The Essential Covalent Structure of Human Fibrinogen Evinced by Analysis of Derivatives Formed during Plasmic Hydrolysis*

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SUMMARY

In an effort to determine the essential structural features of human fibrinogen, plasmic hydrolysis was carried out and degradation products were identified and analyzed. In agreement with previous work, the initial attack was shown to occur in the Aα chains with the release of portions of their COOH-terminal regions (designated /α chains in the terminology developed to describe the present findings), whereas simultaneous, but slower, degradation occurs in the NH₂-terminal region of the Bβ chains and results in the removal of remnants (designated Bβ/ chains) containing peptide B. These changes, plus internal cleavages leading eventually to the separation of Fragments D and E (terminology of Nussenzweig et al.), produce a series of high molecular weight derivatives which can be separated by polyacrylamide gel electrophoresis of unreduced samples in sodium dodecyl sulfate. (These were designated Bands II through VII in order of decreasing molecular size; Band VII was identified as Fragment X in the terminology of Marder et al.) Upon further hydrolysis derivative Band VII can lose the COOH-terminal portion of one of its Bβ chains (as remnant /βa, molecular weight 32,000) to form derivative Band VIII (Fragment Y in the terminology of Marder et al.) or, alternatively, can undergo cleavage to yield early forms of Fragments D and E (designated D1 and E1, respectively). Derivative Band VIII, in turn, is degraded to Fragment E1 plus a later form of Fragment D. Once cleaved from the parent molecule, these Fragments D continue to release /β chains. These degradative pathways lead to a series of Fragments D (D1 through D5) which differ in molecular weight. Additional cleavages occur in Fragment E1 to form a smaller fragment (E2) which, unlike E1, lacks peptide A.

Immunchemical studies of derivatives obtained at successive phases of digestion demonstrated the presence of at least five antigenic determinants on the fibrinogen molecule. One of these is located on /α chains; another, on Fragment E1; three others, on Fragment D. Of the latter, one is associated with /βa chains; a second (designated F) is associated with Fragments F, which arise by further degradation of Fragments D; the third (designated D) is that lost when Fragment F is evolved. Electrophoretic behavior of plasmic derivatives before and after reduction revealed at least one intrachain disulfide bridge in each /α chain and /β chain.

Studies of Fragment D subunit composition and recovery at various phases of digestion indicated that it is a dimeric structure containing substantial portions of the COOH-terminal region of both γ chains (as γγ remnant chains, each having a molecular weight of 42,000) and hence that only one such fragment (linked by at least five interchain disulfide bridges) can be generated by each fibrinogen molecule. These results, plus those reported by others, led to the conclusion that fibrinogen itself has a dimeric structure, the “backbone” of which consists of a pair of γ chains linked directly to each other by disulfide bridging in the NH₂-terminal region and covalently linked (enter directly or indirectly) with the Aα and Bβ chains in at least two regions of the molecule.

Endopeptidases have been of great value in structural studies of a number of proteins (1). In the case of plasma fibrinogen such an approach employing plasmin-catalyzed hydrolysis was used to establish the presence of at least two antigenically distinct regions, named D and E, in the protein “core” (2–4). The antigenicity of these plasmic fragments, which were chromatographically separable from one another, was preserved even after prolonged enzymic degradation. These discoveries provided both a stimulus and a rational basis for additional experimentation.

Blombäck and co-workers (5–7) used cyanogen bromide cleavage to prepare a fragment which contained the NH₂-terminal regions of all three types of fibrinogen subunit chains (Aα, Bβ, and γ), joined by interchain disulfide bridges. This structure was termed the NH₂-terminal disulfide knot (N-DSK). The amino acid sequence of each of its constituents is known (5, 7–13), as are the locations of the interchain and intrachain disulfide bridges (11–13). The initial estimate (6, 7) of the...
molecular weight of the N-DSK, 26,000, led to a premature suggestion that there were two such units for each fibrinogen molecule. More recent estimates indicate a molecular weight in the range of 49,000 to 60,000 (14, 15), and, coupled with other structural studies (11–13), strongly indicate that the NH2-terminal regions of all six chains are involved in its structure.

Plasminic core Fragment E has been conclusively shown to be derived from the same region of the molecule as the N-DSK. Marder et al. (14) and others (16) have demonstrated an antigenic identity between N-DSK and Fragment E, which itself is antigenically unique with respect to other regions of the molecule (3, 17, 18 inter alia). Furthermore Mills (19), employing sodium dodecyl sulfate electrophoretic analyses of Fragment E from relatively early digests, and Marder et al. (14), using NH2-terminal analysis, have demonstrated the presence of thrombin susceptible bonds, probably due to the presence of peptide A.

Information regarding the structure of the remainder of the molecule is indirect and far less conclusive than that cited above for the NH2-terminal region. Two different hypotheses, both based upon analyses of plasminic digests, can be proposed. The first, consistent with the structural symmetry indicated by electric birefringence measurements on fibrinogen (20, 21), suggests that the COOH-terminal regions of fibrinogen chains, like those in its NH2-terminal region, are covalently linked to form a single dimeric structure. Support for this may be drawn from the data of Fletcher and his co-workers (17, 22), who showed that a large variety of intermediate core species was formed during the degradation. Among them were several species immunologically identifiable as Fragment D; these ranged in size from 150,000 or more to the final plasmin resistant core fragment of 88,000. Since Fragment D is derived from the COOH-terminal region of the molecule, the relatively high molecular weight of its early forms supports the “dimeric” hypothesis because only a single structure of this size can be obtained from one molecule of fibrinogen. Though the data of these investigators has been disputed (18), and few subsequent studies have been directly interpreted as supporting such a model (23), the concept remains viable.

The second hypothesis, suggested by Nussenzweig et al. (2) and elaborated upon by Marder et al. (14, 18, 24–28), proposes that there are two monomeric Fragments D derived from each molecule of fibrinogen. The experimental basis for this hypothesis rests upon the recovery of Fragment D’s early forms supports the “dimeric” hypothesis because only a single structure of this size can be obtained from one molecule of fibrinogen. Though the data of these investigators has been disputed (18), and few subsequent studies have been directly interpreted as supporting such a model (23), the concept remains viable.

Terminology employed for fibrinogen subunit chains and their hydrolytic derivatives conforms to the tentative recommendations (August, 1972) of the Subcommittee on Nomenclature of the International Society on Thrombosis and Hemostasis.

The abbreviations used are: N-DSK, NH2-terminal diisulfide knot; DTT, dithiothreitol; dace, 1-dimethylaminonaphthalenedisulfon; DPT, diisopropyl phosphorofluoridate.

The underlying assumptions for many recent structural studies (14, 16, 19, 31–36 inter alia).

As a portrayal of the COOH-terminal region of the fibrinogen molecule the “dimeric” and “monomeric” models are not compatible. The studies to be reported were designed to test these models critically by studying the sequence of degradation catalyzed by plasmin.

Terminology

The degradation of fibrinogen by plasmin has been divided into Stages 1, 2, and 3 according to the terminology introduced by Marder and co-workers (18). Although there is considerable overlapping in the transition from one “stage” to another, each phase can be distinguished from the others by virtue of the products present in the digest. Stage 1 is characterized by the presence of coagulable species; Stage 3, by that of core fragments containing either D or E antigenic determinants, but not both. In Stage 2 there exist core species with both D and E antigenic determinants, but not (or relatively few) coagulable species.

The subfractions comprising core derivatives isolated from Stage 1 digests of Fraction I-4 have been designated in a manner related to that employed for plasma fibrinogen Subfractions I-8 and I-9 (37), which consist primarily of circulating fibrinogen catabolites (38). They are identifiable by their relatively high solubilities and delayed aggregation rates (39). To differentiate an in vitro plasminic derivative subfraction from the fraction of corresponding solubility isolated from plasma, the suffix “D” is added to the name of the former, and the clottability of the digest mixture from which it was isolated is shown as a superscript. For example, a derivative subfraction essentially equivalent in solubility to plasma Fraction 1-8, but obtained from a digest whose clottability had fallen to 88% at the time of sampling, is designated 1-8D9. (The clottability of the isolated subfraction itself is not implied by this term.)

Terminology for the plasminic core Fragments D and E identifiable in intermediate and advanced digest mixtures is the same as that proposed by Nussenzweig et al. (2–4). Forms of D and E fragments distinguishable by their electrophoretic migration in the presence of sodium dodecyl sulfate are indicated by numerical subscripts, the higher the number, the smaller the fragment (e.g. D1, D2, etc.). Core species larger than Fragment D, also identifiable by their migration in sodium dodecyl sulfate-containing gels, have been designated with Roman numerals (I, largest; II, next largest, etc.) as suggested by Mills and Karpatkin (33). Because of structural implications resulting from this as well as our previous studies, the X, Y terminology applied to intermediate derivatives has not been utilized for fragments identified here. Nevertheless, because of its present wide usage, we have attempted (cf. “Discussion” and Fig. 12) to relate this terminology to our own.

