A Re-examination of the Cleavage of Fibrinogen and Fibrin by Plasmin*

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Three Fragment D species (D1, D2, D3) were isolated with time from a plasmin digest of fibrinogen and had molecular weights of 92,000, 86,000, and 82,000 by summation of subunit molecular weights from sodium dodecyl sulfate polyacrylamide gel electrophoresis. Their molecular weights by sedimentation equilibrium ultracentrifugation were 94,000 to 87,000, 88,000 to 82,000, and 76,000 to 70,000 depending on the values calculated for the partial specific volumes. Each of the Fragment D species contained three disulfide-linked subunits derived from the Aα, Bβ, and γ chains of fibrinogen and differed only in the extent of COOH-terminal degradation of their γ chain derivatives. Plasmin cleaved Fragment D1, to release the cross-link sites from its γ′ subunit of 38,000 molecular weight; however, the β′ subunit of 42,000 molecular weight and the α′ subunit of 12,000 molecular weight were resistant to further digestion by plasmin. Fragment D isolated from highly cross-linked fibrin had a dimeric structure due to cross-link formation between the γ′ subunits of two fibrinogen Fragment D species. The molecular weight of fibrin Fragment D was 184,000 by summation of subunit molecular weights and 190,000 to 175,000 by sedimentation equilibrium. Cross-linking the γ chain, as well as incorporating the site-specific fluorescent label monodansyl cadaverine into the γ chain cross-link acceptor site, prevented its COOH-terminal degradation by plasmin. Therefore, only one species of fibrin Fragment D, as well as only one species of monodansyl cadaverine-labeled fibrin Fragment D monomer, was generated during plasmin digestion. These results show unequivocally that each fibrinogen Fragment D contains only three subunit chains and therefore the digestion of fibrinogen by plasmin must result in the production of two Fragment D molecules from each fibrinogen molecule. The recently proposed model of fibrinogen cleavage that postulates the generation of a single Fragment D with three pairs of subunit chains from each fibrinogen molecule is incorrect.

Incorporation of monodansyl cadaverine into the cross-link acceptor sites of the α chain did not alter its cleavage by plasmin detectably. A series of monodansyl cadaverine-labeled peptides, which ranged in molecular weight from 40,000 to 23,000, were cleaved from the α chain of monodansyl cadaverine-labeled fibrin monomer during the early stages of plasmin digestion. These peptides were degraded progressively to a brightly fluorescent plasmin-resistant peptide of 21,000 molecular weight and a weakly fluorescent peptide of 2,500 molecular weight. Thus both α chain cross-link acceptor sites are contained within a peptide segment of 23,000 molecular weight.
Mosesson et al. (11) which gives one Fragment D and one Fragment F as terminal products. The molecular weights and symbols for the subunit chains are from that publication. Their model is depicted schematically like the model in A to facilitate comparison. The major difference between the two models is the presence of at least one disulfide bridge between the Fragment D regions of the γ chains in B.

**MATERIALS AND METHODS**

**Reagents**—Except where specified, all reagents were analytical reagent grade and were obtained from commercial sources. Tris-NaCl buffer refers to 0.05 M Tris-0.15 M NaCl buffer, pH 7.4, unless stated otherwise. Monodansyl cadaverine was made 7.5 mM in Tris-NaCl buffer and stored at 4°C. A 0.01 M solution of p-nitrophenyl-p-guanidinobenzoate was prepared according to the method of Chase and Shaw (12). Ultrapure guanidine hydrochloride (Heico) was used for ultracentrifugal analyses.

**Fibrinogen**—The human fibrinogen used in these studies was 90% clottable and contained sufficient fibrin-stabilizing factor as a contaminant to catalyze complete cross-linking during fibrin formation. The plasmid formed from urokinase activation of the contaminant plasminogen in the fibrinogen was enough to digest fully the fibrinogen or fibrin made from this preparation.

**Enzymes**—Human urokinase was dissolved in Tris-NaCl buffer to a concentration of 5000 Ploug units/ml and stored at -20°. Human thrombin, kindly supplied by Dr. D. L. Aronson (Bureau of Biologics, FDA) was dissolved immediately before use in Tris-NaCl buffer at 90 NIH units/ml. Fibrin-stabilizing factor (Factor XIII) was measured immediately prior to use by the [14C]putrescine incorporation assay (14).

