# Primary Soluble Plasmic Degradation Product of Human Cross-linked Fibrin. Isolation and Stoichiometry of the (DD)E Complex†

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ABSTRACT: The formation of the (DD)E complex and fragments DD and E upon proteolysis of human cross-linked fibrin was studied by timed digestions using varying amounts of plasmin. The (DD)E complex was the primary soluble degradation product released from cross-linked fibrin. This complex contained fragments DD and $E_1$. Upon further digestion (DD)E complex was cleaved to (DD)$E_2$ complex whereby only the fragment E moiety was affected. However, when fragment $E_2$ was digested to fragment $E_3$, dissociation of the complex occurred. Thus, fragments DD and $E_3$ are the terminal plasmic digestion products of cross-linked fibrin. This pattern was consistent regardless of the plasmin to fibrin ratio; however, the rate of production of the terminal degradation products was directly dependent upon enzyme concentration. Digestion conditions were modified so that either the (DD)E complex or fragment DD was the predominant degradation product, allowing for the purification of these species by one-step gel filtration. The molar ratio of fragment DD to fragment E in the (DD)E complex was investigated by dissociation of the complex and by reassociation of the purified components. The (DD)E complex contains one molecule of fragment DD and one molecule of fragment E.

Digestion of human cross-linked fibrin by plasmin results in the formation of fragments DD and E and $\alpha$ polymer remnants as the major high-molecular-weight degradation products (Gaffney & Brasher, 1973; Hudry-Clergeon et al., 1974; Kopeč et al., 1973; Marder et al., 1976; Pizzo et al., 1973a,b). A complex between fragments DD and E, as first observed by Gormsen & Feddersen (1973), was distinctly demonstrated by Gaffney & Brasher (1973) and by Hudry-Clergeon et al. (1974).

Plasmic digests of cross-linked fibrin were reported to contain between 25% (Gaffney et al., 1975) and 50% (Gaffney et al., 1977) of the fragment E antigen in the (DD)E complex. However, the relationship between the (DD)E complex and uncomplexed fragments DD and E was not clarified. The question remained whether the complex and the free moieties are formed simultaneously or if the latter species are formed by degradation of the former. It has been suggested that the number of fragment D and E moieties in the (DD)E complex is the same as in fragment X, based on the similar mobilities in Tris–glycine polyacrylamide gel electrophoresis (Hudry-Clergeon et. al., 1974; Gaffney et al., 1975). Direct demonstration of this ratio was hampered by the difficulty in purifying the complex (Olexa et al., 1979).

The purpose of the present work was to ascertain the sequence of formation of soluble degradation products during plasmic digestion of cross-linked fibrin and to isolate the (DD)E complex and fragment DD. In order to directly demonstrate the ratio of fragment DD to E in the complex, the latter was dissociated and protein content of each species determined by three different methods. The complex has also been re-formed from its purified constituents to support the stoichiometric data.

## Materials and Methods

*Preparation of Cross-linked Fibrin.* Human fibrinogen (Grade L, A. B. Kabi, Stockholm, Sweden) was enriched with Factor XIII and clotted as previously described (Marder et al., 1976). This fibrin was insoluble in 2% acetic acid overnight and after reduction and electrophoresis on $NaDodSO_4$ gels (7%) showed bands corresponding to the $\beta$, $\gamma\gamma$ and $\alpha$ polymer chains. Noncross-linked $\alpha$ or $\gamma$ chains were absent.

*Digestion of Cross-linked Fibrin.* One gram of cross-linked fibrin was suspended in 20 mL of prewarmed (37 °C) 0.15 M Tris-HCl buffer, pH 7.8, containing 5 mM calcium chloride and 0.02% sodium azide. Aliquots of 0.1, 0.3, 0.6, 1.0, or 10.0 mL of human plasmin (kindly supplied by Dr. David Aronson, Bureau of Biologics, Food and Drug Administration, Rockville, MD, containing 10.0 CTA[1] units/mL; 12.7 CTA units/mg of protein) were added with gentle magnetic stirring at 37 °C. At 10 min, 30 min, and 1, 2, 4, 6, 12, 18, 24, 36, 48, 60, and 72 h, 0.5-mL aliquots of the supernates were removed, and trasylol (aprotinin 10000 KIU/mL, Mobay Chemical Corp., New York, NY) was added to each aliquot in the ratio of 250 KIU/1 CTA unit of plasmin. Each digest was done in triplicate.

