Proteolytic Fragmentation of Fibrinogen. I. Comparison of the Fragmentation of Human and Bovine Fibrinogen by Trypsin or Plasmin†

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ABSTRACT: The purpose of the investigations was to obtain sufficiently accurate data of the composition of the digests, at various well-defined stages of the reaction, to enable kinetic modeling of the fragmentation process. The extent of the reaction was expressed as moles of base consumed per mole of fibrinogen, during digestion in the pH stat. All the pH stat curves could be described by the sum of two first-order reactions. From the pH dependence of the base consumption corresponding to these two reactions, the pK's of the α-NH_2 groups generated by the digestion were determined. These were not significantly different for the slow reaction in three of the cases studied, their average being 7.79. The base consumption at pH 8.0 was converted with these pK's into the actual number of bonds cleaved. The values found were the following: trypsin digestion of bovine fibrinogen, 13.2 and 91.3; trypsin digestion of human fibrinogen, 25.7 and 79.1; plasmin digestion of bovine fibrinogen, 3.1 and 54.3; and plasmin digestion of human fibrinogen, 5.2 and 50.1. For the quantitative estimation of the fragmentation products, gel filtration on Sephadex G-200 columns and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate were used. The gels were scanned directly at 280 nm. It was proved that the two methods yielded identical results. The fragmentation patterns obtained with the four systems were similar. Notable difference was in the X and Y fragments of the plasmin digests of bovine fibrinogen, which appeared as doublets. Also, human fibrinogen was fragmented more slowly by trypsin than bovine fibrinogen. As a result of these studies, curves are given that express the composition of the digests as a function of base consumed during the digestion in the pH stat. The digestion of bovine fibrinogen by trypsin was investigated in more detail than the other digestions. The large fragments, X, Y, D, and E, as well as the peptide fractions P1 and P2, were isolated. Their specific optical densities were determined and with these the optical density distribution among the fragments was converted into mass fractions. At any point of the reaction adherence to the mass conservation law was established. Molecular weights of the isolated fragments were determined by high-speed sedimentation equilibrium. The values obtained were 220 000, 134 000, 84 000, 48 500, and 14 400 for fragments X, Y, D, E, and P1, respectively. These values are in close agreement with those derived from the stoichiometry based on the mass distribution in the digests. The mass distribution and molecular weights also suggest that there are two D, one E, and two P1 fragments and a large number of small peptides (P2) produced per molecule of fibrinogen. Molecular weight data of the fragments of all four systems were obtained also from sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Each fragment, in all four systems investigated, had practically the same molecular weight, and these were in good agreement with those obtained by sedimentation equilibrium for the tryptic fragments of bovine fibrinogen. The R_f values of the peaks were constant during digestion for all fragments, with the exception of fragment D, which showed an increase in mobility corresponding to a decrease of approximately 10 000 in its molecular weight. According to the mass distribution and molecular weight data plasmic digests of human fibrinogen also yielded two D and one E fragments.

A large number of publications appeared during the past years on the mechanism of fragmentation of fibrinogen by proteolytic enzymes (for reviews, see Mihalyi, 1972; Doolittle, 1973). Nevertheless, there is considerable controversy on this subject with respect to the pathways followed in the degradation, and to the nature and number of intermediates and final products. It is debated whether the intermediates are sharply defined entities (Marder et al., 1969; Pizzo et al., 1972), or whether they form a spectrum of a large number of progressively lower molecular weight products (Mosesson et al., 1973). Also, the number of the so-called core fragments, fragments D and E according to the nomenclature introduced by Nussenzweig et al. (1961), derived from one molecule of fibrinogen is variously stated as 2D + 1E (Marder et al., 1969), 1D + 1E (Mosesson et al., 1973), and 2D + 2E (Plow and Edgington, 1974). Since any model of the native molecule must accommodate these fragments, it is natural that there is equal quandary as far as the models are concerned.

The controversy could be easily resolved by precise quantitation of the reaction intermediates and products on a series of samples inhibited at various stages of the digestion. For preparing reproducible and well-defined samples, a rigorous scale of measurement of the extent of reaction should first be established. This is obviously important for kinetic studies, but also because even the core fragments are slowly degraded, and for their isolation the proper harvesting time has to be determined. Then, the molecular weights of the fragments should be estimated in a reliable way and correlated with the fraction of mass present in each of the degradation products (Mihalyi, 1972). Strict adherence to the law of mass conservation should be established by a complete balance sheet including all the

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fragments at each point along the reaction path. None of the methods used so far was able to yield data of sufficient accuracy in this respect. The present work was undertaken to furnish these data, followed by their use in kinetic modeling of the degradative process. With sufficiently accurate data, the kinetic analysis may give more refined insight into the sequence of events than the mere qualitative observation of the appearance of the large fragments. Furthermore, the kinetic scheme can be utilized to construct, or to eliminate, some models of the native molecule.

It was ascertained with the trypsin digestion of bovine fibrinogen that all requirements set forth in the preceding paragraph for mass conservation and molecular weight relationships were satisfied. For purposes of comparison three more systems were investigated: human fibrinogen digested by trypsin, and bovine and human fibrinogen digested by plasmin. In view of the extreme similarity of all four processes, it was deemed unnecessary to obtain the same detailed documentation for the latter ones as for the digestion of bovine fibrinogen by trypsin. The fragments derived from fibrinogen can be divided into two classes: high molecular weight fragments, bracketed by native fibrinogen and a fragment of approximately 50 000 molecular weight inclusive (fragment E), and low molecular weight fragments situated below these in the molecular weight spectrum of fragments. There is a clear gap between these two classes; the kinetic analysis also suggested that they are independent in some respect as far as their origin and mechanism of formation are concerned. Only the data for the high molecular weight class will be presented in this paper.

Experimental Section

Materials

Bovine fibrinogen (fraction I from bovine plasma) lot No. B 7907 from Armour Pharmaceutical Co., Kankakee, Ill., and lot No. G 10505 from Reheis Chemical Co., Chicago, Ill., were purified by Laki's procedure (1951). Clottability of the preparations ranged from 94 to 96%. Human fibrinogen (grade L) purchased from AB Kabi, Stockholm, Sweden (lot No. 59843), was used without further purification. Clottability of this batch was 95%. Both proteins were analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis after reduction under the conditions given by Weber and Osborn (1969). There was no evidence in either of them of degradation of the chains in gels stained by Coomassie brilliant blue. The fibrinogen preparations were dialysed exhaustively against 0.3 M NaCl in the cold. The concentration of their solutions was estimated spectrophotometrically using $E_{1cm}^{1\%}$ 15.06 for the corrected optical density at 280 nm (Mihalyi, 1968).