**Polyacrylamide Gel Electrophoresis**—Polyacrylamide gel electrophoresis in the presence of Na dodecyl-SO₄-polyacrylamide gel electrophoresis was performed as previously described (3, 15). Gels of samples with fluorescently labeled cross-link acceptor sites were examined for fluorescence and photographed through a green filter under ultraviolet light (shortwave lamp UVS-11, Ultra-Violet Products) using a Polaroid MP-3 camera and Type 55/Positive-Negative film. Gels were subsequently stained for protein with Coomassie brilliant blue at 65-70°C for 30 min and destained in 14% acetic acid/7% methanol. Photographs of protein-stained gels were taken for comparison with fluorescent gel photographs. Some gels were stained for carbohydrate using the periodic acid-Schiff reagent as previously described (3).

**Isolation of Fibrinogen and Fibrin Fragment D Species**—Fibrinogen Fragment D species were isolated from plasmin digests of fibrinogen as previously described (8). Digestion was initiated, complete γ cross-linking and extensive α polymerization of the fibrinogen was documented by Na dodecyl-SO₄-gel analysis. Urokinase was added to a final concentration of 10 Ploug units/ml in order to convert the contaminant plasminogen to plasmin and thereby initiate fibrin digestion. The reaction mixture was incubated at 37° with stirring for 24 hours, by which time the fibrin was digested completely and the solution was clear. Fibrin Fragment D was then separated from the reaction solution by ion exchange and gel filtration chromatography as described for fibrinogen Fragment D.

**Isolation of Fragment D from Dns-cadaverine-labeled Fibrin**—The
method of Lorand et al. (16) was used to incorporate Dns-cadaverine into the cross-link acceptor sites of fibrin monomer; fibrinogen was eluted off resin except for a small fraction containing fibrinogen, which was made 5 mM in Dns-cadaverine. Under these conditions, only the $a$ and $7212$ chains of fibrin become labeled (16); Dns-cadaverine competitively inhibits most of the $\gamma$ dimerization and virtually all $a$ polymerization. The fibrin clot was washed exhaustively in the Tris-NaCl buffer to remove free Dns-cadaverine and then suspended in the same buffer. Digestion was initiated by adding urokinase to a final concentration of 10 Ploug units/ml; the reaction mixture was then allowed to incubate at 37° with constant stirring. By 12 to 18 hours the fibrin was completely and the Dns-cadaverine-labeled fibrin Fragment D was isolated from the digestion solution as described for fibrinogen Fragment D. A time course digestion of Dns-cadaverine-fibrin was also performed under the above conditions; aliquots of the supernatant fluid, and ultimately the entire digestion mixture, were removed with time and analyzed by Na dodecyl-SDS gel electrophoresis. The same conditions were used except that the reaction mixture was made 5 mM in Dns-cadaverine.

**Experiments**—In the later portion of the peak, as previously observed (8). Fractions containing a Fragment D species in purest form were pooled and then chromatographed on 6% agarose. The gel filtration patterns of fractions from the 30-, 45-, and 60-min fibrinogen digests are shown in Fig. 2. When present, Fragments X and Y always eluted in Peak 1 and Fragment D was always contained in Peak 2. In the 30-min digest Fragments X and Y predominate, but in the 45-min digest about half of the applied sample was Fragment D. After 60 min of digestion, the mixture contained predominately Fragment D and no Fragment X or Y. Fragment D from cross-linked fibrin and Fragment D from Dns-cadaverine-labeled fibrin were isolated similarly. Fig. 3 shows the Na dodecyl-SDS gel pattern for each of the isolated, nonreduced Fragment D species. The Fragment D recovered from the 30-min fibrinogen digest was isolated in very small quantities and was contaminated by significant amounts of Fragments X and Y. The Fragment D species isolated from later digests were single-banded, with the exception of the 60-min digest which showed two Fragment D species. The Fragment D species isolated from the 45-min, 8-hour, and 24-hour digests were arbitrarily designated $D_1$, $D_2$, and $D_3$, respectively. Fragment $D_3$, isolated from the 45-min digest, corresponded in mobility to the earliest and largest Fragment D in the 30-min digest.