*Fragment $E_1$* was obtained from the purified (DD)E complex by incubation in 3 M urea, 0.05 M sodium citrate, pH 5.5, for 4 h at 37 °C and gel filtered on a Sepharose CL-6B column, 2.5 × 190 cm, in a buffer containing 0.05 M Tris-HCl, 0.028 M sodium citrate, 0.1 M sodium chloride, and 0.02% sodium azide, pH 7.4 (Olexa & Budzynski, 1978; Olexa et al., 1979).

*Dissociation of the (DD)E Complex.* To assure complete dissociation the purified complex was incubated in 6 M urea (rather than 3 M urea), 0.05 M sodium citrate, pH 5.5, for 4 h at 37 °C and then gel filtered on a Sepharose CL-6B column, 0.9 × 90 cm, in a buffer containing 0.05 M Tris-HCl, 0.028 M sodium citrate, 1.0 M sodium chloride, and 0.02% sodium azide, pH 7.4. Approximately 2 mg of protein was applied to the column. Flow rate was 60 mL/h. Samples (1 mL) were collected and pooled as indicated.

*Quantitative protein determination* was done by three methods: (1) direct ultraviolet spectrophotometry at 280 nm using the following absorption coefficients at 1 mg/mL: fibrinogen, 1.5; fragment DD, 2.0; and fragment E, 1.2; (2)

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Abbreviations used: CTA, Committee on Thrombolytic Agents; KIU, kallikrein inhibitor units; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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spectrophotometry at 630 nm of $Cl_3CCOOH$-precipiated, Amido Black stained protein by the method of Schaffner & Weissman (1973) (the standard curves for bovine serum albumin, fibrinogen, and fragments DD and E completely overlapped); (3) determination by the method of Lowry et al. (1951). Since in this method the standard curves for fibrinogen and fragments DD and E differed slightly, the standard curve used was determined by the composition of the sample to be tested.

*Binding of Fragments DD* and *and E.* Purified fragments DD and E were combined in various molar ratios calculated from molecular weights of 190000 for fragment DD (Ferguson et al., 1975; Gaffney et al., 1975; Gormsen & Feddersen, 1973; Marder et al., 1976; Pizzo et al., 1973a) and 60000 for fragment $E_1$ (Olexa & Budzynski, 1978). The mixture was diluted to 0.4 mg/mL in 0.15 M Tris-HCl buffer, pH 7.4, and incubated at room temperature for 60 min. An aliquot was diluted with an equal volume of 0.05 M Tris-HCl, 0.38 M glycine, 40% sucrose, pH 8.6, and 50 μL (10 μg) was electrophoresed on 9% Tris–glycine gels.

*Polyacrylamide Gel Electrophoresis.* Proteins were analyzed in two electrophoretic systems: in 7% polyacrylamide gels containing 0.1% $NaDodSO_4$ according to the method of Weber & Osborn (1969) and in nondissociating medium in 9% Tris–glycine gels by the method of Davis (1964). Approximately 10 μg of protein was applied per gel and staining was done using the procedure of Fairbanks et al. (1971). Gels were scanned in a densitometer (Densicord 552, Photovolt, New York, NY).

*Double immunodiffusion* was done in 1% agarose (SeaKem, Bausch and Lomb, Rochester, NY) according to the method of Ouchterlony (1958), and the slides were stained with Coomassie Brilliant Blue by the method of Fairbanks et al. (1971). Antisera against fibrinogen and fibrinogen degradation products fragment D and fragment E were raised in white New Zealand rabbits. The antisera were absorbed repetitively with normal human serum until no precipitate could be detected. Anti-D and anti-E antisera were then absorbed with either fragment E or fragment D, respectively, to insure monospecificity.

## Results

*Timed Digestions of Cross-linked Fibrin.* Cross-linked fibrin was digested by plasmin at a ratio of 6 CTA units/g of fibrin, and samples from the supernate were taken at various times. Each aliquot was electrophoresed on Tris–glycine and $NaDodSO_4$ gels (Figure 1). Three species of fragment E can be seen on $NaDodSO_4$ gels. For the clarity of the presentation they have been designated as fragments $E_1$, $E_2$, and $E_3$ (molecular weights 60000, 55000, and 50000, respectively) in order of increasing electrophoretic mobility (Olexa & Budzynski, 1978).

