exclusive of other models. The calculations reported in our earlier paper (Pandit and Rao, 1974a) and this paper do not lead to such a clear-cut choice.

Acknowledgment

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Degradation of Fibrinogen by Plasmin. Isolation of an Early Cleavage Product†

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ABSTRACT: As part of a project aimed at developing sensitive, specific, and quantitative tests for in vivo proteolysis of fibrinogen or fibrin a peptide which is cleaved from human fibrinogen at an early stage of digestion by plasmin has been isolated and characterized. This peptide, which has been designated fragment H, appears to be fairly resistant to further plasmin digestion and to have a molecular weight of approximately 20,000 as determined by gel filtration and by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. It has a unique amino acid composition consisting of a high content of hydrophilic residues, especially serine, glycine, and proline, and a remarkably low content of hydrophobic residues. Edman degradation shows that it consists mainly of a single peptide chain whose NH_2-terminal sequence is Met-Glu-Leu-Glu-Arg-Pro-Gly-Gly-Asn-Glu-, and in addition there appears to be a minor contaminating chain. A product with the same characteristics has been isolated from a plasmin digest of the reduced carboxymethylated Aα chain of fibrinogen indicating that fragment H is produced by cleavage of the Aα chain. Polypeptides, apparently identical with fragment H, have also been isolated from plasmin digests of different samples of fibrin.

Three enzymes, thrombin, activated factor XIII, and plasmin, play important roles in the formation, stabilization, and subsequent lysis of a thrombus. In each case selected regions of the fibrinogen or fibrin molecule serve as substrates for the enzyme (Doolittle, 1973). Specific and sensitive methods to quantitate the levels of the reaction products of these enzymes in the circulation would be helpful in studying their roles in physiologic and pathologic states. One example of this approach has been the development of a radioimmunoassay for fibrinopeptide A, the 16 amino acid fragment which is released from the Aα chain of fibrinogen by thrombin to initiate the clotting process (Nossel et al., 1971). By means of this assay the plasma level of

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fibrinopeptide A has been shown to be elevated in diseases known to involve intravascular coagulation and thromboembolism (Nossel et al., 1974). In the in vivo states of either disseminated or localized intravascular coagulation, nonclottable degradation products from fibrinogen or fibrin, believed to be produced by plasmin, have been detected by immunologic assays directed against the terminal products of plasmin digestion of fibrinogen or fibrin (Merskey et al., 1966; Plow and Edgington, 1973a,b; Soria et al., 1973; Gordon et al., 1973). In order to develop a sensitive and possibly specific test for plasmin action in vivo by measuring the plasma level of one of the earliest fragments released from fibrinogen or fibrin by this enzyme, we have studied the products of limited exposure of fibrinogen to plasmin. We report here the characteristics of a large polypeptide which is released from the Aα chain and for which we plan to develop a radioimmunoassay. Numerous studies have shown that plasmin cleavage of fibrinogen or fibrin in vitro gives rise to a series of intermediate and end stage products which are usually designated by letters as initially proposed by Nussenzweig et al. (1961). For example, two large molecular weight intermediates have been designated as fragments X and Y, and two terminal products as fragments D and E (for a review see Marder, 1973). In accordance with this practice we have elected to refer to the polypeptide described in this report as fragment H.

Materials and Methods

Human fibrinogen (Grade L) with a clottability of 96% was obtained from AB Kabi, Stockholm, Sweden. Human plasminogen was isolated by affinity chromatography (Deutsch and Mertz, 1970) from Cohn fraction I-III kindly supplied by Dr. Carlos Erich of the New York Blood Center, or was lyophilized human plasminogen from AB Kabi. It was dissolved in 0.1 *M* NH_4HCO_3 or H_2O and stored at −20°. In all cases the activity was between 21 and 30 U/mg and on sodium dodecyl sulfate polyacrylamide gel electrophoresis the enzyme showed a major band corresponding to a molecular weight of 8.4×10^4 with a very faint band of higher molecular weight. Streptokinase was Streptokinase-Streptodornase Varidase (Lederle Laboratories) and was dissolved in H_2O and stored at 2°. Urokinase, reference standard, B Grade, and dithiothreitol were obtained from Calbiochem. The urokinase was dissolved in 0.15 *M* Tris-HCl (pH 7.5) and stored at −20°. Thrombin, topical grade, was bovine thrombin from Parke, Davis and Co. It was dissolved in H_2O and stored at −20°. Sephadex products were from Pharmacia. Ampholines were from LKB Instruments. The reagents used for Edman degradation were from Beckman Instruments. Acrylamide (electrophoresis grade) and *N,N'*-methylenebisacrylamide were from Eastman and the latter was recrystallized before use. Sodium dodecyl sulfate was lauryl sodium sulfate from Schwarz/Mann and was used without further purification. Acetic acid was aldehyde free from J. T. Baker Chemical Co. All other chemicals were reagent grade.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed as described by Brewer and Ashworth (1969) using the standard analytical system. Electrophoreses were run at 1.5 mA/tube in 7.5% gels. Polyacrylamide gel electrophoresis in dodecyl sulfate was performed essentially as described by Weber and Osborn (1969). Samples were incubated at pH 7.5 in solutions containing 1% dodecyl sulfate and 5 *M* urea; no reducing agent was used unless specifically mentioned, in which case the incubation solution was 1 m*M* in dithiothreitol; electrophoreses were run at 4–5 mA/tube; 5% gels were used to study the degradation of fibrinogen, and 7.5 or 10% gels for the smaller molecular weight fragments. The latter technique was used routinely to monitor reactions as well as to help identify and characterize reaction products. Gels were stained with Coomassie Brilliant Blue for 2–3 hr and destained in 7% acetic acid using an acrylamide gel diffusion destainer (Hoefer Scientific Instruments, San Francisco, Calif.).