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2 We have identified at least eight more or less discrete bands of this type in samples containing circulating fibrinogen catabolites or plasmin derivatives. Since the specific structure of the derivative species migrating in Bands I to V is not directly pertinent to the present studies, data relating to those bands will form the subject of another paper (in preparation). However, information relating to the composition of derivative Bands VII and VIII, which are abundant in Stage 2 (cf. Figs. 1 and 8) is included in the present report.
Haemostasis, insofar as guidelines are available. The intact subunit chains of fibrinogen are designated Ax, By, and Cz; those of fibrin, α, β, and γ. Cross-linked chains are designated γ-γ (γ-linker) or α-γ (α-polymer). Cleavage sites other than those attacked by thrombin are indicated by a solidus placed to denote cleavage on the COOH- or NH2-terminal side (consistent with the convention of designating peptide sequence from NH2 to COOH terminus). Thus, a single cleavage of an Ax chain (at a site not within or abutting the A peptide) would be indicated as Ax → Ax/ + /α. Chain fragments are further designated with a numerical subscript (based on previous nomenclature, Reference 38) which, in most cases, increases as the size of the fragment decreases. That is, the Ax remnant designated "Aα band 11" in Reference 38 retains this identification in the present study and is designated Aα/11; a somewhat smaller Ax remnant is designated Aα/15; and so forth. Chain fragments identified since the previous report have been assigned consecutively higher subscript numbers (14 and above for Ax remnants) regardless of electrophoretic position (i.e. size). Remnant chains whose formation could not be precisely localized to a new COOH or NH2 cleavage site are indicated by a bar (e.g. /β/). A remnant chain whose precursor chain (i.e. Aα, Bγ, or Cz) is ambiguous or uncertain is preceded by a question mark. It is anticipated that subunit chain terminology will be of intermediate utility (46) in gels whose final acrylamide concentration was between 7 and 14%. For disulfide bond reduction, when desired, DTT was added directly. After these digestions inhibition of hydrolysis was achieved by addition of Kunitz pancreatic trypsin inhibitor (Worthington Biochemical Corp.) to a final concentration of 10 to 20 μg per ml, a concentration at which it is an extremely effective plasmin inhibitor (43).

S-sulfo derivatives of fibrinogen or of plasmin derivatives were prepared according to the method of Poche et al. (44).

Cross-linking of fibrinogen or its plasmin derivatives was carried out under the conditions previously described (45).

Electrophoretic and Related Procedures—Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed and molecular weights were estimated as described by Weber and Osborn (46) in gels whose final acrylamide concentration was between 5 and 14%. For disulfide bond reduction, when desired, DTT (14 mMr, final concentration) was employed. Samples were prepared for application by mixing with an equal volume of 50% (v/v) glycerol-10 mM sodium phosphate, pH 7. Gels were stained with Amido Schwarz 10B or Coomassie brilliant blue, as previously described (38). Densitometric scans of stained gels were made in a Gilford model 240 spectrophotometer equipped with a Linear Transport apparatus.

Protein markers used were the various fibrinogen subunit chains and their derivatives whose molecular weights had previously been established (38) against standard marker proteins. For molecular weight estimation in the range below 12,000 the following markers were used in their reduced form: cytochrome c (horse heart), 11,700 (46) (Sigma Chemicals, St. Louis); the A chain, 2,300, and B chain, 3,400, of insulin (47) (E. R. Squibb and Sons, New York); ribonuclease A, 13,700 (46) (Worthington Biochemical Corp.); and synthetic salmon calcitonin, 3,000 (48) (a gift from Dr. Richard Baker, SUNY, Downstate Medical Center).

Identification of chains retaining peptide A or B was made by comparison of their mobility in sodium dodecyl sulfate electrophoresis before and after treatment with repilase (a gift from Pentapharm, Ltd., Basel, Switzerland) or thrombin (lot H-1, provided by Dr. D. L. Aronson, Bureau of Biologies, FDA). The procedure followed has recently been described (38).

Preparative clution of unreduced plasmin derivative bands from sodium dodecyl sulfate gels was carried out in the following way. Sample loads of 70 to 150 μg of protein per gel (typically 24 to 36 gels per run) were subjected to electrophoresis in 5% gels. One of every six gels was stained to provide assurance of uniform migration conditions, and the remaining unstained gels were cut into 1.5-mm slices (Lateral Gel Slicer, Camaco, Rockville, Md.). The individual slices were pooled appropriately, then manually homogenized and extracted several times with 0.3 M NaCl. The extract was precipitated by addition of 20% trichloroacetic acid (final concentration, 10% w/v) and the resulting trichloroacetic acid precipitate extracted several times with ether. The precipitate was then dissolved in urea.

MATERIALS AND METHODS

Plasmin Preparations—Human plasminogen (a gift from Dr. Alan Johnson, New York University School of Medicine) was water-soluble, had a specific activity of 20 caseinolytic units per mg of protein, and showed to spontaneous activity in a standard assay (40) (α-casein, Worthington Biochemical Corp., Freehold, N. J.) prior to activation. Complete activation to plasmin (EC 3.4.2.14) was achieved by the addition at room temperature of a small amount of streptokinase (10 units per ml, final concentration) to a solution (50%, v/v) glycerol, 5 mM sodium phosphate buffer, pH 7) of plasminogen (4.5 mg of protein per ml). The active material was stored at −30°C; over a 4-year storage period the activity fell from 90 caseinolytic units per ml to 30 to 35 caseinolytic units per ml at the time these present studies were made.

The other human plasmin preparation employed (10.2 caseinolytic units per ml in 50% glycerol) yielded equivalent experimental results. It had been prepared at the Michigan Department of Health (41). The specific activity of this material was 6 to 7 units per mg of protein.

Preparation of Fibrinogen Subfractions and Derivatives—Plasma Subfraction I-4 (clottability > 98%) isolated by a modification (37) of the Blomback procedure (42) was the starting material for preparation of plasmin derivative Subfraction I-9D from Stage 1 digests. To obtain such subfractions, the contaminating plasminogen in I-4 was activated by addition of streptokinase (10 units per ml, final concentration) to the solution of fibrinogen under the conditions described by Sherman et al. (39). When the digestion had proceeded to the desired degree (e.g. digest clottability of 88% to 40% in this study), DTT (Schwarz-Mann, Orangeburg, N. Y.) was added in the manner previously described (39) for the inhibition of proteolytic activity. Most preparations inhibited in this manner and stored at −30°C underwent only minor changes in clottability (2 to 5%) over storage periods exceeding 1 year. The mixture was then fractionated (37–39) to obtain Fraction I-9D. Increases in the clottability of Subfraction I-9D isolated at any phase of digestion could be achieved by subjecting the subfraction to the reprecipitation procedures recently reported (38), but these manipulations yielded I-9D which was more than 95% clottable only when the clottability of the digest itself was at least 88%. Similar observations were reported by Sherman et al. (39).

Experiments on advanced phases of plasmin hydrolysis required greater rates of degradation than those achieved by activation of plasminogen present in I-4. For these experiments, as well as for further degradation of derivative subfractions like I-9D** (which no longer contained plasminogen), plasmin was added directly. After these digestions inhibition of hydrolysis was achieved by addition of Kunitz’ pancreatic trypsin inhibitor (Worthington Biochemical Corp.) to a final concentration of 10 to 20 μg per ml, a concentration at which it is an extremely effective plasmin inhibitor (43).

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sodium dodecyl sulfate-phosphate buffer for further analysis (46). For experiments in which treatment with reptilase was planned the precipitate was suspended in 20 mM sodium phosphate buffer, pH 7. Reptilase was added and the mixture incubated at 37°C with constant stirring for 2 hours, whereupon solid urea (1 mg per ml of solution) and sodium dodecyl sulfate (50 μl of 20% sodium dodecyl sulfate per ml of solution) were added prior to reduction and electrophoresis.

Agarose electrophoresis (proteins stained with Light green) and immunoelectrophoresis (49) were performed at room temperature in 1% agarose, barbital buffer (pH 8.6), on flexible plastic film (Cronar, 70-mm movie film, Du Pont) 90 to 100 mm in length, for 1 to 2 hours at 60 to 80 volts. Immunodiffusion was carried out at room temperature for 24 to 48 hours with rabbit antihuman fibrinogen serum which had been made specific by absorption with human serum. Absorption of this antifibrinogen serum was done with chromatographically isolated S-sulfo Dβ and γ chains which had been dissolved in 5 M guanidine HCl and dialyzed against 0.15 M NaCl-0.02 M phosphate, pH 7, to form a fine suspension. The completeness of absorption was monitored by immunodiffusion.