**RESULTS**—The purified fibrinogen Fragment D species and fibrin Fragment D were each hydrolyzed in vacuo at 110° for 22, 48, and 72 hours in 1 M HCl and 0.1% (w/v) phenol. Each hydrolysate was then analyzed by standard chromatographic methods (21, 22) on a Spinnco model 119 amino acid analyzer. Methionine and cysteine were determined as methionine sulfoxide and cysteic acid, respectively, from separate 22-hour hydrolyses following performic acid oxidation (23). The $\alpha$-chain content was determined from 22-hour hydrolyses with p-toluene-sulfonic acid (24).

**Isolation of Fragment D Species**—The chromatograms of the fibrinogen digests on DEAE-cellulose were all similar to those previously published (8). Fractions of the peak containing Fragment D as well as variable amounts of Fragments X and Y, depending on the length of digestion, were examined on Na dodecyl-SDS gels. Fractions containing single Fragment D species were pooled. When mixtures of Fragment D species were present the earlier Fragment D species was generally in highest concentration in the initial portion of the peak containing Fragment D, whereas the later species was in higher concentration in the later portion of the peak, as previously observed (8). Fractions containing a Fragment D species in purest form were pooled and then chromatographed on 6% agarose. The gel filtration patterns of fractions from the 30-, 45-, and 60-min fibrinogen digests are shown in Fig. 2. When present, Fragments X and Y always eluted in Peak 1 and Fragment D was always contained in Peak 2. In the 30-min digest Fragments X and Y predominate, but in the 45-min digest about half of the applied sample was Fragment D. After 60 min of digestion, the mixture contained predominately Fragment D and no Fragment X or Y. Fragment D from cross-linked fibrin and Fragment D from Dns-cadaverine-labeled fibrin were isolated similarly. Fig. 3 shows the Na dodecyl-SDS gel pattern for each of the isolated, nonreduced Fragment D species. The Fragment D recovered from the 30-min fibrinogen digest was isolated in very small quantities and was contaminated by significant amounts of Fragments X and Y. The Fragment D species isolated from later digests were single-banded, with the exception of the 60-min digest which showed two Fragment D species. The Fragment D species isolated from the 45-min, 8-hour, and 24-hour digests were arbitrarily designated $D_1$, $D_2$, and $D_3$, respectively. Fragment $D_3$, isolated from the 45-min digest, corresponded in mobility to the earliest and largest Fragment D in the 30-min digest.

**Fig. 2.** Agarose chromatography of the protein peak from DEAE-cellulose which contained fibrinogen Fragments X, Y, and D, depending on length of digestion. Peak 1 contained Fragments X and Y. Peak 2 contained Fragment D. See text for experimental details.
Incorporate Dns-cadaverine—When cross-linked in the presence of Dns-cadaverine, fibrinogen Fragment D species. Fragment D, from the 45-min digest, corresponded to the earliest Fragment D in the 30-min digest. The 8- and 40-min digest contained both Fragments D, and D, The 8- and 40-min digest contained both Fragments D, and D, respectively.

Ability of Fibrinogen Fragment D Species to Cross-Link or Incorporate Dns-cadaverine—Fig. 5 shows that significant cross-linking occurred only when fibrinogen Fragment D was incubated with fibrin-stabilizing factor, thrombin, and calcium. The nonreduced, cross-linked Fragment D, species corresponded in mobility on Na dodecyl-SO₄ gels to fibrin Fragment D prepared from digests of cross-linked fibrin. Fig. 6 shows that the γ dimer in cross-linked Fragment D, had a mobility identical with that of the γ dimer of fibrin Fragment D and Dns-cadaverine-labeled fibrin Fragment D when analyzed after reduction on Na dodecyl-SO₄ gels.