Cross-linked fibrin digested for 6 h produced only the (DD)E complex, as seen on Tris–glycine gels, which on $NaDodSO_4$ gels dissociated into fragment DD and a single band of fragment $E_1$ (Figure 1). At 12 h, again, only a (DD)E complex was seen; however, upon dissociation, fragment DD and fragments $E_1$ and $E_2$ were present. Therefore, both fragments $E_1$ and $E_2$ participated in the (DD)E complex. At 24 h of digestion, in addition to the (DD)E complex free fragment DD appeared, and three distinct species of fragment E were present in $NaDodSO_4$ gels. By 36 h of digestion, fragment $E_1$ dissappeared. After 60 h only fragments $E_3$ and DD were seen on $NaDodSO_4$ gels, and the (DD)E complex was no longer present. Fragments $E_3$ and DD appeared in the digestion mixture simultaneously. This fact taken together

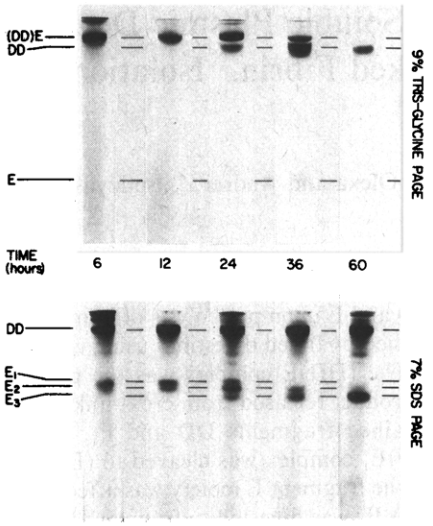


FIGURE 1: Electrophoretic patterns in Tris–glycine 9% gels (top) and in $NaDodSO_4$ containing 7% gels (bottom) of a timed digest of cross-linked fibrin incubated with plasmin at a ratio of 6 CTA units of plasmin per gram of fibrin. The numbers between gels indicate time of digestion in hours.

with the presence of uncomplexed fragment E band on Tris–glycine gels suggested that fragment $E_3$ did not participate in a (DD)E complex. Free and complexed fragment DD had the same mobility on $NaDodSO_4$ gels.

In order to test if the observed digestion sequence was unique to the plasmin–fibrin ratio used in the previous experiment, this ratio was varied from 1 to 100 CTA units/g of fibrin. Aliquots from the supernate were removed at various times and analyzed in $NaDodSO_4$ and Tris–glycine gels.

Regardless of the plasmin concentration, the appearance of fragment DD coincided with the appearance of fragment $E_3$, and the disappearance of fragments $E_1$ and $E_2$ and the (DD)E complex was simultaneous. The sequence of digestion of cross-linked fibrin was consistent regardless of plasmin concentration but the rate of digestion was directly dependent upon the enzyme concentration. The data shown in Table I indicate the time of appearance of fragments DD and $E_3$ as well as the time of complete disappearance of the (DD)E complex from the fibrin digestion mixtures. It is evident that as the plasmin concentration increases the rate of degradation of the complex to fragments DD and $E_3$ also increases.

*Isolation of the (DD)E Complex and Fragment DD.* Data presented in Table I provided a basis for manipulation of the digestion conditions so that either the (DD)E complex or fragment DD was present, but not both species.

Homogeneous (DD)E complex was obtained from cross-linked fibrin digested by plasmin (1 CTA unit/g) for 48 h. The digest was clarified by centrifugation at 3000*g* for 15 min, and the supernate was chromatographed on a Sepharose CL-6B (Pharmacia, Piscataway, NJ) column, 2.5 × 190 cm, in a buffer containing 0.05 M Tris-HCl, 0.028 M sodium citrate, 0.1 M sodium chloride, 25 KIU/mL of trasylol, and 0.02% sodium azide, pH 7.4. Approximately 250 mg of protein was applied and the flow rate was 45 mL/h. Fractions (3 mL) within the peaks were combined, concentrated, and analyzed by gel electrophoresis. This procedure (Figure 2A) separated high-molecular-weight material, peak IA, and low-molecular-weight α polymer remnants (Harfenist & Canfield, 1975; Takagi & Kawai, 1977), peak III A, from the main protein fraction, peak IIA, which contained only the (DD)E complex, as seen on Tris-glycine gels (Figure 3).