**Chromatographic Procedures—DEAE-cellulose** (Whatman DE23) chromatography of plasmic fibrinogen digests or subfractions therefrom was carried out at room temperature on columns (1.5 x 30 cm) with a combined pH and phosphate gradient (38, 50). CM-cellulose chromatography (Whatman CM23) for separation of S-sulfo derivative chains was carried out at room temperature in 8 M urea employing a sodium acetate gradient (38). Fractions corresponding to 1% of the total gradient volume were usually collected.

Gel chromatography was carried out on Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) columns (2.5 x 85 cm) employing upward flow. For chromatography of fractions soluble in saline solutions (e.g. supernatant solutions of early plasmic digests containing β2 chains) the column was equilibrated with 0.15 M NaCl-0.05 M Tris Cl, pH 9.0. For certain fractions, particularly those which were insoluble in saline solutions (e.g. S-sulfo derivatives), the column was equilibrated and eluted with 2 M guanidine HCl (“Ultra-Pure” guanidine HCl, Schwarz-Mann).

**Thermal Denaturation of Core Fragments—Fibrinogen** and certain plasmic core derivatives, such as Fragment D, can be precipitated from a digest at 56-60°C, whereas under the same conditions other plasmic derivatives (e.g. Fragment E) remain soluble (2, 22, 51 inter alia). This procedure was therefore potentially useful for determining the recovery of Fragment D from plasmic digests. However, we found, consistent with observations of Beck and Jackson (51), that precipitation of Fragment D species was not always complete under these conditions. Since recovery values were accurate only when precipitation of D approached 100% sodium dodecyl sulfate gel electrophoresis of supernatant solutions remaining after heating was used to monitor the completeness of precipitation. For early and intermediate Stage 3 digests, heating at 60°C for 10 to 15 min was adequate. For more advanced Stage 3 digests, heating at 80°C for 10 min was useful, although the precipitate which formed was often cloudy or finely granular and did not compact well upon centrifugation. This behavior made complete separation of precipitate and supernatant difficult and rendered heating at 85°C unsatisfactory for assessing recovery from certain advanced digests. Nevertheless, this procedure was highly suitable for obtaining supernatant solutions free of detectable Fragment D for immunoelectrophoretic analysis (Fig. 1).

**Determination of Absorbance Coefficients**—The absorbance coefficient (A1%cm (282 nm)) of certain derivatives was determined by making absorbance measurements (282 nm in 5 M urea-0.1 x NaOH solutions) on solutions prepared from weighed amounts of freeze-dried, salt-free material. Correction for the moisture content of the dried samples (8 to 13% in these experiments) was made in each case by drying an aliquant in a vacuum oven (80°C) to constant weight.

**Tryptic Peptide Mapping**—Mapping was done by a modification (52) of the method of Katz et al. (53). Digestion was carried out with trypsin treated with l-1-tosylamido-2-phenylethyl chloromethyl ketone (Warثington Biochemical Corp.); maps were developed with a cadmium acetate-ninhydrin stain.

**Analysis of Dansyl-labeled Peptides and Proteins—NH₂-terminal analysis** was performed by an adaptation of the dansyl chloride method (54). Present procedures have been sufficiently changed from our previously reported modification (38, 50) of the method to warrant a detailed description at this time. Material to be labeled was dissolved in a freshly deionized urea solution (5 to 8 M, final concentration) buffered at pH 8.5 with N-ethylmorpholine acetate (final concentration, 0.015 to 0.022 M) prior to addition of about 5 μmoles of dansyl-Cl (in methanol-acetone, 1:1 at a concentration of 18.5 or 37 μM) per mg of protein. After labeling, usually overnight, the protein was sequentially precipitated with trichloroacetic acid (final concentration, 7 to 10%), washed with water, dried under vacuum, and hydrolyzed with constant boiling HCl for 24 hours at 105-110°C under reduced pressure. HCl was then removed under vacuum, and the residue was dissolved in 3 M ammonium formate buffer, pH 3.0 ± 0.1, and extracted with ether as suggested by Herzik et al. (55) to isolate the α-dansyl derivatives (with the exception of that of arginine and of histidine). Ammonium hydroxide labeling mixture and, instead of trichloroacetic acid precipitation, was developed with a cadmium acetate-ninhydrin stain.

**Quantitative NH₂-terminal analysis** was performed by carrying exact amounts of authentic amino acids through the entire procedure, with the exception that urea was not added to the labeling mixture and, instead of trichloroacetic acid precipitation, the labeled amino acids were concentrated under vacuum. The dansyl amino acid spots resulting from analysis of each protein or amino acid standard were cut out, eluted with 1.5- to 2.0-ml amounts of methanol-ammonia (85:5, v/v), and their fluorescence was measured in an Ammeco-Bowman spectrophotofluorometer using 335 nm for the excitation wave length and 510 nm for emission.

Dansyl-labeled protein material for NH₂-terminal analysis of individual reduced subunit chains was prepared in N-ethylmorpholine buffered urea solutions as described above. The labeled mixture was then dialyzed against several changes of 2% acetic acid and freeze-dried. For electrophoretic analysis by the method of Weber and Osborn (46) this dansylated protein...
Fig. 1. Sodium dodecyl sulfate gel (9% acrylamide) and immunoelectrophoretic analyses of sequential samples from a plasmic digest of fibrinogen (I-4). Clottability of the digest at the time of sampling is shown beneath the gels; digest stage (1, 2, or 3), above. The results of immunoelectrophoresis of whole (left) and heated supernatant fractions (Δ super, right) are placed beneath the appropriate gel of unmodified digest sample. The ordinate pertains to densitometric scans. The relative scan area of a given region of the gel, normalized for the amount of total sample applied, is placed to the right of the gel (gel and immunoelectrophoretic analysis of 89% clottable sample not shown).

was dissolved to a concentration of 3 to 8 mg per ml in a urea-phosphate-sodium dodecyl sulfate buffer (8 M, 10 mM, 1%, pH 7) and reduced with DTT (14 mM). An alternate labeling method, which did not require DTT reduction immediately prior to electrophoresis, consisted of dissolving the protein in 8.4 M guanidine-HCl buffered with N-ethylmorpholine at pH 8.5, reducing with DTT and subsequently adding dansyl chloride. (The amount of dansyl chloride added was equal to the calculated amount for the protein plus an amount equivalent to the quantity of DTT in the mixture.)

Sodium dodecyl sulfate electrophoresis was carried out (typically 24 gels per run) at sample loads of 150 to 400 μg per gel. After completion of the experiment, the fluorescent bands were identified, sliced out, pooled appropriately, homogenized, eluted with 10 M urea, dialyzed against 2% acetic acid, and freeze-dried. The NH₂-terminal acids of the resulting material were then determined as described above.

RESULTS

Acrylamide gel electrophoresis of unreduced digest samples in the presence of sodium dodecyl sulfate was a precise and reproducible way of establishing which stage of degradation had been reached. In Fig. 1 is shown such an analysis (in 9% gels) of serial samplings from plasmic digests. Gels of this concentration were selected to demonstrate digest components whose apparent molecular size was in the range of 100,000 to 10,000. These included derivative chains released from the core (e.g. /α₁ to /α₂) as well as the smaller core fragments (e.g. Δ, E). Gels of 5% concentration (cf. Fig. 8) were more suitable for resolution of larger core fragments. Fig. 1 also includes digest clottability, parallel immunoelectrophoretic analyses done before and after heat precipitation, and results of certain gel scanning experiments. This figure thus serves not only as a primary data source but also as a reference to which other analyses of digest samples will be compared.

Identification of Peptide A in Fragment E—The usefulness of the enzymic method for identification of remnants depends on the fibrineopeptide remaining with the molecule under study. Since there is a plasmin-susceptible site at position 23 of the Aα chain (12), it became important to determine the phase of digestion at which this bond was cleaved. The method was therefore used to study Fragment E isolated from intermediate (i.e. E₃, Fig. 1, Gel 6) and advanced (i.e. E₄, Fig. 1, Gel 9) Stage 3 digests. (The isolation was carried out by procedures outlined later, see Fig. 11.) The presence of peptide A on Fragment E₃ was demonstrated enzymically in unreduced (Fig. 2) and in reduced samples (not shown, cf. Aα, Table I) and confirmed by a radioimmunoassay technique.² It may therefore be inferred that derivative subfractions (like I-9D40) occurring

² Dr. H. Nossel has analyzed these fragments by a radioimmunoassay procedure (58) specific for individual peptides. Peptide B was not detectable in either Fragment E₃ preparation. Peptide A was not detectable in E₄ but was found in E₅.
at earlier phases than those examined here also have enzymically identifiable $\alpha_4$ core remnants.