It should be emphasized that fibrin monomer or Fragment D, when cross-linked in the presence of Dns-cadaverine, showed labeling of monomeric γ chain and γ dimer or monomeric γ' derivative and γ' dimer, respectively. In the latter instance one acceptor site is assumed to be labeled while the other acceptor site becomes cross-linked to the lysine donor site, thus accounting for the labeled dimer of the γ chain or γ' derivative. When Dns-cadaverine-labeled fibrin monomer was digested, the mobility of the degraded labeled γ chain clearly established that the subunit of 38,000 molecular weight in the reduced patterns of fibrinogen Fragment D was from the γ chain. Since Dns-cadaverine labeling of the γ chain acceptor site apparently prevents its degradation beyond the γ subunit, it appears that the formation of the γ'' and γ''' derivatives results from COOH-terminal degradation of the γ subunit. Similarly, the cross-links between γ chains in fibrin prevent their COOH-terminal degradation by plasmin and therefore fibrin Fragment D contains only γ' dimer.

Molecular Weights of Fragment D Species—Table I shows the molecular weights of fibrinogen Fragments D, D, and D, as well as fibrin Fragment D when determined by sedimentation equilibrium or by summation of subunit molecular weight from Na dodecyl-SO₄-gel electrophoresis. By either method there is a progressive, gradual increase in the molecular weight of the fibrinogen Fragment D species with respect to length of digestion. The molecular weight of fibrin Fragment D is about twice that of fibrinogen Fragment D, whether determined in the centrifuge or by summation of subunit molecular weights from Na dodecyl-SO₄ gels. It should be mentioned that the partial specific volumes (δ and ϕ) used to calculate the molecular weights from the centrifuge data were determined from the amino acid compositions of each Fragment D species. The reliability of equating the calculated δ value (19) with the measured δ value was tested by examining eight proteins whose amino acid compositions are known and whose δ (observed) values have been precisely determined (25). The average error in the calculated values was only 1.3% of the measured δ. While 6 M guanidine hydrochloride may have no measurable effect on the δ value for a given protein (e.g. RNase A), it ordinarily reduces this value due to preferential binding to certain amino acids (25). This correction was calculated to be -0.0125 ml/g for fibrinogen Fragments D, D, and D, and -0.0132 ml/g for fibrin Fragment D, using the method of Lee and Timasheff (20). These correction factors are almost identical with the average observed change, δ'·δ'' recently published for a number of proteins (25). Finally the number average molecular weight was determined by osmotic pressure for a mixture of fibrinogen Fragment D, D, and D, species in 4 M guanidine hydrochloride (26). The molecular weight determined by this method, which is independent of hydrodynamic effects, was 91,000. Hence by three different methods the molecular weights of the two earliest fibrinogen Fragment D species ranged from a minimum of 82,000 to a maximum of 94,000.

Fibrin Fragment D—Central to the issue of whether one or two Fragment D molecules are generated when plasmin cleaves fibrinogen is the structure of the largest product in prolonged digests of highly cross-linked fibrin. On inspection of Fig. 1, it is obvious that the model proposed by Mosesson et al. (11) should result in a fibrin Fragment D which is polymeric as a result of intermolecular cross-links (27) between the γ chains of different fibrinogen Fragment D species. Our studies of fibrinogen Fragment D structure, however, predict that fibrin Fragment D can only have a dimeric structure as a result of cross-linking. Fig. 7 shows the Na dodecyl-SO₄-gel electrophoretic pattern of a nonreduced sample from a terminal plasmin digest of highly cross-linked fibrin; it is evident that the derivative with the highest molecular weight, which is also the major digestion product, has the same mobility as purified fibrin Fragment D. Bands compatible with polymeric forms of fibrin Fragment D were never observed in terminal digests of highly cross-linked fibrin by plasmin. On reduction of the whole digest or of isolated fibrin Fragment D, a subunit band indicative of γ' dimer formation was evident and no other higher molecular weight bands were present. Even after pro-

*Kindly performed by Dr. F. J. Castellino, Department of Chemistry, University of Notre Dame, Notre Dame, Ind.
FIG. 5 (left). Attempted cross-linking of fibrinogen Fragment D (FBGN D) species in the presence of fibrin-stabilizing factor, thrombin, and calcium. By Na dodecyl-SO₄-5% gel analyses of nonreduced samples, only Fragment D₁ (from the 45-min digest) cross-linked to give a higher molecular weight species and it had the same mobility as fibrin Fragment D₁ (FBN D₁) from highly cross-linked fibrin.