Three batches of Tos-PheCH$_2$Cl[1]-treated trypsin from Worthington Biochemical Co., Freehold, N.J., designated TRTPCK-ODA, -3CA, and -2GA by the manufacturer, were employed in these studies. Solutions of 1 mg per ml in 0.001 N HCl were prepared by weighing the enzyme powder without previous drying and were used immediately. Enzyme activity was estimated in the pH stat under the conditions given by Ehrenpreis and Scheraga (1957). One unit was defined as the amount of enzyme that hydrolyzes 1 μmol of Tos-Arg-OMe per min. The solutions had a corrected optical density ($E_{280} - E_{320})_{1cm}^{1\%}$ of 13.31 ± 0.008 and hydrolyzed 224 ± 8 μmol of Tos-Arg-OMe per min per ml. There was no significant difference in this respect between the three batches used.

Crystalline soybean trypsin inhibitor (lot SI-2CA) was obtained from the same manufacturer as the trypsin.

Human plasminogen (lot No. 46541-39) was purchased from AB Kabi, Stockholm, Sweden. It was activated by Urokinase B grade (lot No. 001564) from Calbiochem, San Diego, Calif., for 24 h at room temperature in a Tris-lysine buffer of pH 8.0, containing 25 volume % glycerol. The resulting plasmin was purified by a method based on the procedure of Robbins et al. (1965), by gel filtration through a Sephadex G-200 column, followed by ammonium sulfate precipitation. The precipitate was dissolved in 0.1 M NaCl containing 50 volume % glycerol and dialyzed exhaustively against the same solvent. It is important to remove the last traces of ammonia, otherwise ammonia is carried away by the gas stream during the reaction in the pH stat and a stable baseline cannot be obtained. Enzymatic activity was estimated as described above for trypsin. It was established with a standard plasmin prepared at the Michigan Department of Health (Sgouris et al., 1960) that under these conditions 1 C.T.A. unit hydrolyzed 0.347 μmol of Tos-Arg-OMe per min. Concentration of the solutions was determined by optical density measurements in neutral solution, assuming $(E_{280} - E_{320})_{1cm}^{1\%} = 17.0$ (Robbins et al., 1965). Specific activity of the preparations was 25–30 C.T.A. units per mg.

Methods

Digestion. For obtaining the series of inhibited digests, samples of 10 ml of approximately 2% fibrinogen solution in 0.3 M NaCl were digested at pH 8.0 and 25 °C in the pH stat (Mihalyi and Godfrey, 1963). The reaction was initiated by 0.4 ml of trypsin solution and stopped after the desired level was reached by 0.4 ml of soybean trypsin inhibitor solution of 2 mg per ml. For the plasmin digests, 0.8 ml of enzyme solution of 10.3 C.T.A. units per ml was used, and the reaction was stopped by 0.4 ml of soybean trypsin inhibitor solution of 4 mg per ml. These conditions, referred to in the following as standard conditions, were used in all but the experiments performed to investigate the pH dependence of the digestion reaction.

Gel Filtration Chromatography. Columns of 2.6 × 94 and 5 × 94 cm gel beds were poured from Sephadex G-200, 40–120-μm particle size, obtained from Pharmacia, Uppsala, Sweden. The columns were operated at 4 °C in the down-flow mode. Samples of 75–100 mg of protein in 4-ml volumes were loaded on the narrower column and four times larger samples on the wider column. Eluting buffer was 0.05 M sodium phosphate, pH 7.1, 0.15 M NaCl, for the narrower column used for analytical purposes, and 0.15 M ammonium formate, pH 7.4, containing 0.05 M ε-aminocaproic acid, for the larger, preparative column.

Electrophoresis in Polyacrylamide Gel in the Presence of Sodium Dodecyl Sulfate. A method was used that enabled direct scanning of the gels at 280 nm (Dravid et al., 1969; Watkin and Miller, 1970). Gels of 13-cm height of 5% acrylamide and 0.135% bisacrylamide content were cast in fused silica tubes of 0.47-cm inner diameter. The buffer system contained 0.1 M sodium dodecyl sulfate, 0.1 M Tes adjusted to pH 8.0, and 25 volume % glycerol. For the uv scan, it was essential to use commercially available, specifically purified reagents to ensure a low background absorption. Protein samples of approximately 13 mg per ml in 0.133 M sodium

[1] Abbreviations used are: Tos-PheCH$_2$Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Tos-Arg-OMe, tosyl-L-arginine methyl ester; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Temed, *N,N,N′,N′*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)-aminomethane; uv, ultraviolet.

TABLE I: Analysis of the pH Stat Curves.

No. of Determ.	Digestion	Kinetic Parameters of the Reaction at pH 8.0 and 25 °C				No. of Bonds Cleaved[c]	
		k_1[a]	A_1[b]	k_2[a]	A_2[b]	Fast Reaction	Slow Reaction
6	Bovine fibrinogen–trypsin	0.69 ± 0.05	10.7 ± 0.6	0.055 ± 0.003	58.5 ± 1.2	13.2	91.3
3	Human fibrinogen–trypsin	0.39 ± 0.01	20.8 ± 1.1	0.052 ± 0.001	44.6 ± 0.4	25.7	79.1
2	Bovine fibrinogen–plasmin	0.61 ± 0.30	2.5 ± 0.7	0.024 ± 0.004	34.5 ± 2.4	3.1	54.3
3	Human fibrinogen–plasmin	0.99 ± 0.25	3.2 ± 0.2	0.048 ± 0.005	31.0 ± 0.8	5.2	50.1

[a] Rate constants k_1 and k_2 were calculated with natural logarithms and time in minutes. For trypsin they were converted to 10 U/ml, for plasmin to 1 C.T.A. U/ml enzyme concentration, assuming linear relationship between these two quantities. [b] The amount of base consumed for completion of the reaction class indicated by the subscript. [c] Calculated from the A values at pH 8.0 and the corresponding pK's, or by computer fitting of the A values at various pH values to an ideal titration curve.

dodecyl sulfate–0.033 M Tes buffer of pH 8.0 were incubated for 1 h at 37 °C prior to electrophoresis. After incubation 25 volume % glycerol was added to the samples. An approximately ten times larger load was necessary for the direct scanning than for stained gels. A single component of $E_{1cm}^{1\%}$ 15 gave a peak height of about 1.0 when loaded in an amount of 80 μg per gel. Electrophoresis was conducted at 7 mA per gel for approximately 4 h. After electrophoresis the gels were scanned in the chromatogram scanner of Zeiss, Oberkochen, Germany (Jork, 1968), without removing them from the tubes. The areas were obtained either by the disc integrator of the recorder, or by weighing the patterns transferred to graph paper and cutting them out with scissors. To one set of the digests, bacitracin was added as an internal marker and the R_f values of the peaks were calculated with respect to the marker on twofold expanded scans. Molecular weights were read on a calibration curve obtained under identical conditions with nine unreduced proteins and their polymers, covering the 670 000 to 18 000 molecular weight range. Another set of digests, without bacitracin, was scanned on a fourfold expanded scale. These patterns were used for area determinations.

High-Speed Sedimentation Equilibrium. Runs were performed at 4 °C in the Beckman-Spinco, Model E analytical ultracentrifuge, equipped with the photoelectric scanner. Solutions of approximately 0.2 optical density at 280 nm and 1 cm light path were loaded in a cell with a six-hole centerpiece (Yphantis, 1964). Solvent was 0.05 M sodium phosphate (pH 7.1)–0.1 M NaCl–0.05 M ε-aminocaproic acid. The sedimentation equilibrium patterns were analyzed with the computer program of Roark and Yphantis (1969).