Fragment $E_2$ showed no evidence of peptide $A$ and, in addition, was smaller than $E_1$ even after the latter had been subjected to reptilase or thrombin treatment. This suggested not only that a fragment containing the $A$ peptide had been released, but also that hydrolytic attack had occurred at other sites (cf. $E_1$, Fig. 13). The failure to demonstrate the presence of peptide $B$ enzymically or by radioimmunoassay and the known plasmin resistance of the thrombin-susceptible site on the $B\beta$ chain (7, 10, 12) suggested that a derivative chain containing peptide $B$ is released from the core at a relatively early phase ($B\beta_{1/2}$, Table 1; cf. Fig. 13). Confirmation for this conclusion was obtained from studies of other derivative subfractions like $I-9D_{40}$ (see below).

**Subunit Structure of $I-9D_{40}$**—The elucidation of the degradative sequence, especially as it involved the subunit structure of Fragment $D$, was facilitated by the knowledge that the $\alpha\alpha$ chain is the first to be degraded (31-34, 38, 59) and that the hydrolysis results in the release of peptide fragments from the COOH-terminal region of this chain (38, 39). That is, it was possible to prepare plasmic derivative subfractions whose $\alpha\alpha$ chain population consisted almost entirely of $\alpha\alpha$ remnants smaller than the $\gamma$ chain, but which still retained some or most of its $B\beta$ and $\gamma$ chain populations in an intact form. The subfraction selected for study was one which possessed these properties, namely, $I-9D_{40}$ from a 40% clottable digest ($I-9D_{40}$).

CM-cellulose chromatography (Fig. 3) of the $S$-sulfo derivative of $I-9D_{40}$ yielded an elution profile which was more complex than that obtained for the $S$-sulfo derivative of the precursor material, Fraction I-4. The “$\gamma$” chain position (Zone II) was enriched relative to that of the “$B\beta$” (Zone IV) and “$\alpha\alpha$” (Zones V and VI) chain positions. Furthermore, material eluted between the $\gamma$ and $B\beta$ chain positions (Zone III) did not correspond in position to any peak in the precursor material. Though almost all identifiable $\alpha\alpha$ remnant chains occupied the $\Delta\alpha_4/\gamma_1$ and $\alpha\alpha/\gamma_4$ positions (Fig. 3, Gels 10 to 18), a very small amount of an $\alpha\alpha$ remnant occupying the $B\beta$ position in sodium dodecyl sulfate gels (i.e. $\Delta\alpha_4$) was detectable by enzymic analysis of chromatographic Zones V and VI (e.g. Gels 10 to 12). The relative amount of this derivative chain was too low to be detectable in the starting material (Gels 16 to 18). All other derivative chains which were not reactive with reptilase but were larger than a $\gamma$ chain (namely $\beta_1$, $\beta_2$, $\beta_3$) were therefore remnants of

**Table I**

| Chain designation | Representative plasmic derivative(s) or subfraction | Estimated molecular weight X 10^3 of reduced chain |
|-------------------|---------------------------------------------------|-----------------------------------------------|
| $\alpha_4$        | Intact chain                                       | 70.0                                      |
| $\alpha_4/\gamma_1$ | I-9D_{40}                                        | 57.7                                      |
| $\alpha_4/\gamma_3$ | I-9D_{40}                                        | 25.0                                      |
| $\alpha_4/\gamma_5$ | I-9D_{40}                                        | 22.0                                      |
| $\alpha_4/\gamma_7$ | $E_1$                                             | 8.6                                       |
| $\gamma_1$        | Free peptide                                      |                                            |
| $\gamma_1$        | Free peptide                                      |                                            |
| $\gamma_1$        | Free peptide                                      |                                            |
| $\gamma_1$        | $D_1$, ($F$)                                      |                                            |
| $\beta_4$         | Intact chain                                       | 60.0                                      |
| $\beta_4/\gamma_1$ | I-9D_{40}                                        | 57.8                                      |
| $\beta_4/\gamma_3$ | I-9D_{40}                                        | 54.7                                      |
| $\beta_4/\gamma_5$ | I-9D_{40}                                        | 51.1                                      |
| $\beta_4/\gamma_7$ | I-9D_{40}                                        | 42.0                                      |
| $\beta_4/\gamma_9$ | I-9D_{40}                                        | 41.3                                      |
| $\beta_4/\gamma_1$ | $D_1$                                             | 32.0                                      |
| $\beta_4/\gamma_3$ | Free peptide                                      | 22.0                                      |
| $\beta_4/\gamma_5$ | Free peptide                                      | 19.0                                      |
| $\beta_4/\gamma_7$ | $D_1$                                             | 34.3                                      |
| $\beta_4/\gamma_9$ | $D_1$                                             | 25.7                                      |
| $\beta_4/\gamma_1$ | $D_1$                                             | 22.7                                      |
| $\beta_4/\gamma_3$ | $D_1$                                             | 20.8                                      |
| $\beta_4/\gamma_5$ | Free peptide                                      | 5.7                                       |
| $\beta_4/\gamma_7$ | $D_1$                                             | 49.4                                      |
| $\beta_4/\gamma_9$ | $D_1$                                             | 42.0                                      |
| $\gamma_1$        | $D_1$                                             | 7.4                                       |

* Molecular weight previously determined (38).

Table 1

**Molecular weight estimates of fibrinogen chains and certain of their plasmic derivative chains**

1. The mean molecular weight of the $\beta_1$, $\beta_1/\gamma_1$ position in $I-9D_{40}$ was 42,200, whereas that of the $\gamma_1$ position in more advanced Stage 3 digest mixtures was 41,800. Comparison of these means suggested that the differences were not statistically significant ($P < 0.3$). The molecular weight of the position at all degradative phases has thus been taken as 42,000.

2. Migration in sodium dodecyl sulfate gels was the same whether sample was reduced or not.

3. Estimated by the difference between the intact chain and the larger derivative chain (i.e. $\beta_1$ or $\gamma_1$, respectively).

4. In our previous study (38) the molecular weight of the $\gamma$ chain was probably somewhat overestimated because it was computed as the mean of values obtained by electrophoresis of whole fractions (e.g. I-9) as well as isolated chromatographic peaks (cf. Fig. 3). It seems likely in retrospect that the putative $\gamma$ chain contaminant (Band 5 in those experiments) identified in $\alpha\alpha$ chain preparations was probably of $B\beta$ origin (i.e. $\beta_1$) and therefore of somewhat higher molecular weight than $\gamma$ chains. The present value for $\gamma$ chains has thus been recomputed with these factors taken into account.

5. V and VI chain positions. Furthermore, material eluted between the $\gamma$ and $B\beta$ chain positions (Zone III) did not correspond in position to any peak in the precursor material. Though almost all identifiable $\alpha\alpha$ remnant chains occupied the $\Delta\alpha_4/\gamma_1$ and $\alpha\alpha/\gamma_4$ positions (Fig. 3, Gels 10 to 18), a very small amount of an $\alpha\alpha$ remnant occupying the $B\beta$ position in sodium dodecyl sulfate gels (i.e. $\Delta\alpha_4$) was detectable by enzymic analysis of chromatographic Zones V and VI (e.g. Gels 10 to 12). The relative amount of this derivative chain was too low to be detectable in the starting material (Gels 16 to 18). All other derivative chains which were not reactive with reptilase but were larger than a $\gamma$ chain (namely $\beta_1$, $\beta_2$, $\beta_3$) were therefore remnants of
Bβ chains. The electrophoretic pattern suggests that the /β₁ and /β₂ derivatives arise via the release of a remnant containing peptide B. The /β₂ derivative also lacks peptide B; the demonstration that virtually all early Bβ remnants are subsequently cleaved to form the same remnant (namely /β₁) in Fragment D₁ (cf. Fig. 8, Gel II) suggests that /β₂ also arises by attack in the NH₂-terminal region (cf. Fig. 15). The /β₁ chain is measurably larger (Table I) than a γ chain and migrates in the cathodal region of the γ chain position. Its identity as a Bβ chain derivative is demonstrated not only by its migration in sodium dodecyl sulfate gels but also by its chromatographic difference from the γ chains (Fig. 3; cf. Gels I to S and 10 to 15) and its failure to undergo cross-linking (not shown).

The position designated /β₁, /γ₁, Bβ/γ (Fig. 3) is occupied by at least three types of derivatives from Bβ or γ chains; Bβ/γ is readily identifiable by enzymic criteria (Gels 1 to S). Proof of the γ chain origin of /γ₁ is developed later; however, the presence of this derivative is shown by the fact that the γ chain (except for the cleavage resulting in the removal of its NH₂-terminal region as part of Fragment E, cf. Fig. 13) is plasmin-resistant and /γ₁ continues to occupy this position during digestion of other portions of the molecule (see Fig. 8, Gels I through 15). By contrast /β₁, like other Bβ core derivatives, undergoes progressive degradation (see following section and Figs. 4 and 8).