Amino Acid Analysis. Amino acid analyses were performed using a Beckman automatic amino acid analyzer, Model 121, and a one-column system. Protein samples were hydrolyzed in vacuo in 6 *N* HCl at 109° for 24 hr unless otherwise stated. Tryptophan was determined after methanesulfonic acid hydrolysis (Liu, 1972).

Electrofocusing. An LKB 2117 Multiphor was used for electrofocusing, and the method outlined in the instructions for its use was followed except that the pH 9–11 Ampholine was omitted from the buffer mixture. Protein samples were applied with 10-mm^2 pieces of 3MM Whatman filter paper soaked in solutions containing 3–5 mg/ml of polypeptide in 0.01 *M* NH_4HCO_3. The gels were stained for 20 min at 60°, and the process of destaining was carefully observed since the peptides gradually washed out along with the Ampholines, and an attempt was made to stop destaining at the point of maximum contrast between the bands and the background.

Amino Acid Sequence Determinations. NH_2-terminal amino acid sequences were determined using the automated phenylisothiocyanate degradation method (Edman and Begg, 1967; Edman, 1970) and identifying the Pth[1] derivatives by thin-layer chromatography. In some cases identifications were confirmed by gas chromatography (Pisano and Bronzert, 1969). The aqueous phases from the final Pth extractions were examined by means of specific color reactions for Pth-His (Edman, 1970) and Pth-Arg (Easley et al., 1969).

Course of the Digestion of Fibrinogen by Plasmin. Fibrinogen, dissolved in 0.1 *M* Tris-HCl (pH 7.5) at a concentration of 10 mg/ml, was digested by incubation with plasminogen and streptokinase at 0°. Digestions were conducted using different concentrations of reagents, and at selected times aliquots were withdrawn and added to equal aliquots of 10 *M* urea–2% dodecyl sulfate to quench the reaction. The resulting samples were incubated at 37° for 2 hr, and 20-μl aliquots were studied by dodecyl sulfate–polyacrylamide gel electrophoresis in 5% gels. When suitable conditions were found for isolating what appeared to be an early, relatively stable digestion product, a portion of the reaction solution was acidified to pH 4 with glacial acetic acid. The acidified sample was incubated at 0° and examined by electrophoresis to establish that further digestion did not occur at this pH.

Preparative Digestion of Fibrinogen with Plasmin. Many modifications in the method for limited digestion of fibrinogen were evaluated and the procedure finally adopted was as follows: to 1.5 g of fibrinogen in 0.1 *M* Tris-HCl (pH 7.5), at 0°, were added 15,000 U of streptokinase and 100 U of plasminogen, and the volume was adjusted to 150 ml. After 1.25 hr at 0° the solution was acidified to pH 4 with glacial acetic acid and filtered over Sephadex G-100. De-

[1] Abbreviations used are: Pth, 3-phenyl-2-thiohydantoin; EDTA, ethylenediaminetetraacetic acid.

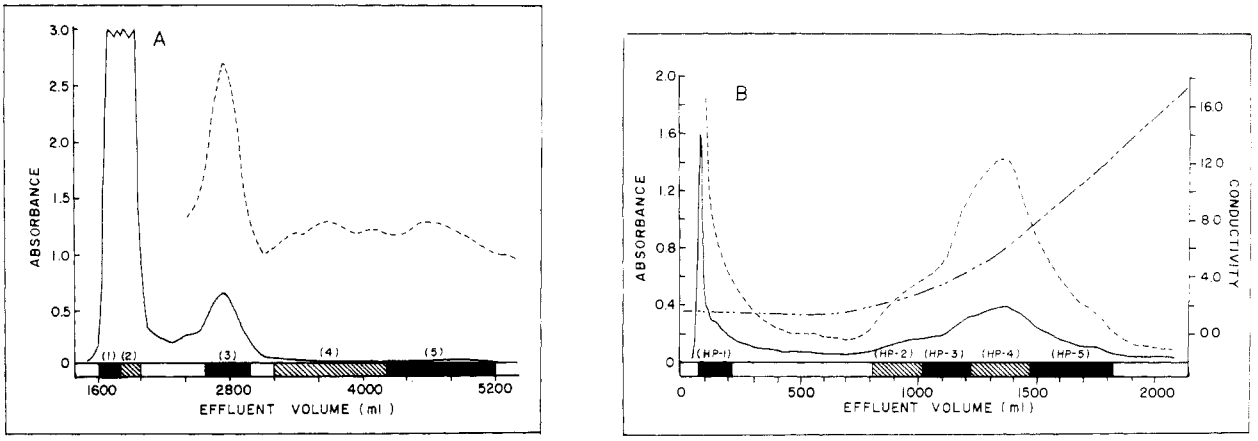

FIGURE 1: (A) Gel filtration on Sephadex G-100 (105 × 8 cm) of a limited plasmin digest of 1.5 g of fibrinogen. Eluent, 0.1 *M* Tris-HCl (pH 7.5) adjusted to pH 4 with glacial acetic acid; sample volume, 150 ml; flow rate, 200–250 ml/hr; fraction size, 20 ml; temperature, 4°. Effluent was monitored by absorbance at 230 nm (---) and at 280 nm (—). (B) Ion exchange chromatography of 182 mg of fraction 3, Figure 1A (denoted fragment H), on DEAE-Sephadex A-25. The column (35 × 2.5 cm) was eluted with 520 ml of 0.025 *M* Tris-HCl (pH 8.0) followed by a gradient composed of 850 ml of each of the following buffers in a series of three identical chambers: 1st chamber, 0.025 *M* Tris-HCl (pH 8.0); 2nd chamber, 0.125 *M* Tris-HCl (pH 8.0); 3rd chamber, 0.125 *M* Tris-HCl–0.425 *M* NaCl (pH 8.0). Sample volume, 12 ml; flow rate, 180 ml/hr; fraction size, 10 ml; temperature, 4°. Effluent was monitored by absorbance at 230 nm (---), by absorbance at 280 nm (—), and by conductivity expressed in mmhos (---). No further absorbing material was eluted when the column was washed with the third buffer.