Total Nitrogen Estimates. Duplicate samples containing 0.2–0.5 mg of N were digested for 90 min with concentrated H_2SO_4 to which K_2SO_4 and HgO were added. Distillations were performed in the micro nitrogen distillation apparatus, ACS specifications (Steyermark et al., 1951).

Specific Optical Densities of the Fragments. These were obtained by recording the ultraviolet absorption spectra in the Cary 15 recording spectrophotometer and determining the total nitrogen content of the same protein solutions of approximately 1 mg per ml concentration. The following preparations were used: bovine fibrinogen was purified from Reheis, lot No. G 10505. Fragments X and Y were prepared from a digest carried to 35.6 equiv of base consumed per 340 000 g of fibrinogen at pH 8.0. The components were isolated on the wide Sephadex G-200 column and recycled on the same column. They were dialyzed free of ε-aminocaproic acid against 0.1 M NaCl, containing 0.01 mg soybean inhibitor per ml. Fragments D and E were isolated from a digest conducted to 54.4 equiv of base consumed per 340 000 g of fibrinogen at pH 8.0 as described recently (Mihalyi and Towne, 1976). Fraction P1 was obtained by pooling peak V of the Sephadex G-200 fractionations. For this preparation ε-aminocaproic acid was omitted from the eluent. The lyophilized powder was dissolved in a small volume of water and salts were removed by a short dialysis against distilled water. Fraction P2 was prepared from the supernatant of trichloroacetic acid precipitates of the digests. For both the isolation of P1 and P2 the digestion was carried to 54.4 mol of base consumed per mol of fibrinogen at pH 8.0.

Human fibrinogen was digested by plasmin to the extent of 32.4 mol of base used up in the pH stat per mol of fibrinogen. Fraction P1 was obtained by gel filtration of the digest on Sephadex G-200 column. Peak V was pooled, lyophilized, and separated from salts and ε-aminocaproic acid by gel filtration on a 2.5 × 85 cm Sephadex G-10 column, using distilled water as eluent. Fraction P2 was isolated from the supernatant of the digest precipitated by 15% trichloroacetic acid.

Results

Kinetics of the Hydrolysis of Peptide Bonds Monitored by the pH Stat. Human fibrinogen is digested more rapidly in the initial stages by either trypsin or plasmin, but eventually both bovine and human fibrinogen reach approximately the same level of base consumption. However, this level with plasmin is only about two-thirds of that obtained with trypsin. All the pH stat curves are accurately described by the sum of two simultaneous first-order reactions, as already reported for the trypsin digestion of bovine fibrinogen (Mihalyi and Godfrey, 1963). The resolution into the constituent exponential curves was performed by the Modelaide program (Shrager, 1970) and the results for pH 8.0 are given in Table I.

For the trypsin digestion the sum of the bonds cleaved in the two reactions is the same with the two proteins, although their distribution between the fast and the slow reaction is different. There are relatively more bonds cleaved in the fast reaction with human fibrinogen than with the bovine one. This causes an apparently faster rate in the initial part of the pH stat curve, even though the actual rate of splitting of the bonds in the fast reaction is slower in human than in bovine fibrinogen. The slow reaction appears to proceed at the same rate with both proteins.

The pH stat curves of the plasmin digestion of human and bovine fibrinogen indicate a faster overall reaction with the human protein. This is apparent also in the rate constants given in Table I. On the other hand, the number of bonds in the fast and slow reactions is nearly the same with the two proteins. Comparison of the rates with trypsin and plasmin, at equimolecular concentrations of the enzymes, indicates a nearly twice as high rate for plasmin. For these calculations it was assumed that the specific activity of pure plasmin is 35 C.T.A.

units per mg. This result is in contrast with the nearly sixfold lower esterase activity of plasmin as compared with trypsin, at equal molar concentrations, on Tos-Arg-OMe at pH 8.0.

The digestions were performed also at various pH values and the pH stat curves were resolved into the constituent reaction curves. The base consumption for each of these was fitted to a titration curve. The computer evaluated the best value of the pK, the asymptotic value of the base consumption reached at high pH, corresponding to the actual number of bonds cleaved, and the electrostatic factor. The pK values found for the α-NH_2 groups produced in the slow reaction were 7.75 ± 0.10, 7.89 ± 0.08, and 7.76 ± 0.11, for trypsin digestion of bovine fibrinogen, trypsin digestion of human fibrinogen, and plasmin digestion of bovine fibrinogen, respectively. There were insufficient data for the slow reaction with plasmin digestion of human fibrinogen and the data for the fast reaction with either of these systems were not accurate enough for the analysis. A value of 7.37, however, was obtained earlier (Mihalyi and Godfrey, 1963) for the pK of the groups formed in the fast reaction of the trypsin digestion of bovine fibrinogen, and this was used for all four digestion reactions. The curve fitting indicated that the values conform to an ideal titration curve, with no indication of any electrostatic effect. The base consumptions (A values), normalized with respect to the asymptotic values, for the slow reaction of the trypsin digestion of human and of bovine fibrinogen, and for the plasmin digestion of bovine fibrinogen, are plotted against pH in Figure 1. The data for the trypsin digestion of bovine fibrinogen are taken partly from a previous study (Mihalyi and Godfrey, 1963). Since the above pK values do not differ by more than the curve fitting error, a titration curve with a pK of 7.79, corresponding to their average, was drawn in the figure. The actual number of bonds cleaved in the reactions, calculated either from the A values at pH 8.0 and the corresponding pK's, or by curve fitting of the A values vs. pH, are also listed in Table I.

In the course of these studies it was found that fibrinogen from Armour (lot B 7907) had approximately 10% fewer bonds cleaved in both the fast and the slow reaction than fibrinogen from Reheis (lot G 10505). The experiments described in this paper, with the exception of the gel filtration studies shown in Figure 2, were performed with the latter lot.

For the validity of a kinetic analysis of an enzymatic reaction, the enzyme activity should remain constant and the products should not inhibit the reaction. Control experiments showed that under the conditions of the experiments described here these two requirements were satisfied: activity of the enzyme at the end of the slow reaction was unchanged when tested in a Tos-Arg-OMe assay; and the low molecular weight digestion products, when added to native fibrinogen, had no appreciable effect on the rate of digestion of the latter.