**Fig. 3.** Elution patterns (lower portion) of S-sulfo chains of fractions 1-9D₄₀ (35 mg) and 1-4 (20 mg) subjected to gradient elution chromatography in 8 M urea on CM-cellulose column (0.9 x 30 cm). The point at which the gradient was begun is indicated by the vertical arrow. Each tube contained 4.5 ± 0.1 ml. In the case of S-sulfo 1-4, peaks in the elution pattern are designated according to the chains present, consistent with previous data and nomenclature (38, 60). In the case of S-sulfo 1-9D₄₀, eluate fractions were pooled as shown in Zones I to VI. Analysis of Zone I material (not shown) yielded very little stainable material, consistent with previous observations relating to S-sulfo derivatives (60). Gels (9% acrylamide) of starting material placed next to the /β₄ position of the cross-linked sample. The /β₃ derivative is demonstrated not only by its migration in sodium dodecyl sulfate gels but also by its chromatographic difference from the γ chains (Fig. 3; cf. Gels I to S and 10 to 15) and its failure to undergo cross-linking (not shown). The presence of a faint band beneath the γ-γ dimer suggests that /γ₁ derivative chains can participate in cross-linking. Nevertheless, the uncertainty of the completeness of such a reaction is indicated by the parenthetical (?) of cross-linked sample.
fragments were removed by heat precipitation. The supernatant in inhibited with Kunitz' pancreatic trypsin inhibitor and the core Tris-phosphate buffer, and subjected to gradient elution chroma-
tography on DEAE-cellulose (Fig. 5). Zone IV, containing the slower migration of derivative chains larger than 32,000 larger thermolabile core fragments by heat precipitation (Fig. 4).

The possible presence of disulfide bridges in these derivative chains was investigated after they had first been separated from the core at an early phase. The major remnants at this stage (/αα to /αθ) ranged in molecular weight from 40,500 to 48,700 and accounted, by their size, for the appearance of /α core remnants like /αα/θ1 and /αα/θ2 (molecular weight 25,000 and 22,600, respectively, see Table 1). At a somewhat later phase several large fragments (/βα/β1 and /βα/β2, Fig. 1) became evident. These first appeared after the /α remnants and continued to increase in amount at a time when /α chains or /α core remnants of sufficient size to generate such fragments had been consumed.

Isolation of the /βα chain on a preparative scale was accomplished in the following way. During plasmic hydrolysis of 700 mg of a mixture of 1-9D7 and 1-9D40, clottability was allowed to fall from 85% (starting mixture) to 62%. Plasmin was then inhibited with Kunitz' pancreatic trypsin inhibitor and the core fragments were removed by heat precipitation. The supernatant solution was freeze-dried, redissolved, dialyzed against 0.005 M Tris-phosphate buffer, and subjected to gradient elution chromatography on DEAE-cellulose (Fig. 5). Zone IV, containing the /βα chain was then chromatographed (not shown) on Sephadex G-100 to separate it from lower molecular weight fragments (Fig. 5, Gel 7).

Demonstration of the /βα chain origin of /βα was as follows.

Sequential sodium dodecyl sulfate gel electrophoresis analyses (analogous to those shown in Fig. 1) were carried out on plasmic digests (not shown) of fractions containing core fragments. Digestion of fractions which were devoid of /α chains or /αα remnants sufficiently large to be the precursor of such a peptide (cf. Fig. 3) nevertheless resulted in the progressive evolution of a chain migrating in the /βα position. This indicated that this derivative was of either /βα or /γ origin. Furthermore, the derivative chain must have been released from the COOH-terminal end of the molecule because the nature of the interchain disulfide bridges in the NH2-terminal region (12) precludes the release of a derivative chain of this size from the NH2-terminal end.

Immunoelectrophoretic experiments (Fig. 1) have demonstrated the appearance during Stages 1 and 2 of thermostable antigenic material.4 The nature of these antigenic species was explored by comparing digest supernatant fractions and purified /βα chains (Fig. 6). All Stage 1 and 2 samples tested formed immunoprecipitates with antifibrinogen serum. The precipitin line

4 In immunoelectrophoretic analyses of sequential digest samples, the precipitin arcs formed by /αα and subsequently by /βα chain remnants released from the core become less intense and finally undetectable (Fig. 1). In the case of /αα remnants it appears (see "Discussion") that the antigenic site is destroyed during plasmic hydrolysis, an occurrence which could account for its transient appearance in the early phases of Stage 1. However, there is also a disparity between the disappearance of the arcs attributable to /βα chain remnants (namely /βα), and the release of such fragments estimated densitometrically. One possible explanation for this disparity is that during intermediate degradative phases relatively small, fast diffusing, immunoactive peptides are formed in increasing amounts. These fast diffusing fragments exhaust available antibody, but do not produce a visible precipitin arc. Though this seems to be a reasonable explanation, it must await further experimental support.
formed by heated digest samples consisting primarily of $\alpha$ remnants (i.e. 89% to 80% digest, Fig. 6) was qualitatively different than that formed by the $\beta$ chain, though the pattern of later samples suggested that these two antigens were immunologically related. When these samples were reacted against antifibrinogen serum which had been absorbed with S-sulfo $\beta$ chains, a precipitin line still formed against the $\alpha$ remnants present in the 89% to 80% digest samples (not shown), but none formed against later digest samples or against the $\beta$ chain (cf. Fig. 9). These results showed a qualitative difference between the antigenic sites on $\alpha$- and $\beta$-chains and indicated that the $\alpha$ antigenic site was lost during plasmic hydrolysis. In contrast to the capacity of S-sulfo $\beta$ chains to absorb antibody against $\beta$ chains, antifibrinogen serum absorbed with S-sulfo $\gamma$ chains still formed a precipitin line against this derivative (not shown), demonstrating antigenic nonidentity of $\beta$ and $\gamma$ chains.

Samples of the $\beta$ chain yielded tryptic peptide maps (not shown) very similar to those given by preparations of $\gamma$ plus $\beta$, though some spots observed in $\beta$ maps were relatively faint in the latter. Whereas peptide maps of $\beta$ preparations exhibited similarities to maps of both the $\beta$ and $\alpha$ chains, they were distinguishable from those of $\gamma$ chains (Fig. 7 and Reference 38). The nonidentity of $\gamma$ chains and $\beta$ shown by immunodiffusion and peptide mapping experiments was further substantiated by studies of the subunit composition of Fragment D, which are described in the next section.

**Plasmin-resistant “Backbone” Structure of Fragment D—Immunoelectrophoretic analyses of digest mixtures showed the separation of the D and E antigenic determinants (Fig. 1) and were used to identify core fragments possessing the D antigen and lacking E. Further identification was made on the basis of characteristic chromatographic behavior on DEAE-cellulose (Fig. 11) coupled with immunodiffusion analyses of eluate fractions which showed the presence of the D determinant and the absence of the E. Sodium dodecyl sulfate gel electrophoretic analysis of unreduced digest samples revealed the presence of at least five discrete bands attributable to Fragment D (i.e. $D_1$ to $D_5$). In certain electrophoretic experiments a given band resolved into a doublet (e.g. Fig. 10, Gel 1), but no additional designation was assigned. The larger core species were abundant during earlier phases, whereas the smaller species were predominant in the more advanced phases of Stage 2 (Figs. 1 and 8).

Sodium dodecyl sulfate gel electrophoresis of reduced samples of Fragment D (Fig. 8) showed a plasmin-resistant derivative chain, molecular weight 42,000, which was identified as a $\gamma$ chain derivative (i.e. $\gamma_1$) on the following grounds. First, the known hydrolytic sites of cleavage of $\alpha\alpha$ and $\beta\beta$ chains (see above) preclude the possibility that a plasmin-resistant chain of 42,000 could arise from either $\alpha$-or $\beta$-chains. Secondly, confirmation of the $\gamma$ chain origin of the derivative was obtained from comparison of tryptic peptide maps of the S-sulfo derivatives of $\gamma$ chains and that of $\gamma_1$ (Fig. 7).

Analysis of reduced Fragment D species (Fig. 8, Gels 11 to 15) showed the absence of derivatives larger than intact $\gamma$ chains. The subunit structure of the most advanced Fragment D species, namely $D_1$ (Fig. 8, Gel 15), includes only two types of derivative chain identifiable by size (i.e. $\gamma_1$ and one of molecular weight 6,700). Since ultracentrifugal studies of Fragment D indicate that its molecular weight is not less than 80,000 (2, 18, 22, 63), it may be inferred that both $\gamma_1$ derivative chains are present in this fragment. Further support for the presence of two $\gamma_1$ chains per molecule of Fragment D was obtained from NH$_2$-terminal analyses.