tails of the gel filtration are given in the legend to Figure 1A. Small samples of the digestion solution and of the acidified solution were reserved for monitoring by means of polyacrylamide gel electrophoresis in dodecyl sulfate, as were aliquots of the peak tubes from the eluate and portions of the final isolated peptides. Fractions were pooled as indicated in Figure 1A and lyophilized after dialyses performed as follows: fractions 1 and 2 were dialyzed in no. 20 Visking tubing against 0.05 *M* ammonium formate (pH 4); fraction 3, ultimately designated fragment H, was dialyzed in no. 23 Visking tubing against 0.05 *M* NH_4HCO_3; fractions 4 and 5 were lyophilized directly. Similar digestions were carried out using a variety of modifications: the streptokinase used for activation of plasminogen was replaced by 884 U of urokinase; the time of digestion was extended to 2 hr and to 4 hr, and in some cases the reaction was not stopped at all, but the solution was applied directly to the Sephadex column after a 2-hr incubation.

The isolated fractions were characterized by their dodecyl sulfate polyacrylamide gel electrophoresis patterns in 5% gels, and fraction 3 (fragment H), both reduced and nonreduced, was also examined in 10% gels. This fraction was further characterized by amino acid analysis, by electrofocusing, and by NH_2-terminal sequence determination.

The following control solutions were incubated at 0° and filtered over Sephadex G-100 under conditions similar to those used for the digestion: (i) a solution containing only plasminogen and streptokinase; (ii) a solution of fibrinogen from which contaminating plasminogen and plasmin had been removed by treatment with lysine-Sepharose prepared according to the method of Cuatrecasas (1970); (iii) a solution of the same fibrinogen to which plasminogen had been added; (iv) a solution of the same fibrinogen to which streptokinase had been added.

Ion Exchange Chromatography of Fragment H on DEAE-Sephadex A-25. Material corresponding to fraction 3, Figure 1A, from several digests was combined and subjected to ion exchange chromatography on DEAE-Sephadex A-25. Experimental details are given in the legend to Figure 1B. Five fractions were collected as indicated, dialyzed in no. 23 Visking tubing against H_2O, and lyophilized. The fractions were characterized by polyacrylamide gel electrophoresis with and without dodecyl sulfate, by amino acid analysis, by electrofocusing, and in three cases by NH_2-terminal sequence determinations.

Isolation of Fragment H from the Reduced and Carboxymethylated Aα Chain of Fibrinogen. Reduced and carboxymethylated Aα chain was isolated from fibrinogen essentially as described by Henschen and Edman (1972). Its identity as the Aα chain was confirmed by determination of the NH_2-terminal sequence as Ala-Asp-Ser-Gly-Glu-Gly-, and polyacrylamide gel electrophoresis in dodecyl sulfate which showed the major component in the position of the Aα chain although there was slight contamination with a band in the region corresponding to the Bβ chain; 90 mg of this preparation in 14 ml of 0.1 *M* NH_4HCO_3 (pH 7.6) was digested with plasmin at room temperature with stirring. The peptide was insoluble in the buffer, but slowly dissolved during digestion. At the beginning of the reaction 1500 U of streptokinase and 17 U of plasminogen were used, and during digestion an additional 9.6 U of plasminogen was added in small portions. The total reaction time was 42 hr. The digest was filtered over Sephadex G-75. The experimental details for the filtration are given in the legend to Figure 2A. Fraction I was pooled as indicated, and lyophilized. The product was characterized by amino acid analysis, by dodecyl sulfate polyacrylamide gel electrophoresis in 10% gels, and by NH_2-terminal sequence determination.

Isolation of Fragment H from Fibrin Clots. (i) Noncross-linked fibrin: 225 mg of lysine-Sepharose treated fibrinogen in 55 ml of 0.1 *M* Tris-HCl (pH 7.5) containing 0.03 *M* EDTA was clotted with 400 U of thrombin for 5 hr at room temperature. The clot was separated by filtration, washed with buffer, and digested by stirring for 16 hr at room temperature in 30 ml of 0.1 *M* Tris-HCl (pH 7.5) with 3000 U of streptokinase and 26 U of plasminogen. The solution was then acidified to pH 4 by the addition of glacial acetic acid, and filtered over Sephadex G-100. Details of the gel filtration are given in the legend to Figure 2B. Fractions were pooled as indicated, dialyzed against 0.1 *M* NH_4HCO_3, and lyophilized. Before plasmin digestion a

