Isolation and Characterization of the Clottable Low Molecular Weight Fibrinogen Derived by Limited Plasmin Hydrolysis of Human Fraction I-4*

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ABSTRACT: As part of an investigation into the origin of a naturally occurring high ethanol solubility, low molecular weight, human fibrinogen (fraction I-8), low solubility, high molecular weight, fibrinogen (fraction I-4) was subjected to limited plasmin degradation. Early in this hydrolysis, a high ethanol solubility derivative was formed, which was then purified to $\geq 95\%$ clottability (I-8D_5). Fractions I-4, I-8, and I-8D_5 were compared. Molecular weights were 347,000 for I-4, 273,000 for I-8, and 262,000 for I-8D_5. Fractions I-8 and I-8D_5 had the same sialic acid and hexose contents (w/w) and both values were significantly greater than those of I-4. The N-terminal amino acids were qualitatively and quantitatively the same (mole/mole) for all three fractions. All three preparations exhibited heterogeneity when subjected to DEAE gradient chromatography.

Tyrosine:tryptophan ratios did not differ significantly between the three fractions. Fractions I-8 and I-8D_5 displayed retarded polymerization of fibrin monomer which resulted in both having longer thrombin times than I-4. When compared with previously described products of *in vitro* fibrinogenolysis, I-8D_5 was considered to be representative of the earliest detectable stage of fibrinogenolysis. The delayed clotting of I-8D_5 was considered to be due to slowed polymerization rather than inhibition relating to the thrombin susceptible site. Further conclusions from the data were that the early plasmin-induced cleavages were from the C-terminal end of the molecule, releasing peptides, and little, if any, carbohydrate, without impairing ultimate clottability. The close resemblance between I-8D_5 and I-8 in these studies supported the concept that I-8 is an *in vivo* proteolytic product derived from a higher molecular weight fibrinogen, such as fraction I-4.

Recently (Mosesson and Sherry, 1966), a method was described for the isolation of a low molecular weight human fibrinogen (designated fraction I-8) by differential ethanol subfractionation of the plasma glycine precipitate (Kazal *et al.*, 1963). Fraction I-8 was 8% ethanol soluble, $\geq 95\%$ clottable, and had certain characteristics (Mosesson *et al.*, 1967) by which it could be distinguished from classically prepared fibrinogen (fraction I-4 of Blombäck and Blombäck, 1956). In addition to lower molecular weight (269,000 *vs.* 325,000) and higher ethanol solubility, I-8 displayed retarded polymerization of fibrin monomer which resulted in prolongation of the thrombin time. Minor differences also existed in DEAE-cellulose gradient chromatographic elution profiles. Carbohydrate contents were the same per mole but higher for I-8 when calculated per gram of protein. The two fractions could be readily identified on acrylamide gel electrophoresis because of the greater mobility of I-8. This separation was related to the molecular sieve properties of this media since electrophoresis in other media (*e.g.*, 1% agarose) did not demonstrate any appreciable charge differences. In contrast, I-4 and I-8 were indistinguishable in their susceptibility to thrombin cleavage of fibrinopeptides and in their qualitative and quantitative N-terminal amino acid determinations.

The data presented was consistent with the hypothesis that I-8 might represent a naturally occurring fibrinogen which was derived from higher molecular weight parent species; that the formation of this species might be mediated by the enzyme plasmin seemed logical on the basis of preliminary studies (Mosesson and Sherry, 1966) which demonstrated the appearance of clottable 8% ethanol-soluble material during plasmin degradation of fraction I-4. Added evidence supporting these conclusions was available from studies of *in vitro* plasmin–fibrinogen degradation which demonstrated, prior to the loss of clottability, that both a highly clottable low molecular weight (265,000) derivative appeared (Fletcher *et al.*, 1966) and a prolongation of the thrombin time occurred (Niewiarowski and Kowalski, 1958).

To test this hypothesis for the origin of fraction I-8, the clottable 8% ethanol-soluble material resulting from limited plasmin proteolysis of fraction I-4 was isolated and purified. Highly clottable preparations were obtained and compared both with the starting fraction I-4 and the naturally occurring "soluble" fibrinogen, fraction I-8. The results are presented in this paper.

