpublished studies.

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Fig. 5. Schematic representation of the reactions proceeding during plasmin digestion of human fibrinogen. The symbols for the degraded polypeptide chains are described in the text and in Table I.

Evidence for Reactions 3 to 8 in Fig. 5, which describe the formation of Fragments Y, D, and E, is provided by the gel analyses shown in Figs. 1 and 2 as well as by earlier observations of others. Fragments Y, D, and E appear in plasmin digests only after formation of Fragment X. The rate of formation of Fragment Y is initially indistinguishable from that of Fragment D. Fragment Y, however, is a transient species and is absent after 60 min of digestion, whereas Fragment D is a major terminal digestion product and readily detected in digests after 24 hours. This time course is consistent with the view of Marder et al. (17) that Fragments Y and D are both derived from Fragment X and accounts for the observation that Fragments X and Y contain the antigenic determinants in Fragment D (16). Fragment E, which is the second major terminal digestion product, appears initially only after Fragments Y and D are formed. For this reason and the fact that Fragment E continues to be formed along with Fragment D, while Fragment Y is disappearing, it is believed that Fragment E is produced by further degradation of Fragment Y.

Before considering the fate of Fragment X on further digestion, it is convenient to consider the polypeptide chain composition of Fragments D and E. On the basis of the polypep-
tide chains present in the digests from 10 to 60 min of digestion, as shown in Figs. 1B and 2B, it is possible to predict the polypeptide chain composition of Fragments Y, D, and E and deduce the nature of Reactions 3 to 8 in Fig. 5. When gels are examined which reveal the polypeptide chains in digests (60 min) containing only Fragments D and E (Fig. 1B), five bands are observed, which correspond to species with molecular weights of 44,500, 42,000, 37,000, 25,000, and less than 15,000. The 44,500 molecular weight species must be a & chain fragment since it contains carbohydrate and begins to form in significant amounts as & chains disappear and before appreciable amounts of $\gamma$ chain are degraded. This species, designated $\gamma''$ chain, also appears in digests about the time that Fragment D is first observed; therefore, it appears to be a major constituent of Fragment D. The 42,000 molecular weight species must be derived from $\gamma$ chains, since it is too large to be derived from $\alpha$ chains, which are extensively degraded early in digestion, and it as well as the 44,500 species could both not be derived from $\beta$ chains. In addition, this polypeptide chain (designated $\gamma'$ chain) appears to form only after $\gamma$ chains are first noted to disappear (15 min). The $\gamma'$ chain, however, decreases in amount on prolonged digestion, and its disappearance coincides with the appearance of the 37,000 molecular weight species ($\gamma''$ chain). The $\gamma$ chain may be degraded further since a species of 25,000 molecular weight (designated $\gamma'''$ chains) is observed in 24-hour digests as the $\gamma''$ chain decreases somewhat. The $\gamma'$, $\gamma''$, and $\gamma'''$ chains are also thought to be components of Fragment D since they are first detected in digests after significant amounts of Fragment D are noted. The assignment of the $\beta''$, $\gamma'$, $\gamma''$, and $\gamma'''$ chains to Fragment D is consistent with the recent observations of Catanzaro et al. (21) who reported that purified Fragment D contained three chains with molecular weights of 44,000, 25,000, and 10,000. By these lines of reasoning, Fragment D must be a three-chained molecule containing a $\beta''$ chain, either a $\gamma'$, $\gamma''$, or $\gamma'''$ chain, and a third chain of molecular weight about 10,000 to 15,000. The 10,000 to 15,000 chain is most likely derived from the degraded $\alpha$ chains in Fragment X and are thus designated $\alpha''$ chains. Thus, Fragment D, just as Fragment X, probably represents a population of species, differing from one another only in the degree of degradation of the residual $\beta$ and $\gamma$ chains. It is of interest that Catanzaro et al. (21) have distinguished at least eight different electrophoretic variants of Fragment D. In addition, after 60 min of digestion, two species are noted on gels with slightly lower molecular weights than Fragment D and may represent some of the structural variants of this fragment. Fragment E is the other terminal digestion product and appears to contain small subunits with molecular weights less than 10,000, since the larger subunits found in terminal digestion products are derived from Fragment D. In order to account for the molecular weight of Fragment E, it is judged to contain portions of the NH₂-terminal sequences of the $\alpha$, $\beta$, and $\gamma$ chains combined through disulfide bonds, which have been designated in Fig. 5 as $\alpha''^b$, $\beta''^b$, and $\gamma''^b$ chains, respectively. This region of the fibrinogen molecule is known to be rich in disulfide bonds (6). In addition, the studies of Budzynski (22) and Caffney and Dobos (23) also suggest a similar subunit structure for Fragment E. It is noteworthy that Fragment E contains carbohydrate, which must be present in the $\gamma''$ chain.