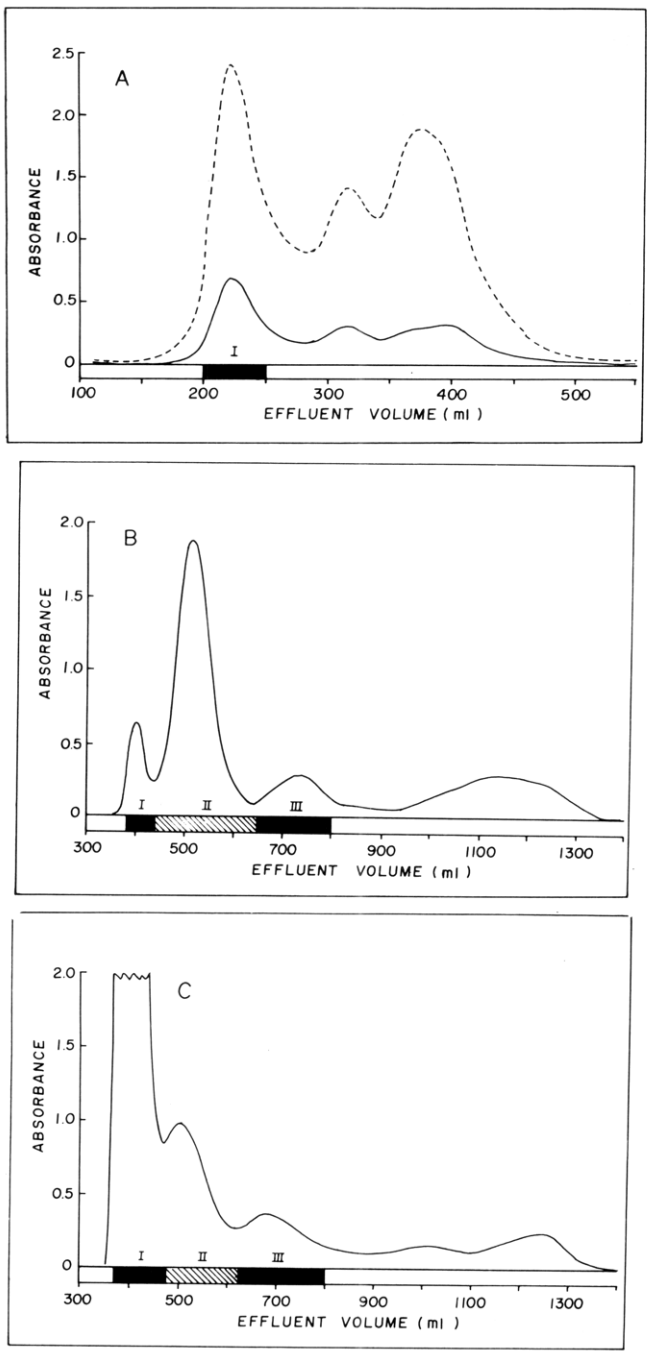

FIGURE 2: (A) Gel filtration of a plasmin digest of 90 mg of reduced and carboxymethylated Aα chain of fibrinogen on Sephadex G-75 (95 × 2.5 cm). Eluent, 0.1 *M* NH_4HCO_3; sample volume, 15 ml; flow rate 60 ml/hr; fraction size, 10 ml; temperature, 4°. Effluent was monitored by absorbance at 230 nm (---) and at 280 nm (—). (B) Gel filtration of a plasmin digest of a clot of non-cross-linked fibrin from 225 mg of fibrinogen on Sephadex G-100 (97.5 × 4 cm). Eluent, 0.1 *M* Tris-HCl (pH 7.5) adjusted to pH 4 with glacial acetic acid; sample volume, 32 ml; flow rate, 80 ml/hr; fraction size, 10 ml; temperature, 4°. Effluent was monitored by absorbance at 280 nm (—). (C) Gel filtration of a plasmin digest of a clot of cross-linked fibrin from 380 mg of fibrinogen on Sephadex G-100. Conditions were the same as for Figure 2B except that the sample volume was 37 ml.

small piece of the clot was cut off, incubated overnight in 10 *M* urea–2% dodecyl sulfate–1 m*M* dithiothreitol at 37°, and examined by electrophoresis in a 7.5% gel. Material from the isolated fractions was also examined by dodecyl sulfate electrophoresis, and fraction III was further characterized by amino acid analysis and NH_2-terminal sequence

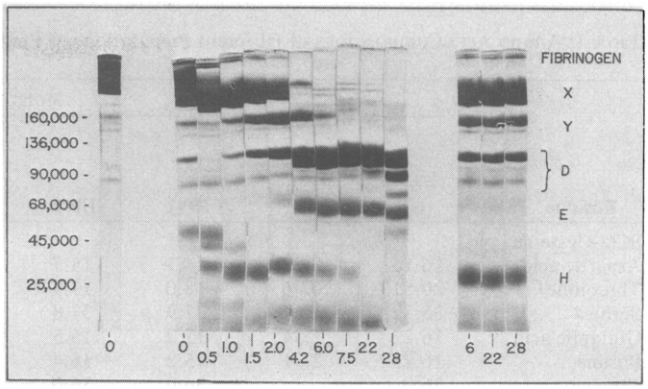

FIGURE 3: Dodecyl sulfate polyacrylamide gel electrophoresis in 5% gels of a plasmin digest of fibrinogen. Digestion conditions: 49 mg of fibrinogen, 3.3 U of Kabi plasminogen, and 500 U of streptokinase in 5.4 ml of 0.1 *M* Tris-HCl (pH 7.5) at 0°; the temperature was raised to 4° after 7.5 hr, and to 37° after 22.5 hr; gel on the left represents fibrinogen before the addition of plasminogen and streptokinase; gels in the center group represent aliquots of the reaction solution quenched with equal volumes of 10 *M* urea–2% dodecyl sulfate at the times (in hours) indicated at the bottom of the figure; gels in the group on the right represent aliquots taken from a portion of the reaction solution which was acidified to pH 4 with glacial acetic acid at 1.5 hr, and then incubated in parallel with the parent solution; figures on left indicate approximate molecular weights estimated from standard proteins run on gels in parallel with the fibrinogen samples; letter notations on the right indicate the fragments believed to be represented by the different bands, using the nomenclature of Nussenzweig et al. (1961) and of Marder et al. (1967).