Fig. 6 (right). Na dodecyl-SO₄-5% gels of reduced samples of cross-linked fibrinogen Fragment D₁ from 45-min digest (45′ FBGN D₁), fibrin Fragment D₁ (FBN D₁) from cross-linked fibrin digest and Dns-cadaverine-labeled fibrin Fragment D₁ (MDC-FBN D₁) from Dns-cadaverine-labeled fibrin digest. Gels were examined for protein, P, carbohydrate, C, and fluorescence, F, to identify the carbohydrate-positive β chain derivative and the fluorescent γ chain derivative.

TABLE I
Comparison of molecular weights of fibrinogen Fragment D and fibrin Fragment D by Na dodecyl-SO₄-gel electrophoresis and meniscus depletion sedimentation equilibrium

<table>
<thead>
<tr>
<th>Fragment D Species</th>
<th>Subunits per Molecule</th>
<th>SDS-gel analysis</th>
<th>Ultracentrifugal analysis of non-reduced Fragment D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Subunit chains</td>
<td>Summation of chains</td>
</tr>
<tr>
<td>Fragment D₁ (45 min)</td>
<td>y''</td>
<td>42,000</td>
<td>92,000</td>
</tr>
<tr>
<td>Fragment D₂ (8 hr)</td>
<td>y''</td>
<td>38,000</td>
<td>86,000</td>
</tr>
<tr>
<td>Fragment D₃ (24 hr)</td>
<td>y''</td>
<td>32,000</td>
<td>82,000</td>
</tr>
<tr>
<td>Fibrin Fragment D</td>
<td>y''</td>
<td>28,000</td>
<td>76,000</td>
</tr>
</tbody>
</table>

The symbols for the subunit polypeptide chains are defined in the text.

a Computed using the native partial specific volumes (v°) determined (19) from the amino acid compositions given in Table II. The v° values obtained for fibrinogen Fragment D₁, D₂, D₃, and fibrin Fragment D were 0.721, 0.723, 0.726, and 0.726 ml per g, respectively.

b Computed using the apparent partial specific volumes (α°) in 6 M guanidine hydrochloride also determined from the amino acid compositions (20). The α° values obtained for fibrinogen Fragments D₁, D₂, D₃, and fibrin Fragment D were 0.709, 0.711, 0.714, and 0.712 ml per g, respectively.

longed digestion, there was never any evidence that the γ′ dimer was degraded to lower molecular weight species.

Amino Acid Analyses—A comparison of the amino acid compositions of highly purified fibrinogen Fragment D species is given in Table II. The amino acid content of Fragment D₁ is 52% hydrophilic and 28% hydrophobic; that of Fragment D₂ is 51% hydrophilic and 31% hydrophobic. The extinction coefficients, E₁₀₀, computed from the compositions of Fragments D₁, D₂, and D₃ were 19.9, 21.2, and 22.2, respectively. Most of the residue values for the later species (D₂ or D₃) were smaller than the values for the earlier species (D₁ or D₂, respectively); within experimental uncertainty, the remaining values were unchanged. In the transition from Fragment D₁ to Fragment D₂, the values for proline, threonine, serine, glycine, and aspartic acid were reduced markedly. Similarly, the main difference between the contents of Fragments D₂ and D₃ was a decrease in residues with polar and acidic side chains and in phenylalanine. Considering the over-all degradation of Fragment D₁ to give Fragment D₂, it is notable that only 14% of all the residues released were hydrophobic whereas 38% were hydrophilic. These observations suggest that the COOH-terminal region of the γ′ subunit that is digested by plasmin to form the γ′′ subunit lies on the surface of the fibrinogen Fragment D₁ molecule.

The composition of fibrin Fragment D₁, also shown in Table...
II, is consistent with our identification of fibrin Fragment D as a dimer of Fragment D₁ molecules. The values for most of the amino acid residues in fibrin Fragment D agreed with twice the Fragment D₁ values to within 10%; moreover all the values were greater than twice the Fragment D₂ values.