To isolate fragment DD, the cross-linked fibrin digested by

Table I: Effect of Plasmin Concentration on the Formation of Fragments DD and $E_3$ and on Degradation of the (DD)E Complex

| plasmin (CTA units/ g of fibrin) | appearance of fragments DD and $E_3$ (h) | disappearance of (DD)E complex (h) |
|---|---|---|
| 1 | >72 | >72 |
| 3 | 48 | 72 |
| 6 | 18 | 48 |
| 10 | 2 | 6 |
| 100 | <0.1 | 1 |

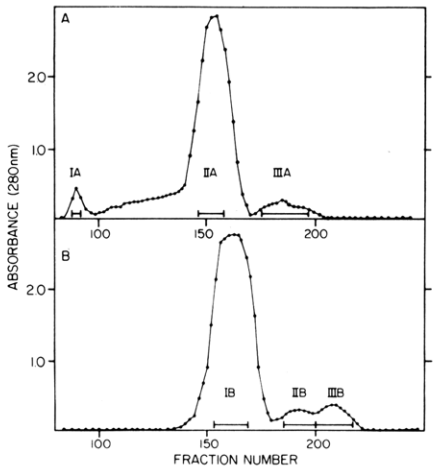


FIGURE 2: Gel filtration profiles on a Sepharose CL-6B column, 2.5 × 190 cm, of cross-linked fibrin digested with (A) 1 CTA unit/g of fibrin for 48 h or with (B) 100 CTA units/g of fibrin for 24 h. Three-milliliter fractions were collected and pooled as indicated.

100 units of plasmin/g of fibrin for 24 h was gel filtered on a Sepharose CL-6B column as for the (DD)E complex with the exception that the buffer contained 1.0 M sodium chloride (Figure 2B). Fragment DD, peak IB, was separated from fragment $E_3$ and $\alpha$ polymer remnants, peaks IIB and IIIB, respectively. On Tris–glycine gels the fragment DD preparation was homogeneous and was not contaminated with a (DD)E complex (Figure 3). Therefore, the adjustment of fibrin digestion conditions enabled purification of the (DD)E complex and fragment DD by simple gel filtration.

Stability of the purified (DD)E complex was examined by double immunodiffusion. The complex, fibrinogen, and a mixture of fragments D and E from fibrinogen were tested against monospecific anti-D and anti-E antisera (Figure 4). Although fibrinogen contains both fragment D and fragment E antigenic domains, it is a single entity; therefore, the immunoprecipitation lines converge, and no spurs are formed. The same result was obtained with the (DD)E complex showing that it was a single entity, the preparation was not contaminated with free fragments DD and E, and the complex did not dissociate into its constituents. The (DD)E complex preparation primarily contained $(DD)E_1$ with a small contamination by $(DD)E_2$. The latter was probably responsible for the formation of the additional faint immunoprecipitation line with anti-E antiserum. It is significant that neither $(DD)E_1$ nor $(DD)E_2$ dissociates under these conditions. In contrast, in the mixture of fragments D and E, the immunoprecipitation lines intersected, showing that two distinct species were present.

Approximately 2 mg of purified (DD)E complex was dissociated in 6 M urea, at pH 5.5, for 4 h at 37 °C. The sample was applied to a Sepharose CL-6B column equilibrated and developed in a buffer containing 0.05 M Tris–HCl, 0.028 M sodium citrate, 1.0 M sodium chloride, and 0.02% sodium

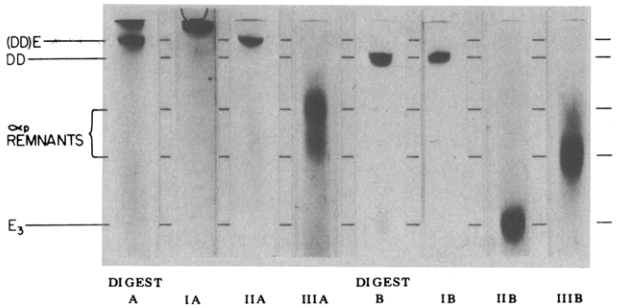


FIGURE 3: Electrophoretic patterns on Tris–glycine gels (9%) of fractions obtained during purification of the (DD)E complex and fragment DD.