determination. (ii) Cross-linked fibrin: 380 mg of lysine-Sepharose treated fibrinogen in 80 ml of 0.1 *M* Tris-HCl (pH 7.5) containing 0.025 *M* $CaCl_2$ and 0.0125 *M* L-cysteine was clotted for 3 hr at 37° with 1200 U of thrombin. The clot was treated in the same manner as the clot of non-cross-linked fibrin except that the plasmin digestion was carried out for 23 hr. Details of the gel filtration are given in the legend to Figure 2C. Fractions were pooled as indicated, dialyzed, lyophilized, and examined in the same manner as the fractions from the non-cross-linked fibrin. Prior to plasmin digestion a piece of the clot was cut off, and the presence of cross-links was established by incubation overnight in 10 *M* urea–2% dodecyl sulfate–1 m*M* dithiothreitol at 37° followed by electrophoresis in a 7.5% gel.

Results

The course of digestion of fibrinogen with plasmin under different conditions was studied by examining several series of polyacrylamide gel electrophoreses in dodecyl sulfate such as the one shown in the center of Figure 3. The conditions described in the legend to the figure were those selected for further investigation because of the early development of a band which seemed relatively resistant to further plasmin digestion. This band appeared in the region corresponding to a molecular weight of 20,000. The gels on the right of Figure 3 show that the reaction was stopped by acidification to pH 4. The 20,000 molecular weight fragment was designated fragment H.

The effluent pattern obtained when a limited plasmin digest of fibrinogen was filtered over Sephadex G-100 is shown in Figure 1A. On electrophoresis in dodecyl sulfate fractions 1 and 2 gave bands in the positions corresponding to the large molecular weight fragments, X and Y, and a small amount of D, whereas the band from fraction 3 corresponded to fragment H. Fractions 4 and 5 appeared to be smaller molecular weight fragments which did not resolve

Table I: Amino Acid Compositions of Different Preparations of Fragment H.[a]

Residue	Molar Ratios of Amino Acid Residues[b]									
	H Pool[c]									
	μ	σ	HP-1	HP-2	HP-3[d]	HP-4	HP-5	H from Aα	H from Non-Cross-Linked Fibrin.	H from Cross-Linked Fibrin.
SCM-Cysteine								0.6[e]		
Aspartic acid	16.16	0.93	14.1	15.7	17.03	15.9	15.7	15.8	16.4	16.7
Threonine[f]	20.51	0.96	18.0	19.9	21.67[d]	20.9	20.2	19.6	19.1	20.9
Serine[g]	35.72	1.64	37.9	37.8	35.55[d]	38.2	39.3	37.4	34.4	34.4
Glutamic acid	16.22	1.54	12.2	15.5	16.91	17.0	18.4	14.5	15.1	16.3
Proline	16.93	2.31	15.2	16.4	16.43	15.9	16.6	17.2	18.9	20.2
Glycine	36.0		36.0	36.0	36.0	36.0	36.0	36.0	36.0	36,0
Alanine	5.56	0.38	4.5	4.7	5.05	4.8	6.3	4.7	5.4	6.3
Half-cystine[h]	0.5[i]		0.2	0.4	1.0	0.4	0.4		0.8	1.1
Valine	5.01	0.80	2.8	4.0	5.79	5.0	4.7	3.7	4.8	5.2
Methionine	2.10	0.64	1.0	2.2	1.85	1.2	2.1	1.6	1.2	1.8
Isoleucine	1.79	0.22	0.9	1.8	2.25	2.0	2.0	1.8	1.6	1.8
Leucine	3.88	0.68	2.2	3.6	3.66	3.8	3.3	3.2	2.4	2.9
Tyrosine	1.76	0.25	1.7	1.6	1.84	1.4	1.7	1.5	2.1	2.2
Phenylalanine	3.01	0.78	1.4	2.2	2.77	3.4	6.8	1.8	2.3	3.7
Histidine	3.29	0.39	2.0	2.7	3.48	3.6	5.0	2.8	3.0	3.8
Lysine	5.34	0.80	3.1	4.1	5.01	4.2	3.7	4.0	4.0	5.5
Tryptophan	6.3[i]		6.1	5.1	6.0	4.9	5.5	*j*	*j*	*j*
Arginine	11.31	0.86	8.7	10.1	12.04	11.0	11.0	10.0	10.6	11.2

[a] Determined from 24-hr hydrolyses unless otherwise noted. [b] Based on Gly = 36.0. [c] Mean values, (μ), and standard deviations (σ), calculated from 12 preparations of fragment H. [d] Average or extrapolated values obtained from 24-, 48- and 72-hr hydrolyses. [e] Value extrapolated to zero time from 24- and 48-hr hydrolyses. [f] Assuming 5% destruction in 24-hr unless otherwise noted. [g] Assuming 10% destruction in 24 hr unless otherwise noted. [h] Determined on oxidized samples as cysteic acid (Moore, 1963). [i] Determined on pooled fragment H. [j] Trp not determined directly; spectrophotometric estimation of the Trp/Tyr ratio (Goodwin and Morton, 1946) indicated Trp content to be similar to that in samples of fragment H obtained from fibrinogen.