**NH$_2$-terminal Analyses of Fragment D and Related Derivative Fractions—**NH$_2$-terminal analysis was carried out on Fragment D species isolated chromatographically from an advanced Stage 3 digest consisting predominantly of Fragments $D_4$ and $D_5$ (cf. Fig. 8, Gel 7). The major NH$_2$-terminal residues were aspartic acid, valine, methionine (in agreement with the findings of Marder et al. (14)), and threonine, plus small amounts of serine, alanine, and glycine. Since sodium dodecyl sulfate gel electrophoresis of reduced, dansyl-labeled samples yielded essentially the same band pattern as did unlabeled, reduced samples, elution and analysis of the fluorescent bands could be used to identify the NH$_2$-terminal acid(s) in any given band. Such determinations were carried out on a Stage 1 derivative (Subfraction I-9D*), a Stage 2 digest of clottability 0 to 10%,
FIG. 8. Determination of the subunit structure of Fragment D species. The gels (5% acrylamide) in the upper portion of the figure are of unmodified Fraction I-4 or sequential digest samples thereof. The samples for the lower gels (9% acrylamide) were obtained by elution of the given band in preparative electrophoretic experiments (Gels 9 to 11), by heat precipitation of digest samples (Gels 12 and 13), by a combination of DEAE-cellulose chromatography and gel chromatography on Sephadex G-100 (Gels 14 and 15), or by these chromatographic procedures after thermal denaturation to remove large core fragments (Gel 16). Gels not obtained from the same electrophoretic experiment were aligned with respect to known bands rather than with respect to the gel origins. Gels of Bands VII and VIII are aligned with that of a reduced sample of Fraction I-10D21 (containing intact γ chains) run in the same experiment (Gels 8 to 10). The recovery of Fragment D determined for the phase of degradation studied is shown above the corresponding gel.

Fig. 1, Gel 6), and the advanced Stage 3 digest having the NH$_2$-terminal residues given above. Alanine and small amounts of aspartic acid were identified in the /γ1, /β4 position in the Stage 1 and Stage 2 samples. However, aspartic acid was the sole NH$_2$-terminal residue in this position in the Stage 3 sample. This suggested that the sites of cleavage in the formation of /β4 and a precursor of /γ1 were Lys-Ala or Arg-Ala bonds or both. The finding of NH$_2$-terminal aspartic acid in this position at advanced phases of Stage 3 indicated that an additional bond (Lys-Asp or Arg-Asp), close to the initial cleavage site, had been broken to form /γ1 (see Footnote b, Table D). NH$_2$-terminal residues in other fluorescent regions of the gels were methionine, valine, and threonine, but no (or only a trace of) aspartic acid was detected. The observation that aspartic acid was the NH$_2$-terminal residue of /γ1, but of no other derivative chain in this type of preparation, indicated that quantitative determination of NH$_2$-terminal aspartic acid could be used as a measurement of the number of /γ1 chains present in Fragment D. Two such analyses indicated the presence of 1 mole of aspartic acid for every 36,000 and 34,000 g of protein, respectively. (In the second analysis bovine albumin, which is known to have 1 mole of NH$_2$-terminal aspartic acid per 67,000 g, was used as a control. The experimentally determined value was 1.0 to 1.5 moles /67,000 g.) The results were thus consistent with the previous conclusion that two /γ1 derivative chains exist in each Fragment D species.

Size Heterogeneity of Fragment D Species—The subunit composition of the various Fragment D species (Fig. 8) suggested that the observed heterogeneity (i.e., D1 to D6) was attributable to the degree of fragmentation (and release) of βγ chains. It had been shown that the /γ1 position in gels obtained by electrophoresis of reduced Fragment D1 was transiently occupied by a large βγ core remnant (/βγ). Since the plasmin-resistant nature of the /γ1 chain had been established, it could be con-
cluded that all subsequently appearing chains smaller than $/gamma_1$ were of B$beta$ origin. Densitometric scans of gels from sequential digests of I-4 showed that both early and intermediate species of Fragment D were capable of releasing substantial amounts of $/beta$ derivatives (Fig. 1). As this release progressed, Fragments D of commensurately smaller size were generated (Fig. 8, Gels 2 to 7; cf. Fig. 1, Gels 8 to 9). The smallest Fragment D species identified, D$_a$, probably represented the ultimate stage of Fragment D heterogeneity attributable to B$beta$ chain fragmentation, because it was virtually devoid of B$beta$ remnants $/beta_2$ and $/beta_3$ (Fig. 8).

**Formation of Antigenic Fragments F from Fragment D**—At least one other discrete process was evident from sodium dodecyl sulfate gels of unreduced digest samples. This process was characterized by the formation of 4 to 5 bands, collectively termed Fragments F, which migrated at a greater rate than did Fragment E (Figs. 1, 8, and 11) and which contained interchain disulfide bridges. These F fragments from an advanced Stage 3 digest were concentrated in a chromatographic peak (Peak F, Fig. 11) which was eluted from DEAE-cellulose after the main Fragment D region. Fragments F from this phase of digestion were prepared free of other antigenic material by thermal denaturation to remove Fragment D species, chromatography of the supernate on DEAE-cellulose and collection of Peak F. Gel chromatography of this material on Sephadex G-100 served to remove lower molecular weight material which had been eluted from DEAE-cellulose in the same region.

Immunoelectrophoresis of whole digest samples from advanced phases of degradation (Fig. 1, Gel 9) revealed a previously unrecognized precipitin arc (F) in a position intermediate between the arcs attributable to Fragments D and E. In sharp contrast to Fragments D, the antigen responsible for arc F remained in the supernatant fraction after heating. Its antigenic nonidentity with Fragment F was evident from the immunodiffraction pattern (Fig. 1) and from immunodiffusion experiments in which chromatographically isolated Fragments F were compared with other plasmic derivatives (Fig. 9). F formed a line of partial identity with D$_a$, showing that these fragments possessed at least one common antigenic determinant (Determinant F). This pattern was not altered by absorption of the antiserum with B$beta$ chains. Thus, neither Determinant F nor the determinant which was unique to Fragment D (Determinant D) was related to that associated with $/beta_2$ (Fig. 9).

Unequivocal evidence that Fragment D was the precursor of Fragment F was provided by an experiment in which isolated Fragment D (Fig. 11) was degraded by the addition of plasmin (Fig. 10). The fact that degradation was associated with a progressive decrease in staining intensity of D fragments (namely D$_d$ and D$_s$) suggested that the formation of the F fragments occurred at the expense of Fragment D and that the transition from D$_d$ to D$_a$ was the consequence of another process (i.e., release of B$beta$ derivative chains).

The main subunit chain of reduced Fragment F preparations migrated at the same rate as the $/alpha_3$ derivative seen in reduced Fragment D (Fig. 8, Gels 11 to 16), suggesting that they both represent the same chain. The slower, faintly staining bands (e.g., Fig. 8 arrows, Gel 16) may have arisen from $/gamma_1$, a possibility which will require confirmation.

**Difference between Derivatives VII and VIII**—Samples of Derivatives VII and VIII were prepared from Stage 2 digests (e.g., Fig. 8, Gel 2) by elution from sodium dodecyl sulfate gels. Analyses of reduced samples showed, in both cases, considerable depletion of the intact $gamma$ chain position as well as an absence of intact B$beta$ and A$alpha$ chains (Fig. 8, Gels 9 and 10). The $/gamma$ and $/beta_3$ chains did not resolve; nevertheless, the presence of some intact $gamma$ chains was established by the finding that $gamma$ dimer, of the size expected from the cross-linking of two intact $gamma$ chains, was formed in cross-linking experiments (not shown) with unmodified samples. The predominant B$beta$ derivative chains in both VII and VIII appeared to be $/beta_2$ and $/beta_3$. Moreover, the band containing $/gamma_1$ was present in both, as were appreciable amounts of A$alpha$/B$beta$ (shown by enzymic analysis). The demonstration of the A$alpha$/B$beta$/B$gamma$ chains established the presence,
in both VII and VIII, of the NH$_2$-terminal region of fibrinogen from which Fragment E is derived.

Although electrophoresis of the reduced samples showed no qualitative differences between VII and VIII, densitometric scans (Table II) indicated quantitative differences between them. The densitometric ratio of [\(\alpha_\alpha/\alpha_\delta + \alpha_\varepsilon/\alpha_\delta\)] to [\(\alpha_\gamma\)] in VII [\(\beta_\delta\)] was virtually the same in both VII and VIII, suggesting minimal changes in these derivative chains during the formation of VIII. However, there was a remarkable difference in the densitometric ratio of [\(\beta_\delta + \beta_\varepsilon\gamma\)] to [\(\gamma_\gamma\)] and [\(\alpha_\gamma\) (i.e. 2.83 for VII, 1.58 for VIII)]. This implied that the essential difference between VII and VIII was the loss of a \(\beta_\delta\) remnant like that previously shown (Figs. 1 and 4) to be cleaved from the core during this phase of digestion (namely [\(\beta_\delta\]).