Gel Filtration. The present studies were limited to the reaction phase characterized by the completion of the cleavage of the molecule into the D and E fragments, that corresponds approximately to the end of the slow reaction observed in the pH stat. The elution patterns obtained with native bovine fibrinogen purified from Armour (lot B 7907) and eight trypsin digests of this material of progressively more advanced degree of digestion are shown in Figure 2. Each of the digest patterns can be resolved into six components, although in some patterns some of the components may show considerable overlapping, or are not separated at all, and also, they may be present at a barely perceptible level. However, whenever they are separated, the position of each peak is the same within an average of two fractions. On this basis, when the curves were analyzed by the Modelaide program (Shrager, 1970), it was postulated

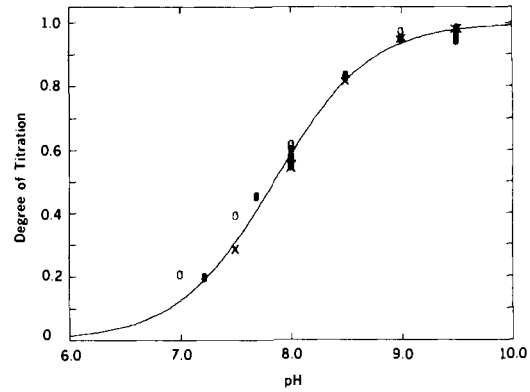

FIGURE 1: Normalized amount of base consumed in the slow reaction of the digestion of fibrinogen plotted against pH. (○) Trypsin digestion of bovine fibrinogen; (●) trypsin digestion of human fibrinogen; (X) plasmin digestion of bovine fibrinogen.

that a component was present at each of the six positions with all the digest patterns. To each of these an ideal Gaussian curve was assigned and the computer was allowed to find the best fitting position of these, as well as their half-width and relative areas. The correspondence between observed and computed curves was exceptionally good, except for slightly higher experimental values between peaks IV and V, with the more advanced digests. Although this indicated another small component at this position, no attempt was made at this point to improve the fit by adding an extra component here.

The components were identified by analogy with those described by other authors with the plasmin digestion of human fibrinogen (Marder et al., 1969), and by their molecular weights as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by sedimentation equilibrium, to be described in the following sections. In order of descending molecular weights, the first component is obviously native fibrinogen. This had the same elution volume as the undigested sample. The following two correspond to the intermediate fragments, fragments X and Y characterized by Marder et al. (1969). The fourth component is the counterpart of fragment D of Nussenzweig et al. (1961), which is not resolved from their fragment E. The latter appears only as the slight difference between experimental and calculated values in the region immediately following fragment D. The fifth and sixth components are peptidic material. The former, which will be denoted P1 here, is a peptide of fairly high molecular weight, described in a previous publication (Mihalyi, 1970), whereas the latter represents a collection of low molecular weight peptides and will be called P2.

The relative areas of the high molecular weight components will not be discussed here, because equivalent data were obtained by the sodium dodecyl sulfate–polyacrylamide gel electrophoresis method, that will be described in the next section. However, the low molecular weight P1 and P2 components were more accurately determined by the gel filtration method and their evolution is depicted in Figure 3. The area of P1 reaches a maximum very early and then remains constant for the entire duration of the experiment. This occurs for all practical purposes while fibrinogen is transformed into fragment X. P2 is built up much more slowly and reaches its plateau at about the time when most of the intermediate fragments disappear. Both the formation of P1 and P2 appear to follow a first-order reaction course, with rate constants of 0.48/min and 0.11/min, respectively, at a trypsin concentration of 0.0446 mg/ml (10 units/ml).

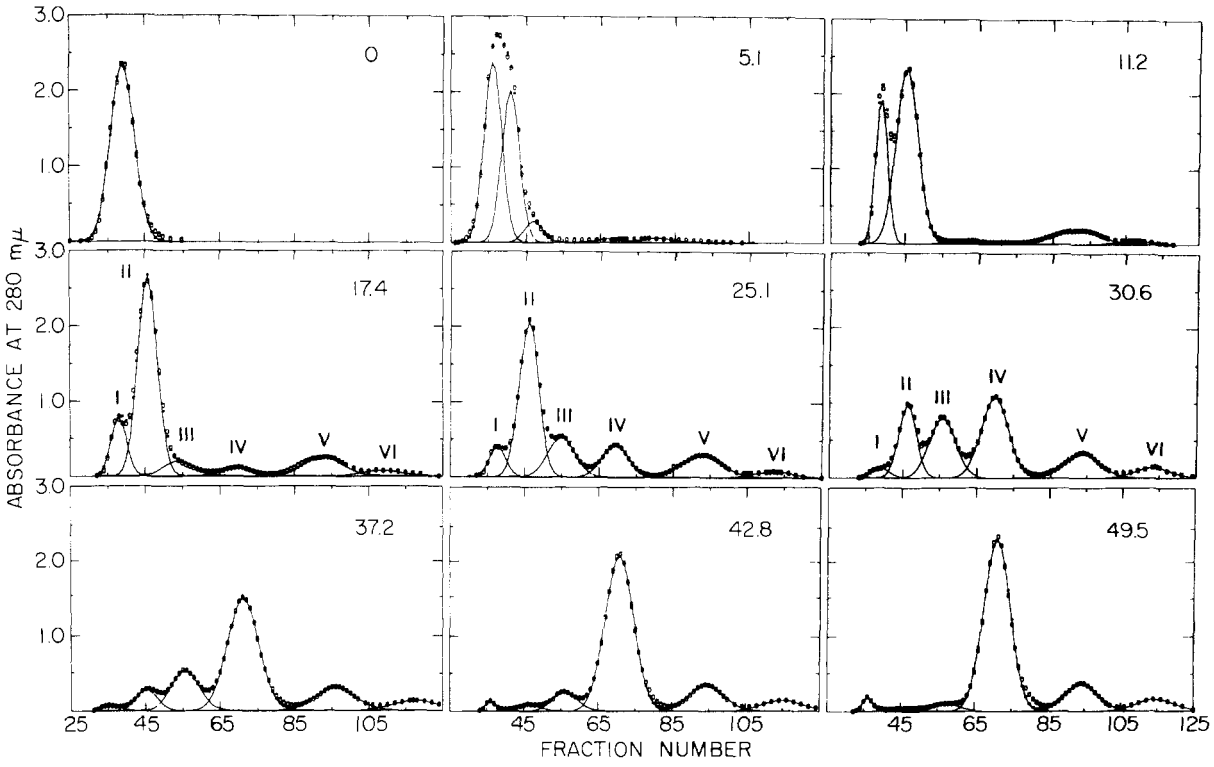

FIGURE 2: Elution diagrams of native bovine fibrinogen and of eight of its tryptic digests obtained with a Sephadex G-200 column. Arabic numerals in the frames indicate moles of alkali per mole of fibrinogen consumed in the pH stat up to the moment of arrest of the reaction. Roman numerals identify the individual peaks. Empty symbols are experimental points; filled symbols are computed values. From Mihalyi (1972), by permission.

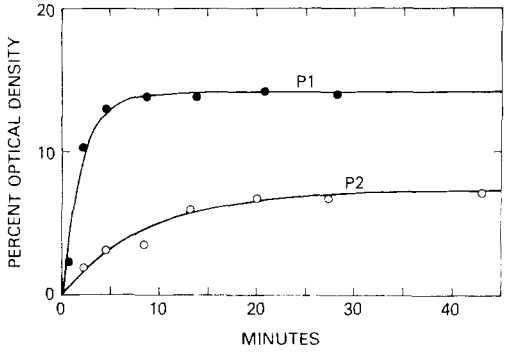

FIGURE 3: Formation of the two peptide fractions, P1 and P2, during trypsin digestion of bovine fibrinogen. Peptide fractions estimated by the areas of peaks V and VI of the diagrams in Figure 2.