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Materials and Methods

Human fibrinogen fractions I-4 and I-8 were prepared from outdated ACD human plasma by the procedure of Mosesson and Sherry (1966). In some instances, I-4 was prepared from commercially available human fibrinogen.[1] The commercial material was considered to be equivalent to fraction I-0 and further purified by the method of Blombäck and Blombäck (1956). All preparations of I-4 were $\geq 98\%$ clottable, those of I-8 were $\geq 95\%$ clottable.

Initial analytical studies (Figure 1) to determine the relationship between fibrinogen clottability and ethanol solubility during plasmin degradation were carried out in a pH-Stat (0.3 M NaCl, pH 8.5) at 37°. Aliquots were removed at timed intervals for fractionation and related determinations. SBTI[2] was used in the clottability assay during the preliminary studies. For studies relating to the production and isolation of a clottable 8% ethanol-soluble derivative of plasmin degradation of fraction I-4, the following protocol was followed. To a 1.8% solution of fraction I-4 in 0.225 M NaCl–0.025 M Tris-Cl (pH 8.6), plasmin[3] was added to a final concentration of 0.1 CTA[4] unit/ml and the mixture was incubated at 37°. When the digest clottability had fallen to between 67 and 92%, the reaction was effectively stopped by the rapid addition of two parts chilled (0°) 0.1 M sodium phosphate (pH 6.2) buffer and one part chilled 0.3 M NaCl (final pH 6.6 $\Gamma/2 = 0.20$); the temperature of the mixture was maintained at 0–2° in a cold bath. Permanent inactivation of plasmin was achieved within 60 min in the presence of DFP (2×10^{-3} M). The fractionation procedure (Mosesson and Sherry, 1966) for the isolation of fraction I-8 from plasma was then employed. First, ethanol fractionation was performed and the fraction precipitating between 8 and 16% ethanol (v/v) was retained (fraction I-5D). Fraction I-5D was refractionated with ethanol and the resulting 8–16% fraction (I-7D) was redissolved in 0.1 M sodium phosphate (pH 6.4). At this point, preparations were retreated with DFP (2×10^{-3} M) so as to prevent any subsequent loss of clottability, as tested by incubation at pH 8.6 and 37° for 4–5 hr or longer. Fraction I-7D was then reprecipitated by 2.1 M glycine at 2° at a protein concentration of 0.5–1%, and the precipitate obtained was designated I-8D_0. The starting preparation of I-4 from which each "derivative" was obtained is referred to as the "parent" I-4. The clottability of the digest mixture at the time the reaction was stopped is termed "digest clottability." The concentration of dialyzed preparations, in 0.3 M NaCl, was measured in a Brice-Phoenix differential refractometer, assuming a specific refractive increment of 0.188 ml/g at 546 mμ (Armstrong *et al.*, 1947) and the corresponding absorbance coefficient ($A^{1\%}_{1\,cm}$ 280 mμ) was determined in a Beckman DU spectrophotometer.

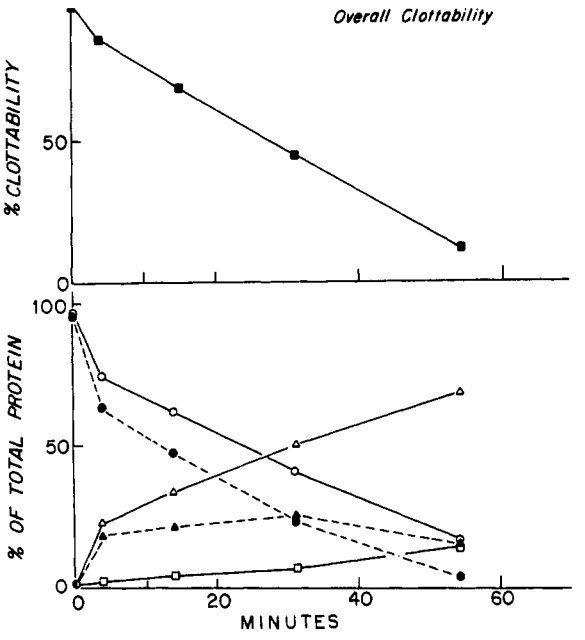

FIGURE 1: Relationship between loss of clottability and ethanol solubility during plasmin breakdown of fraction I-4. On the upper graph is shown over-all clottability (■—■) plotted as per cent clottability. On the lower graph are the total amounts and clottable portions of the respective fractions plotted as per cent of total protein; 0–8% fraction (○—○), 8–16% fraction (△—△), >16% fraction (□—□), clottable 0–8% fraction (●—●), and clottable 8–16% fraction (▲—▲).