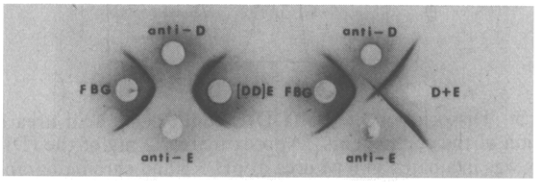


FIGURE 4: Demonstration of the purity and stability of the (DD)E complex by double immunodiffusion. (DD)E, the complex, 500 μg/mL; Fbg, fibrinogen, 500 μg/mL; D + E, a mixture of fragments D (120 μg/mL) and E (380 μg/mL). Immunodiffusion done in a 1% agarose using monospecific anti-D and anti-E antisera.

azide, pH 7.4 (Figure 5). All fractions containing protein were pooled as indicated, concentrated by ultrafiltration, and tested on $NaDodSO_4$ and Tris–glycine gels. This procedure separated fragments DD and E (peaks I and II, respectively). To analyze for cross-contamination, peaks I and II were tested in double immunodiffusion against monospecific anti-D and anti-E antisera (Figure 6). Peak I precipitated only with anti-D antiserum while peak II reacted only with anti-E antiserum. The fuzzy zone between peak II and anti-E antiserum is probably caused by a small contamination of fragment $E_1$ by fragment $E_2$. Four different preparations of the (DD)E complex (2 mg/sample) were dissociated by acid urea, separated by gel filtration, and tested by double immunodiffusion. The same results were obtained in all experiments. The protein content of peaks I and II for the four experiments were determined using three different techniques. The data (Table II) show the mean protein content in nanomoles and the standard deviation. The variation in the amount of protein as determined by the three techniques is statistically not significant and the molar ratio of protein in peak I to that in peak II consistently approaches 1. Therefore, the (DD)E complex consists of one molecule of fragment DD and one molecule of fragment E.

*Reassociation of Purified Fragments DD and $E_1$.* Purified fragments DD and $E_1$ can combine to form the (DD)E complex (Olexa & Budzynski, 1978). To confirm the ratio of fragment DD to fragment E in the complex, the purified components were mixed in molar ratios of 3:1, 2:1, 1:1, 1:2, and 1:3, allowed to interact, and then visualized on Tris–glycine gels (Figure 7). In the mixtures of fragments DD and $E_1$ at the molar ratios 3:1 and 1:3, all fragment $E_1$ or fragment DD, respectively, was in the complex as demonstrated by gel scanning. Therefore, most if not all of the molecules in the purified preparations were capable of forming a complex. Combining fragments DD and $E_1$ in a 1:1 molar ratio produced the maximum amount of the complex with the minimum amount of free components. This result further supports the conclusion that one molecule of fragment DD binds to one molecule of fragment $E_1$ to form the (DD)E complex.

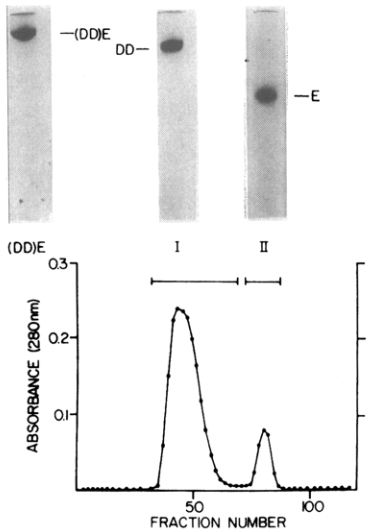


FIGURE 5: Dissociation of the (DD)E complex by acid urea and separation of the components. Approximately 2 mg of the (DD)E complex was incubated in 6 M urea at pH 5.5 and chromatographed on a Sepharose CL-6B column in 0.05 M Tris-HCl, 0.028 M sodium citrate, 1.0 M sodium chloride, and 0.02% sodium azide, pH 7.4. Samples (1 mL) were pooled as indicated. The starting material, (DD)E, was analyzed on Tris–glycine gels (9%) and peaks I and II were tested on $NaDodSO_4$ gels (7%).

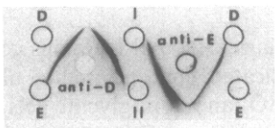


FIGURE 6: Double immunodiffusion patterns of peaks I and II (Figure 5) and fragments D and E (250 $\mu$g/mL) against monospecific anti-D and anti-E antisera.

Table II: Total Protein Content of Peaks I and II Determined by Three Techniques

| | total protein[a] (nmol) | | |
|---|---|---|---|
| | $A_{280}$ | Amido Black | Lowry |
| peak I (DD) | 7.81 ± 0.74 | 7.67 ± 0.64 | 7.85 ± 0.51 |
| peak II ($E_1$) | 7.73 ± 0.34 | 7.53 ± 0.32 | 8.05 ± 0.35 |

[a] The total protein concentration was determined by the three methods. Each measurement was done in triplicate, in four different preparations. The mean value and standard deviation are presented. The total moles of protein in peaks I and II (Figure 5) were calculated from the total protein concentration and the molecular weights, 190 000 for fragment DD and 60 000 for fragment $E_1$.