The tryptic digests of bovine fibrinogen were the only complete set studied by gel filtration chromatography. Sporadic checks were made on digests of the other three systems and these were in agreement with the data furnished by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Electrophoresis in Polyacrylamide Gels in the Presence of Sodium Dodecyl Sulfate. Scans of a series of digests of bovine and of human fibrinogen with either trypsin or plasmin were obtained. The general features in all four groups were strikingly similar and corresponded to those seen in the gel chromatography patterns. The high molecular weight group displayed the familiar stepwise procession of the digested material through fragments X and Y into the terminal products of this phase of the digestion, fragments D and E. But, whereas in gel filtration the latter were not resolved, in the gel scans they were clearly separated. Each peak was identified on the basis of its molecular weight. This was derived from the R_f values calculated from these runs and also checked by comparison with scans of the isolated pure tryptic fragments of bovine fibrinogen, whose molecular weights were known from the sedimentation equilibrium studies that will be described later.

It was ascertained that the area of peaks obtained with the uv scanning of the gels, with purified proteins, was a linear function of the amount of protein loaded. Further, with the same fibrinogen digest, the areas of the components corresponded within ±2.0% to those obtained by gel filtration. Whereas in the elution patterns the heavy components overlap to a considerable degree, in the gel scans they are well separated and the computer resolution of the curves is unnecessary. Thus, the gel electrophoresis method appears to be of equal accuracy as the much more laborious and time consuming gel chromatography. Difficulty was encountered only with the quantitation of the P2 fractions, whose low and broad peak occurs in the region of the salt boundary where baseline anomalies are frequent. For these, the optical density fractions in the supernatants of digests precipitated by 15% trichloroacetic acid were used.

The areas of the high molecular weight peaks are plotted against degree of digestion in Figure 4. There is a remarkable similarity between all four systems investigated. Fibrinogen and fragment X disappear fast as the digestion proceeds. If the two were separated, one could see a transient accumulation of fragment X followed by its decay. As it stands, all that is seen in the curve of their sum is an inflection corresponding to the peaking of fragment X. Fragment Y has a similar transient existence. A "triple point", where the optical density fractions in fragments X, Y, and D are nearly equal, appears in all four sets of curves. Also, fragments D and E reach a plateau at approximately the same level in all four cases. Whereas the mechanism of formation of the high molecular weight fragments of human and bovine fibrinogen is similar, there is an appreciable difference between their rate of formation. Human fibrinogen appears to be fragmented with more difficulty by

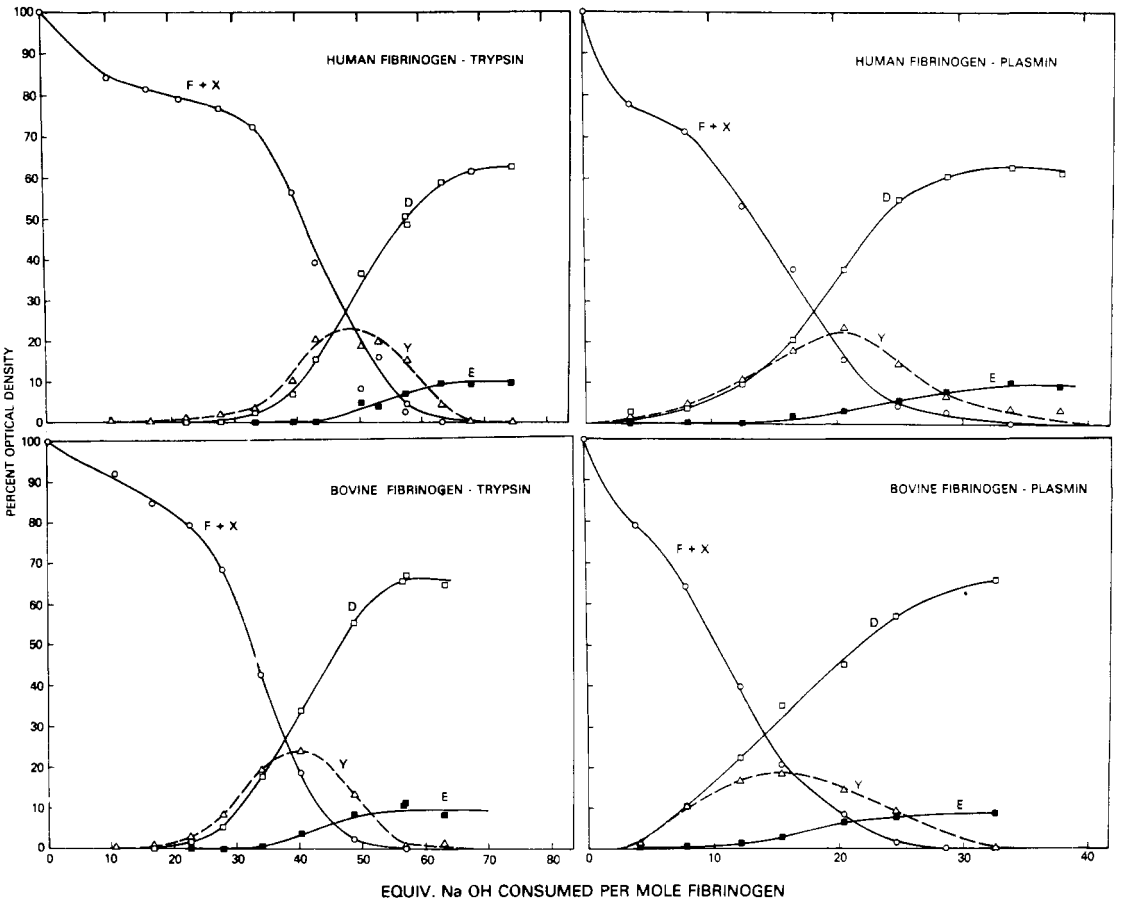

FIGURE 4: Fraction of total optical density contributed by each fragment in the mixture, determined from the areas under the peaks of the gel electrophoresis scans, plotted against degree of digestion, expressed by the number of moles of base consumed in the pH stat. Each fragment identified by a different symbol and by the letters placed next to the curves.

TABLE II: Molecular Weights of Fragments from Mobility in Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.