**Early Ac Chain Degradation**—Fig. 8 shows that the initial plasmin cleavages of Dns-cadaverine-labeled monomeric fibrin result in the release of about five Dns-cadaverine-labeled peptides. Since the β chain did not incorporate the Dns-cadaverine label, these peptides must be of α chain origin. Moreover, their appearance coincided with the disappearance of the α chain. Since these peptides were released into the supernatant fluid, it is apparent that they are not disulfide-bound into the monomeric fibrin molecule; furthermore their appearance coincided with the disappearance of reduced, single-chain polypeptides on 5 to 7.5% polyacrylamide gels with molecular weights less than 21,000.

**TABLE II**

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Fibrinogen D Species</th>
<th>Fibrinogen D Species²</th>
<th>Fibrinogen D Species³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan²</td>
<td>24.2</td>
<td>23.8</td>
<td>23.3</td>
</tr>
<tr>
<td>Lysine</td>
<td>56.3</td>
<td>54.9</td>
<td>51.6</td>
</tr>
<tr>
<td>Histidine</td>
<td>16.1</td>
<td>13.5</td>
<td>11.4</td>
</tr>
<tr>
<td>Arginine</td>
<td>35.6</td>
<td>34.6</td>
<td>33.4</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>103.5</td>
<td>96.1</td>
<td>86.8</td>
</tr>
<tr>
<td>Threonine²</td>
<td>45.4</td>
<td>36.9</td>
<td>32.3</td>
</tr>
<tr>
<td>Serine²</td>
<td>56.5</td>
<td>46.4</td>
<td>41.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>101.9</td>
<td>97.9</td>
<td>94.2</td>
</tr>
<tr>
<td>Proline</td>
<td>32.3</td>
<td>23.8</td>
<td>23.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>72.5</td>
<td>64.9</td>
<td>55.4</td>
</tr>
<tr>
<td>Alanine</td>
<td>36.4</td>
<td>32.3</td>
<td>29.2</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>15.2</td>
<td>14.3</td>
<td>14.6</td>
</tr>
<tr>
<td>Valine²</td>
<td>39.2</td>
<td>36.8</td>
<td>35.1</td>
</tr>
<tr>
<td>Methionine²</td>
<td>10.8</td>
<td>10.8</td>
<td>18.2</td>
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<tr>
<td>Isoleucine³</td>
<td>35.9</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>47.5</td>
<td>47.0</td>
<td>45.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>32.5</td>
<td>33.1</td>
<td>29.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>24.8</td>
<td>22.2</td>
<td>17.8</td>
</tr>
</tbody>
</table>

**Discussion**

Major differences between the two models under discussion pertain to the following: (a) the molecular weights of fibrinogen Fragment D species; (b) the structure of fibrin Fragment D; and (c) the chain of origin of each subunit in Fragment D.

**Molecular Weight Studies**—Although analyses of nonreduced samples of Fragment D species by Na dodecyl-SO₄ gel electrophoresis have given molecular weights of 67,000 to 130,000 (6, 5, 28, 29), the validity of these values can be questioned because of potentially decreased Na dodecyl-SO₄ binding and a decreased Stokes radius due to intact disulfide bonds (30). On the other hand, the Na dodecyl-SO₄ gel electrophoretic method for determining the molecular weights of reduced, single-chain polypeptides on 5 to 7.5% polyacrylamide gels is accepted as accurate and reliable (7, 31). By complementing the molecular weight data from subunit analyses of Fragment D species on Na dodecyl-SO₄ gels with molecular weight data obtained by sedimentation equilibrium ultracentrifugation of the same nonreduced Fragment D species, the changes in subunit structure can be correlated with changes in the native molecular weight. Except for minor differences, our Na dodecyl-SO₄ gel patterns for both nonreduced and reduced species of Fragment D are essentially the same as those published recently by Mosesson et al. (11). Our fibrinogen Fragment D₁ is identical with their Fragment D₁ whereas our D₂ and D₃ species appear identical with their D₂ and D₃ products, respectively. The molecular weights of our Fragments D₁, D₂, and D₃ as determined by sedimentation equilibrium ultracentrifugation are 94,000 to 87,000, 88,000 to 82,000, 76,000 to 70,000, respectively, depending on the value used for the partial specific volume. These compare favorably to the sums of the subunit molecular weights determined by Na dodecyl-SO₄ gel electrophoresis, which are 92,000, 86,000, and 82,000, respectively. Using the dimeric fibrinogen Frag-
molecular weight values for Fragment D which were actually determined by less accurate methods, e.g. nonreduced Na dodecyl-SO₄-gel electrophoresis (5) and then for incompletely characterized higher molecular weight intermediates, most likely Fragments X and Y (32, 33).