## Discussion

The present work shows that the (DD)E complex is the primary soluble plasmic degradation product released from cross-linked fibrin (Figure 1). The complex is susceptible to further action of plasmin according to the following:

$$\text{cross-linked fibrin} \rightarrow (DD)E_1 \rightarrow (DD)E_2 \rightarrow DD + E_3$$

The initial complex contains fragments DD and $E_1$. Upon further digestion fragment $E_1$ is cleaved to fragment $E_2$ without disruption of the complex. Digestion of fragment $E_2$ to fragment $E_3$ results in the dissociation of the complex. There is no apparent change in fragment DD structure as digestion proceeds. The terminal plasmic digestion products of cross-linked fibrin are fragments DD and $E_3$. This pattern of digestion is consistent regardless of the plasmin to fibrin ratio; however, the rate of formation of the terminal degradation products differs significantly with the plasmin concentration (Table I). Thus, the variation in the composition of cross-linked fibrin digests may be explained by different amounts of the enzyme and reaction time. From the data presented here, the enzyme–substrate ratio and incubation time can be adjusted to obtain a high yield of a specific degradation product (Table I). This enables the isolation of the (DD)E complex and fragments DD and $E_3$ from the digest by one-step column gel filtration (Figures 2 and 3).

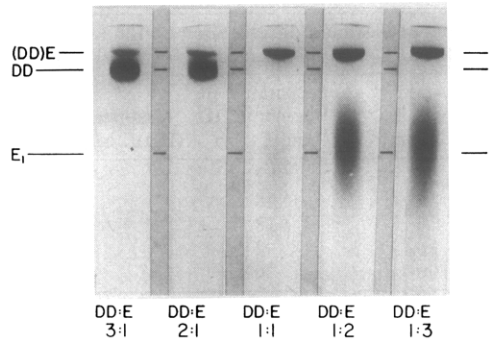


FIGURE 7: The stoichiometry of fragments DD and $E_1$ binding. The electrophoretic patterns were obtained in Tris–glycine gels (9%) using fragments DD and $E_1$ which were mixed in molar ratios of 3:1, 2:1, 1:1, 1:2, and 1:3.

Fragment E present in cross-linked fibrin digests can be distinguished as three distinct species (Figure 1) which appear to be sequential degradation products. The presence of fragments $E_1$ and $E_2$ in the digest coincides with the presence of the (DD)E complex, while fragment $E_3$ appears simultaneously with free fragment DD. This indicates that fragments $E_1$ and $E_2$ are involved in the complex, but fragment $E_3$ is not. After extensive digestion, e.g., 72 h of digestion at 100 CTA units/g of fibrin, the electrophoretic mobility of fragment DD is unchanged and in our experience unaltered fragment DD is not degraded to fragment D species (Olexa et al., 1979) as suggested by some investigators (Gaffney et al., 1975; Gaffney et al., 1977; Haverkate & Timan, 1977; Purves et al., 1978).

Evidence is provided that the (DD)E complex is a quite stable molecule which does not visibly dissociate during column gel filtration (Figure 2), Tris–glycine gel electrophoresis (Figure 3), and double immunodiffusion in agarose gel (Figure 4). The same stable complex can be re-formed from fragments DD and $E_1$ or $E_2$ (Figure 7), indicating the presence of functionally active complementary binding sites on the fragments DD and E isolated in this study. The complex was, however, completely disrupted in the presence of 3 M urea at pH 5.5 (Figures 5 and 6), as was indicated previously (Olexa et al., 1979). In the present work only the soluble degradation products were examined. High-molecular-weight species corresponding to cross-linked forms of fragments X or Y may be formed during digestion of cross-linked fibrin (Alkjaersig et al., 1977). These degradation products are probably not readily released from the clot at the very early stage of digestion (Figure 1) and are rather degraded to the soluble (DD)E complex. After limited plasmic proteolysis of cross-linked fibrin only the (DD)E complex is present in the digest and free fragments DD or E are absent (Figure 1 and Table I). These data imply that the (DD)E complex is released from a clot as a complex rather than as free species which associate in the solution. It is therefore possible that in fibrin the monomers are so arranged that each fragment E moiety is in close contact with a cross-linked fragment DD moiety. Since the (DD)E complex consists of one of each moiety, their arrangement in the complex may be similar to that in the original fibrin fiber.

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