Physicochemical Studies. Sedimentation velocity experiments were performed in a Spinco Model E analytic ultracentrifuge using schlieren optics. The partial specific volume was assumed to be 0.725 cc/g (Armstrong *et al.*, 1947).

Diffusion constants were determined by the method of Allison and Humphrey (1960). A constant antigen/antibody ratio (to avoid excess of either antigen or antibody) was used for all of the preparations. Measurements were made using high magnification on a Nikon Shadowgraph magnifier.

Agarose gel electrophoresis was carried out on microscope slides 75 × 25 mm (proteins were stained with ponceau S). Microimmunoelectrophoresis was done by the method of Scheiddeger (1955). Antifibrinogen sera was prepared in rabbits and then absorbed with human serum. Acrylamide gel electrophoresis was performed by a modification of the method of Davis (1964), as previously outlined (Mosesson *et al.*, 1967).

DEAE-cellulose gradient chromatography of fibrinogen fractions was accomplished at 3–5° employing a 0.9 × 22 cm column with a Tris-phosphate, pH gradient from 0.005 M phosphate (pH 8.6) to 0.5 M phosphate (pH 4.1–4.2) (Finlayson and Mosesson, 1963; Mosesson *et al.*, 1967).

Analytical gel filtration was performed using a 1.5 × 30 cm column for cross-linked dextran (Sephadex

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[2] Abbreviations used: CTA, Committee on Thrombolytic Agents; SBTI, soy bean trypsin inhibitor; DNS, 1-dimethylnaphthalene-5-sulfonyl.

[3] Plasminogen (Kline, 1953) was activated by the method of Troll and Sherry (1955).

[4] One CTA unit is approximately equivalent to 0.66 Remmert and Cohen unit.

G-200[5]) and acrylamide (P-300[6]) and a 1.5 × 50 cm column for agarose (Sagarose 6[7] and Sepharose 4B[5]). For preparative procedures, a 2.5 × 60 cm column was used. Dextran Blue[5] and/or lyophilized *E. coli*[7] were used to determine void volume (V, void).

Carbohydrate analyses were performed by a thiobarbiturate method for sialic acid (Warren, 1959), a modified Elson–Morgan technique for hexosamine (Winzler, 1955) and an orcinol method for total protein-bound hexose (Winzler, 1955).

Tyrosine/tryptophan ratios were determined from the ultraviolet spectra by the method of Bencze and Schmid (1957).

N-Terminal amino acid analysis was performed by the DNS method (Gray and Hartley, 1963); quantitative estimation of N terminals was made by comparison with standard amino acids carried through the procedure. Optimal labeling of protein N terminals (7–9 × 10^{-3} μmol of protein in 1.2–1.4 ml of 0.5 M $NaHCO_3$ and 5 M urea solution) was achieved by overnight incubation in the presence of a 5–10-fold molar excess of DNS-Cl (based upon theoretically available amino and phenolic groups). Optimal labeling of standard amino acid solutions of alanine, glycine, aspartic acid, and tyrosine (in 0.5 M $NaHCO_3$) occurred in this range of DNS excess as well. Significant amounts of the degradation products described by Neadle and Pollitt (1965) did not appear in the amino acid experiments below a molar ratio of 10.

Under the conditions of labeling some protein, particularly fibrin, precipitated slowly from solution; trichloroacetic acid (20% w/v) added to a final concentration of 5–7% completed precipitation. The precipitated protein was then washed consecutively with water and acetone and dried under vacuum. Hydrolysis of proteins and labeled amino acid standards with constant-boiling HCl was carried out for 6 hr at 105° in sealed evacuated containers. After hydrolysis, HCl was removed under vacuum and the residual material was taken up in measured amounts of methanol–ammonium hydroxide (95:5, v/v).