**Recovery of Fragment D from Plasmic Digests**—The following experiments were designed to examine the basis for the monomeric D hypothesis by determination of the recovery of Fragment D at successive phases of degradation. During early and intermediate phases of the third stage of degradation of Fraction 1-4 (e.g., Gels 3 to 6, Fig. 8), thermal denaturation resulted in complete precipitation of Fragment D species free of other digest components. Determination of the recovery of Fragment D from these mixtures could therefore be made spectrophotometrically by comparing the absorbance of the washed, solubilized (in alkaline urea) precipitate fraction obtained from a thermally denatured digest sample with that of an equivalent amount of starting material (i.e. Fraction 1-4). Corrections were applied for the absorbance coefficient of 1-4 (\(A_{\text{abs}}^1\), 282 nm = 18.6) and that of a preparation of Fragment D isolated from this phase of degradation (\(A_{\text{abs}}^4\), 282 nm = 17.9). Under these conditions, the recovery ranged from 60% at an early phase of Stage 3 when Derivatives VII and VIII had been virtually all consumed and D$_1$ was the main species of Fragment D (Fig. 8, Gel 3), to 48.1% at an intermediate phase of Stage 3 when considerable amounts of smaller species (i.e. D$_2$ to D$_3$) had formed (Fig. 8, Gel 6).

At more advanced degradative phases thermal denaturation procedures were inadequate. Estimation of recovery was therefore made by gradient elution chromatography (Fig. 11) on DEAE-cellulose in a manner similar to the technique originally employed by Nussenzweig et al. (2). Fragments D and E were identified by immunodiffusion experiments (see insets, Fig. 11) and by their positions in sodium dodecyl sulfate gels. The absorption of the chromatographic peaks containing these fragments was used to estimate their apparent recovery (Fig. 11, bracketed numbers). Subsequently, for each peak, the proportion of absorbance which was not due to core Fragments D and E was determined by gel chromatography on Sephadex G-100 and appropriate correction of the apparent recovery was made. It seems likely that the F fragments which had formed at this phase were also included in the estimated recovery of D (Fig. 11, Gels 3 and 4). For the peak containing both Fragments D and E (i.e. Peak G), an additional minor correction was made by visual estimation of the relative distribution of D, E, and F (85% D plus F, 15% E) in sodium dodecyl sulfate gels (Fig. 11, Gel 4). Finally, a correction was made for the absorbance coefficients of Fragments D and E from this digest (\(A_{\text{abs}}^4\), 282 nm = 18.6 and 17.8, respectively) and that of 1-4. After these corrections the estimated recovery of Fragment D from this digest was 28.7%; that of Fragment E, 10.5%. Analysis (not shown) of a similar advanced Stage 3 digest gave values of 34.3% and 12.7% for Fragments D and E, respectively.

**DISCUSSION**

**Considerations of Structure of Fibrinogen** (Figs. 12 and 13)—The foregoing information regarding the sequence of events during plasmic hydrolysis, plus data available in the literature, has permitted elucidation of essential features of the structure of fibrinogen. It points to a covalently linked dimeric structure of which the backbone is a pair of \(\gamma\) chains linked directly to each other by disulfide bridging in the NH$_2$-terminal region (12)
Fig. 12. Schematic diagram depicting the major events in the formation of plasmic derivatives. The diagram is not intended to imply the molar content of any given fragment but rather is intended to indicate the region(s) of the molecule whose cleavage results in the formation of another derivative. The derivative species shown are those identified in our sodium dodecyl sulfate gel electrophoretic and related experiments (e.g. D1 to D5, /LY, etc.). For clarity the analogous derivatives X and Y identified by Marder et al. (14) in acidic gels are indicated parenthetically.

Fig. 13. Schematic diagram of the structure of fibrinogen and of certain plasmic derivatives. In the upper portion of the figure is shown a longitudinal representation of the half-molecule. Also shown to the left is an end-on view of the NH2-terminal region of the molecule (N-DSK) to indicate its dimeric structure. Disulfide bridges whose precise location has been reported (12) are indicated by solid lines (—); those whose presence has been established by the present studies but whose precise location is still uncertain are indicated by hatched lines ( ). Certain of the known cleavage sites are indicated by vertical arrows (↑) which are numbered to correspond to sites previously established (38) (e.g. numbers 2, 4, 6, 8, 11, 13 on the Aα chain) or which have been established by the present studies. Thrombin cleavage sites are also indicated (T). In the lower portion of the figure are shown the essential structural features of certain representative plasmic derivatives (i.e. Bβ/13, E1, D5, /α11, and /β6). The minimum number of interchain disulfide bridges (five) required for the postulated structure of Fragment Ds is suggested by the end-on representation of this derivative.
not participated in interchain disulfide linkages and that they originated from the COOH-terminal region of the molecule.

Evidence for the symmetrical, covalently linked dimeric structure of the NHi-terminal region of fibrinogen was provided by the identification of the NHi-terminal portions of all six chains in the N-DSK (11–15). Further evidence included establishment of the structural overlap of the N-DSK with plasmic Fragment E (14, 16) and the demonstration of one Fragment E per fibrinogen molecule (16, 18). Additional support for this structure and identity has been obtained by showing the presence of peptide A in early and intermediate forms (i.e., $E_3$) of Fragment E (Fig. 2, Footnote 3, and References 14, 19, and 66). The finding that Fragment E$_3$ isolated from more advanced digests lacked this peptide (Fig. 2 and Footnote 3) confirms the observations of Mills (19) and is consistent with known plasmin-susceptible sites (12, 13).

The following results indicated that the backbone of fibrinogen includes both $\gamma$ chains linked in a second region to form a dimeric structure which appears as Fragment D during plasmic hydrolysis. Tryptic peptide maps of S-sulfonamidated $\gamma_1$ chains prepared from Fragment D and those of intact $\gamma$ chains were very similar (Fig. 7) and were unlike those of B$\beta$ or $\alpha$ chains. Furthermore, the degradative sequence of B$\beta$ and $\alpha$ chains elucidated in this study precluded the possibility that the plasmogen-resistant chain identified as $\gamma_1$ (Fig. 8) could arise from any other chain than $\gamma_1$. Since aspartic acid was shown to be the NHi-terminal residue of $\gamma_1$, but of no other derivative chain present in advanced Stage 3 digests, the finding of 1 mole of NHi-terminal aspartic acid for each 34,000 to 36,000 g of protein was evidence that two such chains were present in each Fragment D. That is, data available in the literature indicate that the smallest Fragment D species (i.e., D$_2$) is not likely to have a molecular weight of less than 80,000 (63); it may prove to be considerably larger. Sodium dodecyl sulfate gels of reduced Fragment D$_2$ exhibited only two bands (Fig. 8, Gel 15), one attributable to $\gamma_1$ (molecular weight 42,000) and the other, to $\alpha_1/\gamma_1$ (molecular weight 6,700).

A molecular weight of 80,000 or higher thus does not appear possible unless two such $\gamma$ chain derivatives are present in the fragment. The same conclusion arises even when molecular weights obtained by sodium dodecyl sulfate gel electrophoresis of unreduced Fragment D (16, 19) are used, despite the fact that these conditions almost certainly lead to an underestimate of the molecular weights of disulfide bridged proteins (69–70).

In addition to the disulfide bridges known to exist in the N-DSK (12), intrachain bridges have now been demonstrated in other regions of both $\alpha$ and $\beta$ chains (Fig. 4; cf. Fig. 13), thus accounting for at least four of the 28 to 29 disulfide bridges in fibrinogen (71). Moreover, at least five interchain bridges must exist in that portion of the molecule which forms Fragment D$_2$ (cf. Fig. 8, Gels 15 to 18 and Fig. 13). Comparison of the present results with those of Gårdlund et al. (16, 72) reveals some relationships between the disulfide-containing fragments they isolated after CNBr cleavage of fibrinogen or Fragment D (H-I, H-II, H-III) and the disulfide bridged regions we have identified. Fragments H-I and H-II were formed from both Fragment D and fibrinogen; Fragment H-III, only from fibrinogen. This observation permitted the speculation that H-III represents an intrachain disulfide-containing region of the $\alpha$ chains. The size of H-I and the number (four to six) of bridges present in this fragment suggest that it contains a multichain portion of the molecule present in Fragment D$_2$. The H-2 fragment may arise from the intrachain disulfide bridge region of the B$\beta$ chain.