Digestion	Mol Wt of Fragments								
	X_1	X_2	Y_1	Y_2	D[a]	E	Pl_a	Pl_b	Pl_c
Bovine fibrinogen-trypsin		213 000		133 000	88 000-79 000	42 000			16 200
Human fibrinogen-trypsin		206 000		132 000	86 000-78 000	47 000	26 000	21 600	16 200
Bovine fibrinogen-plasmin	275 000	241 000	191 000	142 000	89 000-82 000	47 500	27 000		18 000
Human fibrinogen-plasmin	267 000	223 000	179 000	136 000	88 000-78 000	44 500	26 500		18 100

[a] Range of values. Apparent molecular weight decreased during digestion progressively from first to last value.

both trypsin and plasmin. Comparison of the curves for the trypsin digestion of the two proteins reveals that each component of the human fibrinogen digests is delayed equally with respect to those of bovine fibrinogen, so that the whole family of curves is shifted to the right by about 10 mol of base consumed, without any change in the shape or interrelationships of the curves of the individual components. The difference is even more conspicuous when the relative areas of the fragments are plotted against digestion time. A similar shift is apparent also in the curves of the plasmin digests; however, it is more modest, amounting to 4 mol of base consumed per mol of fibrinogen.

Digests at any desired level of digestion can be prepared in a highly reproducible manner by stopping the reaction at the appropriate base consumption, read on Figure 4. Also, the base consumption can be correlated easily, by consulting this figure, with the previously introduced scales of the reaction. For example, the stages defined by Marder et al. (1967), for the plasmin digestion of human fibrinogen, correspond to base consumption in the following way: stage I at 5–12, stage II at 13–29, and stage III at 30–40 mol of base consumed per mol of fibrinogen at pH 8.0 and 25 °C.

The molecular weights of the fragments estimated from the R_f values are given in Table II. The peak positions of the various fragments, with the exception of that of fragment D, are constant through the whole reaction and are the same within experimental error with fragments from either of the four systems studied. The position of fragment D appears to shift slightly during the reaction period considered here, and this shift corresponds to a decrease in molecular weight of about 10 000. All the values in the table are based on the position of the apex of the peak which presumably represents the most abundant component in the case of heterogeneity. Thus, the heavier species of fragment X, unless it is clearly separated, does not appear here, and only the molecular weight of its lightest component is estimated. Similarly, the shift of the

TABLE III: Stoichiometry from Optical Density Distribution in Digests Carried to Completion of Formation of Fragments D and E.

Fragment	% Optical Density (280 nm)	% OD/ Spec. OD	% Mass	Apparent Mol Wt
	Bovine Fibrinogen–Trypsin			
D	69.7 ± 1.7	3.48	52.0	176 700
E	8.6 ± 1.2	0.96	14.4	48 900
P1	14.3 ± 1.2	0.66	9.8	33 300
P2	7.4 ± 0.4	1.59	23.8	81 000
Sum	100.0	6.69[a]	100.0	339 900
	Human Fibrinogen–Plasmin			
D	65.3 ± 0.2	3.14	50.0	170 000
E	10.5 ± 0.1	1.03	16.4	55 700
P1	16.2 ± 1.1	1.13	18.0	61 200
P2	8.0 ± 1.2	0.98	15.6	53 000
Sum	100.0	6.28	100.0	339 900

[a] The theoretical value for the sum is either 6.76 or 6.64, depending on whether 14.78 or 15.06 is taken for the specific optical density of native fibrinogen.

position of the peak of fragment D suggests the presence of a number of closely spaced subspecies, with the weight shifting from the heavier toward the lighter components as the digestion proceeds.

Specific Optical Densities of the Fragments. These were determined for the complete set of fragments of the tryptic digests of bovine fibrinogen. The $(E_{280} - E_{320})_{1cm}^{1\%}$ values are as follows: fibrinogen 14.7_8, fragment X 16.6_2, fragment Y 15.6_1, fragment D 20.0_4, fragment E 8.9_7, peptide fraction P1 21.7_3, and peptide fraction P2 4.6_0. For the plasmic fragments of human fibrinogen only the values for the P1 and P2 fractions were obtained in the present studies and these were 14.3_8 and 8.0_6, respectively. The specific optical densities of the high molecular weight fragments of this system were determined by Marder et al. (1969).

Stoichiometry of the Fragmentation Products. The distribution of optical density among the degradation products, obtained either by gel filtration chromatography or by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, can be converted into distribution of mass by dividing each fraction by the corresponding specific optical density. It has been proven experimentally that the optical density of the system at 280 nm remains constant within experimental error during the digestion. In consequence, the sum of the above ratios should also remain constant and equal 100 divided by the specific optical density of fibrinogen. The constancy of the sum appears to be a sensitive indicator of the accuracy of the estimates of both the composition of the digests and of the specific optical densities of the fragments.

The agreement of the sum with the expected value, for all the tryptic digests of bovine fibrinogen, was surprisingly good, in view of the experimental errors involved in two sets of independent and entirely different kind of determinations.

The mass distribution in the digest at the moment when fragments X and Y vanish and only fragments D, E, P1, and P2 are present can be utilized to estimate the molecular weights of the latter. At this point the total mass of the original fibrinogen molecule is distributed among these fragments and the mass fraction of each of these related to the molecular weight of the parent molecule will give the molecular weight present in the respective fraction. This, however, will be equal to the actual molecular weight only if a single fragment of a kind is formed from one molecule. If more than one is formed, then their number can be found from the molecular weight fraction and the actual molecular weight determined by some other method. These calculations for tryptic digests of bovine fibrinogen are summarized in Table III. The values for fragments D and E are averages of their determination in five separate digests at their plateau region. Those for fragments P1 and P2 were taken from the computer fitting of the kinetic curves shown in Figure 3. The above method can be applied only to these fragments because they are relatively stable, approaching a plateau value close to their true stoichiometry. It is not applicable to fragments X and Y, that are short lived intermediates, further degraded before they can reach a stoichiometric quantity.

Of the other three digests investigated only the plasmin digests of human fibrinogen can be analyzed by this method, because specific optical densities of the fragments are available only for this system. Calculations of the stoichiometry were performed with the average of the data obtained by gel filtration published previously (Mihalyi and Towne, 1976) and those obtained by gel electrophoresis described in this paper. The data near the plateau region were corrected for the small amount of fragments X and Y present, by assuming that X → 2D + E and Y → D + E. The results are listed also in Table III.

Sedimentation Equilibrium Studies. Molecular Weights of the Fragments. Only bovine native fibrinogen and its tryptic fragments were investigated in these studies. The preparations were the same as those used for the determination of the specific optical densities. Number and weight- and Z-average molecular weights of fragments X, Y, D, and E are plotted against concentration in the cell in Figure 5. These plots were obtained by the Roark–Yphantis (1969) computer program. The first three show a moderate degree of self-association, whereas that for fragment E indicates nonideal behavior. That the curves show association and not the presence of heavier species can be inferred from the sodium dodecyl sulfate–polyacrylamide gel electrophoresis data. With all the preparations studied, only one major component was present, with insignificant contamination from the heavier or lighter fragments. All the molecular weight averages extrapolate at zero concentration to the same value: 220 000, 134 000, 84 000, and 48 500, for fragments X, Y, D, and E, respectively. This corresponds to the monomeric weight, or if heterogeneity unresolved by gel electrophoresis was present, then to that of the lightest species. The sedimentation equilibrium curves for native fibrinogen and fragment P1 are not shown. Fibrinogen exhibited associating behavior similar to fragment X, whereas