Table II: Amino-Terminal Sequence of Fragment H.[a]

Sequence of Four Different Preparations of Fragment H Isolated from Fibrinogen; H pool, HP-1, HP-3, and HP-4:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Met-Glu-Leu-Glu-Arg-Pro-Gly-Gly-Asn-Glu-Ile-Thr-Arg-Gly-Gly-

{ Gly-Gly-Ser-Thr-Ser-Tyr-xxx-Thr-Gly-Thr-Glu-xxx-Glu-xxx-Pro-
{ Ala-

Sequence of Fragment H Isolated from Reduced and Carboxymethylated Aα Chain, Non-Cross-Linked Fibrin, and Cross-Linked Fibrin:

1 2 3 4 5 6 7 8

Met-Glu-Leu-Glu-Arg-Pro-Gly-Gly-

Gly-Gly-xxx-Thr-Ser-Tyr-xxx-Thr-

[a] Underlined residues indicate major sequence; minor component is listed below major component at each step. xxx indicates no second component was detected.

or stain well on 5% gels, and they were not characterized further. The substitution of urokinase for streptokinase as plasminogen activator, or a moderate extension of the digestion time did not affect the yield of fragment H, which varied from 115 to 127 mg in preparations from 1.5 g of fibrinogen. The longer reaction times did, however, affect the shape of the peak containing fractions 1 and 2. As the reaction proceeded, this peak became somewhat more retarded and appeared from examination by electrophoresis to contain less X and Y, and a larger proportion of D and some E. In the control experiments no peak corresponding to fragment H appeared in the effluent of the Sephadex columns when fibrinogen was incubated in the absence of plasmin, or when streptokinase and plasminogen were incubated together in the absence of fibrinogen. The only uv absorbing material eluted was in a sharp peak at the void volume.

The averages of the amino acid compositions of different samples of the fragment H isolated from 12 digestions, run under the variety of conditions described, are listed in the first two columns of Table I. Since the composition was unique and remarkably reproducible, material from these experiments was combined into a pool. The NH_2-terminal sequence of material from the pool is given in Table II, and the patterns from electrophoresis in dodecyl sulfate, in both the absence and presence of dithiothreitol, are presented in Figure 4. A typical effluent pattern obtained from ion exchange chromatography of the H-containing pool is shown in Figure 1B. The amino acid compositions of the fractions are listed in Table I, where they can easily be compared with those of other preparations of fragment H. Figure 4 shows the bands obtained from electrophoresis in dodecyl sulfate of fraction HP-3. The other fractions gave bands in the same position. A comparison of the patterns obtained on electrophoresis without dodecyl sulfate of all the fractions can be seen in Figure 5. In addition, the NH_2-terminal sequences of HP-1, HP-3, and HP-4 are given in Table II. The sequences of the pooled H and of the three fractions isolated from ion exchange chromatography were identical except in the relative amounts of the minor components which generally appeared to be in the range of 10–20% of those of the major components. In the case of HP-1 the two components at each step were present in comparable amounts and could be assigned to separate chains only on the basis of the assignments for the other fractions.

In the electrofocusing experiments fragment H gradually washed out during the destaining procedure, so that it was necessary initially to overload the gel slab, resulting in bands which were not as sharp as those usually seen with this method. However, careful observation revealed multiple bands from the pooled material, with the main ones cor-

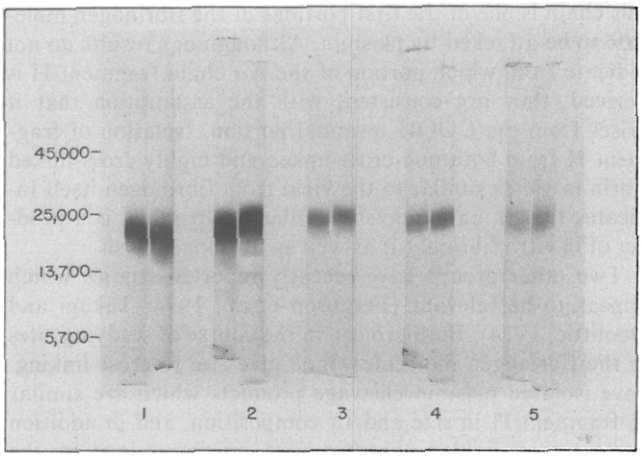

FIGURE 4: Dodecyl sulfate–polyacrylamide gel electrophoresis in 10% gels of fragment H from different sources. The left-hand member of each pair represents the nonreduced sample; that on the right represents the reduced sample; numbers on the left indicate approximate molecular weights estimated from standard proteins run in parallel with the samples of fragment H. The samples are identified as follows by the numbers at the bottom of the figure; 1, H Pool; 2, HP-3; 3, H from reduced and carboxymethylated Aα chain; 4, H from non-cross-linked fibrin; 5, H from cross-linked fibrin.

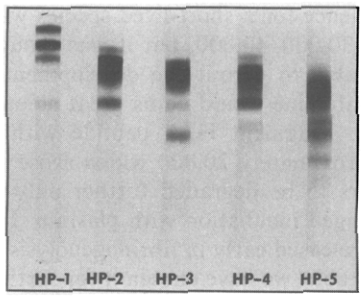

FIGURE 5: Polyacrylamide gel electrophoresis in 7.5% gels of samples of fragment H isolated from ion exchange chromatography on DEAE-Sephadex A-25. The samples are identified by the legend at the bottom of the figure.

responding to isoelectric points between 5.2 and 7.0. Electrofocusing of the five fractions obtained from ion exchange chromatography were consistent with the results from polyacrylamide gel electrophoresis given in Figure 5, showing a partial resolution of the components with the most basic in HP-1 and the most acidic in HP-5.