Though hydrolytic separation of Fragments D and E requires treatment of at least one peptide bond in each of the six constituent chains, the subunit structure of derivative Bands VII and VIII (Fig. 8) suggests that of these critical bonds, those in the $\gamma$ chains ($\gamma_1\rightarrow\gamma_1/\gamma_1$) are cleaved faster than those in the other chains. Furthermore, the separation of D and E occurs more rapidly than the release of $\beta_\alpha$. Therefore, chains of B$\beta$ origin continue to be released from the COOH-terminal region after all core species which could have generated Fragment E have been consumed (Figs. 1 and 8). This combination and sequence appears to account for the occurrence of virtually all possible major species intermediate between those possessing Fragment E plus both COOH-terminal regions of B$\beta$ chains (derivative Band VII) and those containing only the $\gamma$ chain backbone plus small covalently linked remnants of $\alpha$ and B$\beta$ chain origin (D$_2$).

The largest of these intermediate species (Band VIII) corresponds to the Fragment Y band identified by Marder et al. (14) and by other investigators (32, 34) and appears to have been formed via release of a $\beta_\alpha$ chain from derivatives like Band VII (Fig. 8, Table II). Release of Fragment E prior to removal of $\beta_\alpha$ or related derivative chains (i.e., from Band VII) results in fragments migrating in the region of D$_2$, whereas release of E from derivatives like Band VIII gives rise to faster migrating species (e.g., D$_2$ to D$_3$). Heterogeneity of Fragment D (manifested as D$_1$ to D$_3$) is thus the consequence of the separation of Fragment E from precursors possessing different portions of the COOH-terminal regions of B$\beta$ chains plus continuing release of $\beta_\alpha$ derivatives from early and intermediate D species (Fig. 12).

In addition to size heterogeneity of Fragment D, electrophoretic heterogeneity has also been observed under conditions in which charge was an influential factor (23, 28, 62, 73, 74). These findings may have reflected transitional heterogeneity created during fragmentation because of nonuniform charge distribution within the parent molecule. Furthermore, there are charge heterogeneities, such as those recently described in $\gamma$ chains (60, 75), which are intrinsic to fibrinogen itself. Such heterogeneities may have been expressed in these analyses not only in terms of the charge differences in the fragments but also in the possible influence of charge in directing cleavages. An unambiguous demonstration of the presence (or absence) of intrinsic heterogeneity within Fragment D would therefore necessitate comparison of D species which had been degraded to the same extent.

**Consideration of Monomeric Fragment D Model**—The rationale for the monomeric D model of fibrinogen, first proposed by Nussenzweig et al. (2) and subsequently by Marder and co-workers (14, 18, 24), rests upon the estimated molecular weight of Fragment D and upon the recovery of such fragments from plasmic digests. Data provided by the present study plus information in the literature indicate that the monomeric D hypothesis is incorrect. First, the core fragments identifiable as D have a covalently linked dimeric structure which contains the major portion of both $\gamma$ chains, indicating that there can be only 1 mole of such a fragment derived from each mole of fibrinogen. Furthermore, molecular weight estimates of Fragment D considerably in excess of those cited in support of the model have been reported. Most ultracentrifugal estimates of the molecular weight of Fragment D have been in the range of 80,000 to 88,000 (2, 17, 18, 24, 62, 63), though Mihalyi and Godfrey (76) found the molecular weight of bovine tryptic Fragment D to lie between 93,700 and 103,500. Gårdlund et al. (16) estimated the molecular weight range of Fragments D recognizable as bands in sodium dodecyl sulfate gels to be 86,000 to 96,000. Mills (19),
who employed unreduced proteins as markers in sodium dodecyl sulfate gel electrophoresis, computed molecular weights ranging from 95,000 to 130,000. On the basis of gel electrophoresis and immunological analyses, Fisher et al. (17, 22) concluded that there were early forms of Fragment D with molecular weights of 150,000 or more. Our results suggest that early forms of Fragment D have a molecular weight in the range of that reported by Mills (19) and Fisher et al. (17) or higher.

The present studies have demonstrated (Figs. 8 and 11) that the recovery of core Fragments D varies widely (i.e., from 60% to 28.7% or potentially even lower) and is a function not of the molar content of D but rather of the degree of enzymic degradation which has taken place. Therefore, any valid calculation of the number of core fragments must take into account the molecular weight of the species of Fragment D existing at that phase of degradation.

There has been no direct evidence to support the structure proposed for Fragment Y, i.e., lacking one Fragment D. Our analyses of Band VIII (Y) indicate that this derivative is formed by the release of a single /β chain from a core fragment of higher molecular weight (Band VII). The expected size of such a derivative is consistent with recent estimates for the molecular weight of Fragment Y reported by Plow and Edgington (36) and Mills (19), i.e., over 200,000.

Consideration of Other Studies of Plasmic Hydrolysis of Fibrinogen—The monomeric D model has been an implicit or direct assumption in the structural interpretations of many recent studies which utilized electrophoresis in sodium dodecyl sulfate gels to investigate the plasmic hydrolysis of fibrinogen (16, 19, 31–35, 77, inter alia). Apparent discrepancies between these studies and our present work are primarily a function of constraints imposed by the assumed monomeric model. The main differences can be resolved by the following considerations. In certain studies, the molecular weight of Fragment D was calculated by summing molecular weights of the subunit chains detected in sodium dodecyl sulfate gels, a calculation which involved the assumption of a monomolecular contribution by each subunit chain (16, 19, 31, 35, 77). Molecular weight estimates based on the migration of unreduced fragments have also been considered compatible with the monomeric model (16, 32, 77) or, as in the case of Mills (19), were discounted when the estimated upper value for D was found to be above 100,000 (namely 130,000). The latter investigator discounted the values because he assumed that these disulfide bridged species behaved as reduced protein (i.e., a relatively noncompact structure). The unlikelihood of such behavior has been emphasized in several recent studies (68–70) as well as ours (e.g., Fig. 4), by the observation that disulfide bridged molecules migrate as if reduced protein (i.e., a relatively noncompact structure). The recognition of three separate antigenic determinants in Fragment D (namely /βα, D, F) raises the question of the relationship between these determinants and those described recently by Plow and Edgington (36, 82). Though these investigators have assumed the monomeric D hypothesis, their data are also consistent with the dimeric structure proposed above. That is, Plow and Edgington were able to identify two distinct antigenic regions in Fragment D (36). One of these, termed “neoacontgen” (36, 82, 83), was detected with an anti-D serum which had been absorbed with fibrinogen; the other, detected with unabsorbed anti-D serum was termed “total D-fragment-associated antigen” (36). They found two of the latter antigenic sites in Fragment X and only one in Fragment Y. However, they found only a single neoacontgen site per molecule of Fragments X, Y, or D, although two should have been found in X to conform to the monomeric hypothesis. To account for this apparent paradox they proposed that only one of the neoacontgen sites in X was expressed, whereas the other was “latent.” Our results would suggest that the total D-fragment-
associated antigen corresponded to the \( / \beta _3 \) antigenic determinant of which there are two in X (Derivative VII, Fig. 8) and one in Y (Derivative VIII, Fig. 8). The neoantigenic site(s) probably corresponds to the region in Fragment D containing the F or D determinant(s) or both. A covalently linked dimeric fibrinogen molecule would be expected to generate Fragments X, Y, and early and intermediate Fragments D which contained only one such region.

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Addendum—Since this manuscript was submitted, Kowalska-Loth et al. (84) have reported the sequence of the first 78 residues of the \( \gamma \) chain. No potential plasmic cleavage site which could give rise to NH\(_2\)-terminal aspartic acid was evident.

Pizzo et al. (85) have recently reported that degradation of cross-linked fibrin by plasmin yields a resistant derivative of \( \gamma \) (\( \gamma’-\gamma’ \)). The ultimate Lys-Gln bond (86) at the COOH-terminal cross-linked fibrin by plasmin yields a resistant derivative of \( \gamma \) whose structure overlaps that of \( \gammaJI \) (Table 1, Fig. 8). These investigators had previously assumed (32) that the plasmatic-resistant chain of fibrinogen digests corresponding in size to \( \gammaJI \) of \( \beta _3 \) origin (\( \beta’ \) in their nomenclature), and they therefore concluded that the introduction of cross-links at the COOH-terminal end of the molecule must have conferred plasmatic resistance on a more distal portion of the \( \gamma \) chain (i.e., \( \gammaJI \)). Our present demonstration of the plasmatic-resistant nature of the \( \gammaJI \) chain of fibrinogen, plus the relative plasmatic resistance of isolated S-carboxymethyl \( \gamma \) chain (89), indicate that such a conclusion is as yet unwarranted. Nevertheless, this (89) and other recent studies (90, 91) suggest that this approach (viz. analyses of plasmic derivatives from cross-linked fibrin) may provide another method of testing the two structural models of fibrinogen.

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