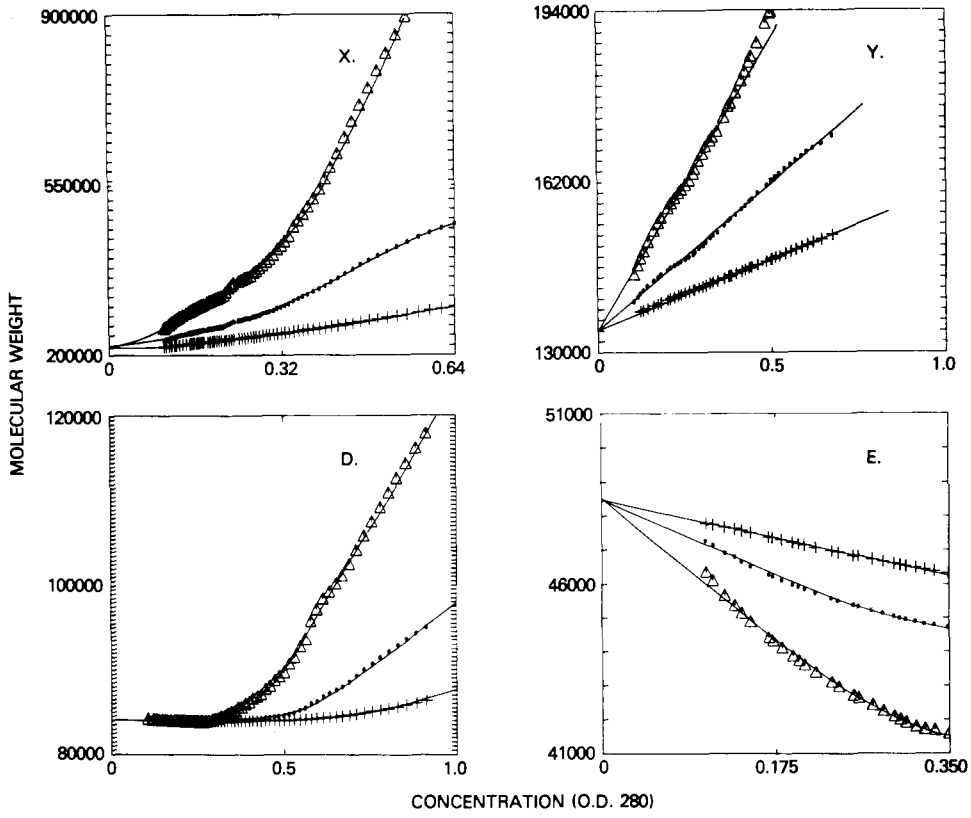

FIGURE 5: Sedimentation equilibrium patterns for fragments X, Y, D, and E. Number average (+), weight average (○) and Z average (△) molecular weights plotted against protein concentration (OD 280 nm) in the ultracentrifuge cell.

fragment P1 displayed a low degree of association. The respective molecular weights were 335 000 and 14 400. In all the molecular weight estimations the same value for the partial specific volume was used, namely 0.72. This is an average of that determined for native bovine fibrinogen (Scheraga et al., 1954) and for bovine D fragment (Mihalyi, 1970).

Discussion

The base consumption during the digestion, recorded by the pH stat, was utilized in these studies to define the extent of the reaction. Digests arrested at various levels of digestion were then analyzed by either gel filtration chromatography or sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The members of the high molecular weight group are characterized by a fixed position of their peaks in the elution diagrams and only a small shift, if any, confined to fragment D, in the gel electrophoresis patterns. The peaks are symmetrical with the exception of that of fragment X. In gel electrophoresis they are well separated and the tracing comes close to the baseline between the peaks. All this indicates that the fragments are well-defined molecular species, with no evidence of material being slowly shifted from one peak to another by continuous degradation. Naturally, the resolution of the methods is not sufficient to disclose a narrow distribution of molecular weights around an average value. However, the symmetry of the peaks, their invariable position, and their adherence to an ideal Gaussian curve in the gel filtration pattern all indicate that the distribution must be narrow indeed.

In gel electrophoresis scans of tryptic digests of human fibrinogen, fragment X is present as a single peak. In the other three systems investigated this component exhibits a double peak of variable degrees of resolution. With the tryptic digests of bovine fibrinogen a doublet of two closely spaced species is observed only on expanded scans of the earliest digests. On the other hand, with plasmin digests of the same protein, the two peaks are clearly visible through the entire life cycle of fragment X, and the ratio of the two areas is shifted in favor of the lighter one as the digestion proceeds. In contrast to this, with plasmic digests of human fibrinogen, only the scan of the first digest shows a shoulder corresponding to the heavier subspecies of fragment X and on the following ones only a fast disappearing skewing of the peak betrays its presence.

The heterogeneity of fragment X was first recognized by Mills and Karpatkin (1972). This is caused by the stepwise removal of approximately 30% of the mass of the molecule, predominantly from the C-terminal portion of the two Aα chains. There are some 100 sites with trypsin and 60 sites with plasmin that are cleaved in this process, according to the pH stat studies. With a random distribution, and equal rates of cleavage at these sites, a very heterogeneous population would result and the decrease of the average molecular weight would be nearly continuous. Actually, distinct subspecies are observed, due to the fact that the sites are not equally spaced, and a few are cleaved at nearly ten times higher rate than the others. Also, significant quantities of high molecular weight peptide segments arise, ranging from 40 000 to 15 000 molecular weight, together with a substantial amount of small peptides (Mills and Karpatkin, 1972; Lahiri and Shainoff, 1973; Furlan and Beck, 1972; Mosesson et al., 1973). The main difference in the four systems studied here resides in the variability of these sites and of their rate of cleavage. Simplest appears to be the bovine fibrinogen-trypsin case, in which only one heavy peptide segment is observed (P1) and the small peptides presumably are removed one by one, or in larger pieces that are immediately broken up into the small peptides. In the other cases, the P1 fraction appears as a triplet, or doublet, that

is slowly degraded to a final product of smaller size. Unfortunately, neither the resolution of the subspecies of fragment X, nor of the large peptides formed (P1), was sufficient to construct a more detailed mechanism of the process. As it will be apparent later, the molecular weight of the last subspecies of fragment X corresponds to the original molecule stripped of all these peptide segments. This process is faster than the cleavage into the D and E fragments, so that it is largely completed when the latter are formed. Also, as the kinetics will suggest, the scissile segment may have a protective effect on the segments connecting the D and E regions, in the sense that these cannot be cleaved before the C-terminal portion of the $A\alpha$ chain is removed. Therefore, as the molecular weight data clearly suggest, very little if any peptidic material is lost in the fragmentation following the formation of fragment X. Thus, the separation of fragments D and E has the earmarks of a surgically clean operation. Plasmin, because of its restricted specificity is more likely to produce large peptides, and also a more heterogeneous fragment X, and this is actually what is observed. Also, some of the prospective large peptides may be carried over into fragment Y, causing the latter to appear as a doublet, as it is clearly seen in the plasmic digests of bovine fibrinogen, but the amount of the heavier species is relatively small.