The effluent pattern obtained from gel filtration over Sephadex G-75 of a plasmin digest of the reduced and carboxymethylated Aα chain of fibrinogen is shown in Figure 2A. Polyacrylamide gel electrophoresis in dodecyl sulfate of the digestion solution before gel filtration gave a pattern which was entirely consistent with this effluent curve and was entirely consistent with this effluent curve and which which was composed of at least four bands, the largest of which corresponded to fragment H. Amino acid analysis (Table I), dodecyl sulfate electrophoresis (Figure 4), and NH_2-terminal sequence determination (Table II) of fraction I gave results almost identical with those for fragment H from fibrinogen. Comparison with protein markers of known molecular weights showed that fraction I eluted from Sephadex G-75 in a region consistent with a molecular weight of 20,000.

Electrophoresis of a reduced sample of the non-cross-linked fibrin clot in dodecyl sulfate showed bands in the positions expected for the α, β, and γ chains of fibrin with no evidence of α polymer or γ dimer. Figure 2B shows the effluent pattern obtained from gel filtration over Sephadex G-100 of a plasmin digest of the clot. Electrophoresis of fractions I and II showed bands corresponding to D and E and a small amount of a larger molecular weight product. Amino acid analysis (Table I), dodecyl sulfate electrophoresis (Figure 4), and NH_2-terminal sequence determination (Table II) of fraction III gave results almost identical with those for fragment H from fibrinogen.

Electrophoresis of a reduced sample of the cross-linked fibrin clot in dodecyl sulfate showed bands in the positions expected for α polymer, β monomer, and γ dimer chains. No bands corresponding to α or γ monomers were visible, indicating that cross-linking had occurred to a significant extent. Figure 2C shows the effluent pattern obtained from gel filtration over Sephadex G-100 of a plasmin digest of the clot. Electrophoresis of fractions I and II showed bands corresponding to a large molecular weight product, probably D dimer, and to E. Amino acid analysis (Table I), dodecyl sulfate electrophoresis (Figure 4), and NH_2-terminal sequence determination (Table II) of fraction III gave results almost identical with those for fragment H from fibrinogen.

Determinations of the yields of material in peaks corresponding to fragment H from different digestions of fibrinogen, calculated from the total absorbance in the effluent peaks and using the experimentally determined value, $E_{280nm}(1\%)$ 18.6, gave a mean of 80 mg of fragment H/g of fibrinogen, corresponding to 68% of the theoretical yield. The yields of fragment H from the reduced and carboxymethylated Aα chain, the non-cross-linked fibrin, and the cross-linked fibrin were comparable, all approximately 60% of the theoretical value.

Discussion

The chemistry of human fibrinogen has been extensively studied (Blombäck, 1972). The molecule has been shown to have a molecular weight of 3.4×10^5 and to consist of three pairs of chains (two each of Aα, Bβ, and γ) connected by disulfide bridges. The molecular weights of the chains are approximately 7×10^4, 6×10^4, and 5×10^4, respectively. The general scheme for fibrinogen digestion by plasmin proposed by Marder et al. (1967, 1969) has been supported by the results of other investigators (Furlan and Beck, 1972; Gaffney, 1972; Mills, 1972; Pizzo et al., 1972), and, although agreement on certain aspects has not been reached (Mosesson et al., 1973; Plow and Edgington, 1974), it seems apparent that fibrinogen is degraded to an intermediate, which has been termed fragment X (molecular weight 240,000), and then further through another intermediate, Y (molecular weight 160,000), to the final large molecular weight products, D (molecular weight 80,000–100,000) and E (molecular weight 60,000). All these products, reflecting the lack of clear-cut specificity of plasmin, represent heterogeneous populations of proteins rather than discrete compounds, and the molecular weights given indicate approximate ranges. At each stage smaller, and as yet largely uncharacterized, peptides are split off. The course of plasmin digestion illustrated in Figure 3 is consistent with this scheme and it was on the basis of the molecular weight ranges stated above that the bands seen on dodecyl sulfate polyacrylamide gel electrophoresis were identified.

The polypeptide, fragment H, which we have isolated and characterized is one of the smaller peptides which arises early in the digestion of fibrinogen by plasmin. During the electrophoresis studies of the earliest events in the digestion

there was evidence for a short-lived species with a molecular weight of 30,000–40,000, but it was thought not to be sufficiently stable to permit the development of an assay whose practical value would be as great as one for a more stable species. Fragment H, a peptide with a molecular weight of approximately 20,000 which arose slightly more slowly, appears to be degraded further only under conditions of prolonged incubation with plasmin. In view of the fact that it is released early in fibrinogenolysis and seems to be relatively stable, we have chosen it for further characterization with the subsequent aim of developing a radioimmunoassay to measure its level in blood samples.