With knowledge of the structures of Fragments X, D, and E, it is possible to deduce the structure of Fragment Y and understand not only how it and Fragment D are derived from Fragment X but also how degradation of Y leads to formation of Fragment E and another molecule of Fragment D. In order to obtain Fragment D from Fragment X it is necessary to cleave at least one additional bond in each of the chains of Fragment X. This is thought to proceed as shown in Steps 3, 4, and 5 in Fig. 5. Clearly, Fragment D would not be released from Fragment X until each of the bonds in all three of the chains are cleaved, irrespective of the order of cleavage of the chains. Thus, on cleavage of the last of the three bonds, Fragments D and Y would be formed. Further degradation of the $\alpha'''$, $\beta'''$, and $\gamma$ chains in Fragment Y then leads to its disappearance and the formation of Fragments D and E.

It is evident that Fragment X must also contain structural variants of the kind indicated by Fragments X₂ and X₃ (Fig. 5). For simplicity, only the minimal number of possible variants of this kind are shown. It is evident from the nature of the reactions leading to the formation as well as the destruction of Fragment Y, that Fragment Y also is structurally heterogeneous. For example, Reaction 6 indicates that the $\alpha'''$, $\beta'''$, and $\gamma$ chains remaining in Fragment Y must each be cleaved to produce Fragments D and E. Any one or two of the chains could be split before the reaction is complete; thus, several intermediate forms of Fragment Y may exist which are indistinguishable in size from one another.

Ability of Plasmin Fragments to Clot—The studies summarized in Fig. 5 allow some insight into the ability of plasmin-degraded products to form clots. Clearly, the thrombin times for the di-
gested fibrinogen were altered very little during the first 6 to 8 min of digestion, when Fragment X was present in maximal amounts in forms corresponding to Fragments X1 and X2. These species contain intact \( \gamma \) chains and \( \beta' \) chains, and analysis of the fibrin clots from these species (Fig. 4) reveals that the \( \gamma \) chains are readily converted to the \( \gamma \) dimers of 90,000 molecular weight. This indicates that clots form despite extensive digestion of \( \alpha \) chains and cleavage of \( \beta \) chains to a smaller extent. The \( \gamma \) chains are beginning to be degraded at this time and decreasing amounts of the \( \gamma \) dimer are produced, as shown in Fig. 4. In addition, Fragment X is beginning to disappear and Fragment Y is increasing in amounts. These results are in accord with the earlier observations that Fragment Y is not clottable (17).

Degradation of \( \alpha \) Chains—The rapidity with which \( \alpha \) chain is digested from native fibrinogen deserves further comment. As shown in Fig. 6, purified preparations of fibrinogen made from outdated plasma or commercial sources of Cohn Fraction I often showed marked diminution of the \( \alpha \) chains when reduced and electrophoresed on SDS polyacrylamide gels. It was also observed that plasma fibrinogen from a patient being given intravenous streptokinase for arterial thromboses lacked \( \alpha \) chains and contained slightly degraded \( \beta \) chains. The studies reported here suggest that the Fraction I-8 (mol wt 273,000) of Mosesson and coworkers (43, 44) has a structure similar to Fragment X, and has lost parts of both \( \alpha \) chains. Fraction I-8Ds of Sherman et al. (45) and “fibrinogen first derivative” of Fletcher et al. (12) (mol wt 262,000 to 265,000) may have structures similar to Fragment X, which has lost a peptide of approximately 4,000 molecular weight from each \( \beta \) chain. Since the starting material for the preparation of Fractions I-8 and I-8Ds, was outdated plasma or commercial Fraction I (43-45), it seems reasonable to assume that these species lacked substantial portions of \( \alpha \) chains and may even have lost the small peptide cleaved from \( \beta \) chain due to in vitro digestion. Loss of the \( \alpha \) chains would also explain the increased carbohydrate content reported for both I-8 and I-8Ds (45). Moreover it is possible that partial \( \alpha \) chain digestion could account for the occasional low molecular weights (269,000 and 298,000) (46, 47) reported for native fibrinogen. Finally, the lability of the \( \alpha \) chains could explain the poor reproducibility of tryptic peptide maps for normal fibrinogen (48-50) and emphasizes that fingerprint patterns reported as evidence for an abnormal fibrinogen must be interpreted with caution (49). As a safeguard, we urge that the SDS gel electrophoretic pattern of reduced fibrinogen be examined to ensure the presence of intact \( \alpha \) chains before any type of characterization studies are begun.

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REFERENCES

The Effect of Plasmin on the Subunit Structure of Human Fibrinogen
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