Earlier estimates of the molecular weight of Fragment D by summation of subunit molecular weights from Na dodecyl-SO₄-gel studies have ranged from 73,000 to 100,000 (3, 8, 9, 34) and are consistent with our values. Previous values obtained for the molecular weight of Fragment D determined by sedimentation velocity or equilibrium have ranged from 75,000 to 88,000 (2, 6, 32, 35) and are in excellent agreement with our values. Unfortunately, these earlier ultracentrifugal analyses were performed without defining which species of Fragment D was being examined and therefore are of limited value for correlations with subunit analyses.

Structure of Fibrin Fragment D—Examination of fibrin Fragment D isolated from a terminal digest of highly cross-linked fibrin (Fig. 7) affords further evidence against the degradation model suggested by Mosesson et al. (11). If the latter model was correct, then fibrin Fragment D should be a polymer as shown in Fig. 9, since γ dimer formation only occurs intramolecularly (27). In our experiments, there was never any suggestion that a “polymer” of Fragment D had formed. Furthermore we found that the largest fragment present in terminal digests of cross-linked fibrin has a structure consistent with the dimerization of two fibrinogen Fragment D₁ species. Our Na dodecyl-SO₄-gel analyses indicate that dimerization occurs by the formation of cross-links between the γ' subunit. In the present study, isolated fibrin Fragment D had a molecular weight of 190,000 to 175,000 by sedimentation equilibrium and 184,000 by summation of subunit molecular weights from Na dodecyl-SO₄-gel analyses, the latter of which is in accord with previous reports (8, 36, 37). Using a less accurate ultracentrifugation method, bovine fibrin Fragment D was estimated recently to have a molecular weight of about 160,000 (38). Based on our molecular weight values, fibrin Fragment D contains one pair of ε-(γ-glutamyl)lysyl cross-links and fibrinogen Fragment D₁ contains none when quantitated by the cyanoethylolation method (39).³

Fibrin Fragment D was also formed by incubation of fibrinogen Fragment D₁ with thrombin, fibrin-stabilizing factor, cysteine, and calcium as previously reported from our laboratory (8); later species of fibrinogen Fragment D did not cross-link. Since the lower molecular weights of these species are due primarily to degradation of the γ chain derivative, it seems likely that this occurs in its COOH-terminal portion where the cross-link sites are located. This proposal is further supported by the observations that fibrin Fragment D from whole digests of cross-linked fibrin is resistant to further degradation by plasmin and that incorporation of Dns-cadaverine into the γ chain cross-link acceptor sites prevents further cleavage of the γ subunit in fibrinogen Fragment D₁. Thus only one species of fibrin Fragment D can be generated when highly cross-linked fibrin is digested by plasmin.

³A. P. Ball and P. A. McKee, unpublished observations.

Chain of Origin of Fragment D Subunits—Mosesson et al. (11) concluded that during the digestion of fibrinogen by plasmin, the Bβ chain is cleaved extensively in its COOH-terminal portion and that its various stages of degradation are responsible for the heterogeneity of Fragment D. They found that a peptide of 32,000 molecular weight (βᵣ in their nomenclature) was generated from the digestion of “fibrinogen” fractions said to be devoid of intact Aa chains or Aa chain derivatives large enough to account for such a peptide. Therefore they believed that /βᵣ was from either the Bβ chain or γ chain. Tryptic peptide maps of /βᵣ were very similar to those of intact Aα or Bβ chains; however, it was clear that /βᵣ was not derived from the γ chain. While immunodiffusion studies also excluded a γ chain origin for /βᵣ, they suggested that /βᵣ was related immunologically to peptides derived from the Aα chain. Our data clearly show that the peptide which Mosesson et al. (11) called /βᵣ as well as its derivatives /β₁ and /βᵣ contain cross-link acceptor sites and therefore must be derived from the Aα chain. Hence, the “fibrinogen” fractions which Mosesson et al. (11) digested to demonstrate /βᵣ must have been contaminated by Aα chain derivatives which migrated in the Bβ and γ chain regions on Na dodecyl-SO₄-gel electrophoresis.