The most striking and unique feature of fragment H is its amino acid composition with its high content of serine, glycine, and proline and its extremely low content of hydrophobic residues. One would expect such a hydrophilic peptide to be situated on the surface of the fibrinogen molecule and thus be readily accessible to attack by plasmin. This is consistent with its appearance early in the digestion. Despite the constancy of the composition of preparations of fragment H from all sources, most of our studies indicate that it is not a single species, but that it consists of a family of closely related polypeptides, probably arising as a result of the lack of complete specificity of plasmin. Such heterogeneity is common in products of plasmin digestion. An additional cause may be heterogeneity already present in the COOH-terminal region of the Aα chains of Kabi fibrinogen. Results of sequence determinations show some heterogeneity in the NH_2-terminal region of fragment H, indicating that it probably contains two overlapping chains of similar molecular weights produced by cleavages at sites fairly close together on the Aα chain. The molecular weight of fragment H appears to be approximately 20,000 as judged from its behavior on dodecyl sulfate electrophoresis in gels of different degrees of cross-linking and from the position of its elution from Sephadex columns, as well as from the yield of Pth derivative obtained in the first step of the Edman degradation. Although the electrophoreses in dodecyl sulfate were performed primarily to monitor reactions and identify products rather than to determine molecular weights, it was evident from calculations based on the patterns shown in Figure 4 that all the samples of fragment H, whether reduced or nonreduced, have molecular weights in the same range. Minor size heterogeneity is probably indicated by the width of the bands. The low cystine values indicate that most of the molecules must lack this residue while a small proportion contains a disulfide bond. The failure to change the molecular weight by reduction with dithiothreitol suggests that the disulfide bridge, where present, is intrachain rather than interchain. Although the preparations of fragment H from ion exchange chromatography have remarkably similar amino acid compositions (Table I) and NH_2-terminal sequences (Table II) they have considerable charge heterogeneity as indicated by the broad peak in Figure 1B and by the multiple bands seen on electrofocusing and polyacrylamide gel electrophoresis even after chromatography (Figure 5). These differences in charge may be due to variations in amide content as well as the nonspecificity of plasmin cleavage.

Isolation of fragment H from the reduced and carboxymethylated Aα chain of fibrinogen in a yield similar to that from fibrinogen shows that it is derived from the Aα chain, which is consistent with the proposals of previous investigators (Mills and Karpatkin, 1970, 1972; Mosesson et al., 1972; Pizzo et al., 1972) that the COOH-terminal region of this chain is one of the first portions of the fibrinogen molecule to be attacked by plasmin. Although our results do not indicate from which portion of the Aα chain fragment H is derived, they are consistent with the assumption that it arises from the COOH-terminal portion. Isolation of fragment H from both non-cross-linked and highly cross-linked fibrin in yields similar to the yield from fibrinogen itself indicates that it, or some very similar polypeptide, is a product of in vitro fibrinolysis as well as fibrinogenolysis.

Two other groups have recently reported studies which appear to be relevant (Ferguson et al., 1974; Takagi and Doolittle, 1974). Both groups, in the course of studying sites in the fibrinogen molecule which give rise to cross-linking, have isolated plasmin cleavage products which are similar to fragment H in size and/or composition, and in addition possess cross-linking acceptor sites, as determined by the ability of the corresponding region of fibrinogen to incorporate monodansyl cadaverine in the presence of activated factor XIII. The fact that we have been able to obtain fragment H with the same molecular weight from highly cross-linked fibrin as from fibrinogen and non-cross-linked fibrin could be explained in several ways. (i) The region corresponding to fragment H may have been cross-linked in the original clot and the cross-linking site split off during the extensive plasmin digestion which produced fragment H. (ii) Monodansyl cadaverine may be incorporated into sites which are not involved in the actual cross-linking of fibrin. (iii) Although no α-monomer chains were visible on electrophoresis of the reduced cross-linked fibrin clot used in the preparation of fragment H, there may have been incomplete cross-linking under the clotting conditions used. Regardless of the uncertainty about the presence of a cross-linking site in fragment H it is apparent that a radioimmunoassay for this polypeptide might be used to estimate either fibrinolysis or fibrinogenolysis in vivo.

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Isolation and Characterization of Coupling Factor F_B from Bovine Heart Mitochondria†

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ABSTRACT: An unusually efficient B-type coupling factor with essential sulfhydryl groups has been isolated from bovine heart mitochondria and characterized. This protein, denoted by F_B, consists mainly of molecules with approximate molecular weight of 330,000. F_B catalyzes ATP-driven reduction of NAD^+ by succinate in the presence of A-particles (submitochondrial particles prepared at pH 9.2 in the presence of ammonia and EDTA) with a specific activity of over 1000 μmol of NADH per min per mg of F_B per 0.5 mg of A-particles at 38° and pH 7.8. After incubation with sodium dodecyl sulfate and 2-mercaptoethanol, F_B showed a single subunit protein band of approximate molecular weight 44,000 in disc gel electrophoresis. These data suggest that the principal molecular species of F_B is made up of eight subunits of equal molecular weight. The kinetic data and amino acid composition of F_B are presented.

At least two mitochondrial coupling factors with essential sulfhydryl groups have been reported in the literature. Factor B of molecular weight 29,200 was first isolated by Lam, Sanadi, and their coworkers (1967, 1969). It catalyzes ATP-driven reduction of NAD^+ by succinate in the presence of A-particles with a specific activity of 2–6 μmol of NADH per min per mg of coupling factor per 0.5 mg of A-particles at pH 7.8 and 38°. Racker and coworkers (1970) concluded from their enzymatic and immunological measurements that factor B is probably identical with a coupling factor F_2 prepared from bovine heart mitochondria (BHM)[1] by a different procedure (Fessenden et al., 1967). Lam and coworkers (1970) also found another sulfhydryl coupling factor from BHM, factor B′ of approximate molecular weight 45,000, which had a specific activity 20-fold lower than that of factor B and hence was regarded as less pure. The study of these coupling factors is of basic biochemical importance, since they seem to serve as the functional link (Sanadi et al., 1968) between the electron

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[1] Abbreviations used are: BHM, bovine heart mitochondria; HBHM, heavy bovine heart mitochondria; PMB, *p*-mercuribenzoate.