Heterogeneity of fragment D has been described by several authors (Jamieson and Gaffney, 1968; Mills, 1972; Furlan and Beck, 1972; Mosesson et al., 1973; Kudryk et al., 1974; Ferguson et al., 1975). In all the plasmin digests of both human and bovine fibrinogen, fragment D was homogeneous, even on expanded scans. The formation of the subspecies of fragment D is a slower process than the cleavage between the D and E regions of the molecule (Eisele and Mihalyi, 1975). Therefore, the amount of secondary degradation products must have been small in these samples and obscured by the broad main peak. However, it is possible that in stained gels the resolution would have been better, because of the much smaller load of protein. At any rate, the drift of the peak during digestion indicates a shift of the material into lower molecular weight species. The tryptic digests, especially those of human fibrinogen, show more distinctly the presence of the subspecies. In the bovine fibrinogen digests this is indicated only in the last one studied by a broadening of the base of the peak on the ascending side and a barely visible inflection point on the descending limb. With human fragment D a shoulder is present on the descending limb from the moment this fragment appears in noticeable amount. The difference in behavior is caused by combination of the delayed fragmentation of human fibrinogen by trypsin with nearly equal rates for the secondary degradation of the two proteins. As a result of this, human fragment D is degraded already into its subspecies at the stage of its full development, whereas its bovine counterpart is nearly homogeneous, because its formation was achieved much earlier. At a more advanced stage of digestion, e.g., at 65 mol of base consumed per mol of fibrinogen (not shown), there is little difference between these fragments from the two species. Both show partial resolution into two components of 82 000 and 72 000 molecular weight in about the same amount, indicating that the secondary process has about equal rates with human as well as bovine fibrinogen. The molecular weights correspond to those determined by Ferguson et al. (1975) for the D_2 and D_3 species.

Comparison of the stoichiometry of the fragmentation of bovine fibrinogen by trypsin and of human fibrinogen by plasmin (Table III) reveals that the sum of the P1 and P2 fractions is the same in both cases. Thus, the same segment appears to be removed from either molecule when the core fragments are formed. Then, the remaining mass is distributed somewhat differently between the core fragments, human plasmic fragment D being slightly smaller, E slightly larger than the corresponding bovine tryptic fragments. These conclusions, however, need further confirmation.

The molecular weights of the heavy fragments, as obtained in these studies, are nearly identical with the four systems investigated. This indicates that the essential structure of the molecule must be very nearly the same with bovine as well as human fibrinogen and also the two enzymes, trypsin and plasmin, must cleave at the same, or very closely spaced sites. The latter is not surprising at all, in view of the similar specificity of the two enzymes. The molecular weights of the fragments obtained with three fundamentally different methods are in excellent agreement. Of the methods employed, gel electrophoresis in the presence of sodium dodecyl sulfate is the least accurate (Frank and Rodbard, 1975) and with inadequate theoretical foundation. Agreement of this with the other methods may have been fortuitous since most authors reported higher values especially for fragments X and Y (Mills, 1972; Plow and Edgington, 1973; Fletcher et al., 1966). Sedimentation equilibrium is less open to criticism; however, the molecular weights derived suffer from uncertainties connected to the choice of partial specific volume. This is particularly true for solvent systems containing high concentrations of urea or guanidine, when also the binding of the unfolding agent should be considered. Also, with associating systems, higher molecular weights are obtained when association is neglected in the treatment of the data. The molecular weights obtained by sedimentation and diffusion, with data extrapolated to zero protein concentration, should be free of the last mentioned effect, and indeed, the data reported by Marder et al. (1969) are close to the ones determined here. The values of Albert et al. (1975) are slightly higher, presumably because the self-association of the molecules was neglected. Neither of the theoretical or experimental ambiguities applies to the values derived from stoichiometry, but unfortunately, this method cannot be applied to fragments X and Y.

Keeping in mind that fragment X is heterogeneous and what will be given here is the molecular weight of its lightest species, the approximate molecular weights can be listed as 220 000, 135 000, 85 000, and 48 000 for fragments X, Y, D, and E, respectively. These are the same for either of the four cases studied here. The numbers, in conjunction with the data in Table III, indicate that late fragment X corresponds to native fibrinogen stripped of all the peptide fragments P1 and P2. Also, its molecular weight corresponds to 2D + 1E. Similarly, fragment Y corresponds to 1D + 1E. These relationships have been deduced already by Marder et al. (1969) and Mills (1972). They follow also from the simplified scheme of the kinetics of the fragmentation process presented by Albert et al. (1975). The differential scanning calorimetry experiments of Donovan and Mihalyi (1974) provide additional evidence for the correctness of the above composition of the native molecule and its intermediate fragments in terms of the D and E domains they contain.

A comparison of the molecular weights of fragments D and E from the stoichiometry (Table III) with those calculated from sedimentation equilibrium or gel electrophoresis leads to the conclusion that two fragments D and one fragment E are formed from each molecule of bovine or human fibrinogen. Similarly, tryptic fragment P1 of bovine fibrinogen appears to be produced in duplicate. The molecular weights given for fraction P2 in Table III naturally represent the sum of all the

small peptides liberated. Physicochemical estimates for their average molecular weight have not been obtained.

The molecular weight data obtained in these studies are in disagreement with those postulated by the dimeric model of Mosesson et al. (1973). The latter calls for a molecular weight of larger than 200 000 for fragment Y, and larger than 150 000 for the earliest D fragment. These molecular weights were not seen, neither were the various intermediates that should arise in the stepwise transition between these fragments. This should have resulted in heterogeneity in these peaks and a much more pronounced shift in their average molecular weight than what was actually observed. The stoichiometries presented in Table III also clearly contradict the above model.

The molecular weight data, as well as the kinetics of the formation of peptides, suggest that fragments D and E are formed by a clean cut through the connecting segments of the two domains, with very little, if any liberation of peptidic material. The kinetic analysis to be presented in part II (Mihalyi et al., 1976) also indicates that each connecting chain is cleaved at a single, specific site. Summation of the molecular weights of the chain segments (Mills, 1972; Pizzo et al., 1972) supports also this contention, but final proof for the contiguity of the chain termini in the sequence of the intact chains should come from the determination of these sequences. This has been obtained now for the γ chain. Takagi and Doolittle (1975) and Collen et al. (1975) have shown that for the remnants of this chain the N-terminal sequence in the earliest D species and the C-terminal sequence in fragment E are contiguous. In later fragments the chain ends are shortened with the resultant formation of the subspecies of these. It is not known whether in this process new sites are uncovered as the chain ends unwind after the initial cleavage, or that sites cleaved at a much slower rate are present originally.

Acknowledgments

The authors are grateful to Drs. Roark and Yphantis for permitting the use of their computer program for the analysis of the sedimentation equilibrium data. We also thank Dr. H. P. Blau from the Institute of Applied Mathematics, University of Berne, for the initial curve fittings of the pH stat curves of the trypsin digestion of human fibrinogen, and Miss Geraldine Secor from Western Regional Research Center, USDA, Albany, California, for the micro-Kjeldahl determinations.

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