Another major point of contention between the two models under discussion is the chain of origin for the plasmin-resistant peptide of 42,000 molecular weight found in all Fragment D species. Mosesson et al. (11) reasoned that this peptide must be of γ chain origin since (a) early, extensive COOH-terminal degradation of the Aα and Bβ chains precluded the possibility that such a large peptide could come from either of these chains; and (b) tryptic peptide maps of the peptide of 42,000 molecular weight (γᵣ in their nomenclature) and of the S-sulfold γ chain were similar. Their first premise, however, is incorrect because extensive COOH-terminal degradation of the Bβ chain does not occur, as discussed in the previous paragraph, and their second premise is very tenuous due to the questionable similarity between the peptide maps of /γᵣ and γ chain (Fig. 7 in Ref. 11).

Our data demonstrate directly that the peptide of 42,000 molecular weight present in all Fragment D species is derived from the Bβ chain. First, all the carbohydrate present in Fragment D is located on the β chain derivative (40). Thus the carbohydrate staining of the peptide of 42,000 molecular weight in our present study substantiates its derivation from the Bβ chain. Second, the presence of this peptide in fibrin Fragment D provides additional evidence for its Bβ chain origin since the γ' subunits are cross-linked and therefore present in dimeric forms. Third, the peptide of 38,000 molecular weight present in fibrinogen Fragment D₁ cannot be from the Bβ chain as proposed by Mosesson et al. (11) since this peptide incorporates Dns-cadaverine and disappears to give the γ' dimer when Fragment D₁ species are cross-linked.

In contrast to its effect upon γ chain cleavage, Dns-cadaverine incorporation into the cross-link acceptor sites of the α chain does not appear to alter its cleavage by plasmin. The free α peptides released from the α chain during the digestion of Dns-cadaverine-fibrin monomer have the same pattern on Na dodecyl-SO₄-gel analysis as those from unlabeled fibrinogen; in addition, the same Na dodecyl-SO₄-gel patterns were observed by Mosesson et al. (11), although they incorrectly concluded that the three smallest α peptides were of β chain origin. Our results show that α cross-link acceptor sites are all released from fibrinogen during its degradation to Fragment X. The α...
peptides which contain the cross-link acceptor sites are then degraded sequentially from a molecular weight of about 40,000 to 21,000. The α peptides of molecular weight 40,000 to 23,000 each contain both α chain cross-link acceptor sites; however, one site may be lost when the plasmin-resistant peptide of 21,000 molecular weight is formed. Thus all cross-link acceptor sites on the Aα chain appear to be contained within a 23,000 molecular weight segment of the initial peptide (or peptides) cleaved from fibrinogen during its digestion. On direct examination, Fragment X contains no cross-link acceptor sites in its Aα chain derivative.

Our results, which thoroughly refute the model proposed by Mosesson et al. (11), can only be consistent with the production of 2 Fragment D molecules from each fibrinogen molecule. Fig. 10 summarizes the asymmetric cleavage of fibrinogen by plasmin and the subunit structures of its cleavage products. Recently this model has received additional support from the investigations of Donovan and Mihalyi (10). Using totally different methods, they found that solutions of fibrinogen and of Fragment Y gave two independent thermal denaturing transformations, at 60.7 and 96.6°, which corresponded to the thermal transitions of isolated Fragment D and Fragment E, respectively. A comparison of the ratio of the enthalpy of denaturation for Fragment D to the enthalpy of denaturation for Fragment E in fibrinogen to that in Fragment Y is consistent only with the presence of two Fragment Ds and one Fragment E in fibrinogen, and one Fragment D and one Fragment E in Fragment Y.

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