Two-dimensional chromatography using benzene–pyridine–acetic acid (40:10:1, v/v) for the first dimension (Morse and Horecker, 1966) and chloroform–benzyl alcohol–acetic acid (70:30:3, v/v) for the second (Deyl and Rosmus, 1965) was routinely performed on 5–25-μl amounts spotted on 20 × 20 cm silica gel G plates.[8] Additional brief runs in chloroform–*t*-amyl alcohol–formic acid (70:30:1, v/v) (Morse and Horecker, 1966) superimposed upon the first dimension served to separate DNS-aspartic acid more clearly from fluorescent material (*e.g.*, DNS-OH and *o*-DNS-tyrosine) near the origin.[9]

Separated DNS-amino acids were eluted from silica gel with methanol–ammonium hydroxide (95:5) as suggested by Seiler and Wiechmann (1966).[10] In one experiment, known DNS-glycine, -tyrosine, -alanine, and -aspartic acid, hydrolyzed and chromatographed as described above, gave the following over-all yields of eluted fluorescence, respectively, 61, 60, 75, and 74. Furthermore, the standard deviations for this determination from six samples of mixtures of these four amino acids were ±9.0–11.2%. To obtain this yield and degree of reproducibility, elution of fluorescent material was accomplished as rapidly as possible after removal of the plates from the chromatographic solvent, usually within 40 min. Fluorescence was measured in an Aminco filter fluorimeter (primary filter, Corning 760 peaking at 360 mμ; secondary filters, Wratter 2A and 58 with 415-mμ cutoff and peak at 525 mμ, respectively).

Biologic Activity. Thrombin times were measured at 37°, 0.1 ml of thrombin (10 NIH units/ml) was added to a mixture of 0.1 ml of fibrinogen (4 mg/ml) in 0.27 M NaCl–0.02 M Tris-Cl (pH 7.3) plus either 0.1 ml of H_2O or 0.1 ml of 0.025 M $CaCl_2$ and the time of clot appearance was recorded. Bovine thrombin (Parke-Davis) further purified by the method of Rasmussen (1955) was used.

Studies of the polymerization of fibrin monomer were performed by a modification (Latallo *et al.*, 1962a) of the method of Donnelly *et al.* (1955). Clottabilities were done by a modification (Mosesson and Sherry, 1966) of the method of Laki (1951).

Results

Isolation and Purification (to High Clottability) of a High Solubility Derivative of Fraction I-4 Produced by Plasmin Digestion

As over-all clottability fell after the addition of plasmin, partially clottable derivatives of greater than 8% ethanol solubility appeared (Figure 1); *e.g.*, when over-all clottability was reduced to 85%, 24% of the fibrinogen became "soluble" (most of which was clottable). After isolation of I-8D_0, additional purifica-

[5] Pharmacia Fine Chemicals, Piscataway, N. J.

[6] Calbiochem., Los Angeles, Calif.

[7] Gallard Schlesinger Mfg., Carle Place, Long Island, N. Y.

[8] Brinkman Instruments, Westbury, Long Island, N. Y.

[9] DNS-arginine and -histidine remain close to or at the origin in these systems and are therefore difficult to distinguish from *o*-tyrosine and ε-lysine which, of course, are present in relatively high amounts in protein hydrolysates.

[10] These authors have pointed out that methanol–ammonium hydroxide (95:5, v/v) was the only solvent system they found which eluted DNS-amino acids successfully from silica gel. They also observed, as have we, that there was a progressive loss of elutable fluorescence after the plates had been removed from the chromatographic solvent. For example, in one experiment with known DNS-glycine and -alanine chromatographed in benzene–pyridine–acetic acid (40:10:1, v/v), we obtained the following per cent recovery of fluorescence eluted in methanol–ammonium hydroxide:

	Time after Removal from Solvent			
	0–10 min	40 min	3 hr	23 hr
Glycine (%)	93	87	58	1.2
Alanine (%)	94	84	58	1.3

Since the fluorescence (as measured by thin-layer chromatography scanning) may be regenerated by spraying the plates with triethanolamine–isopropyl alcohol (20:80) (Seiler and Wiechmann, 1966), we suspect that the time-dependent loss of elutable material is related to binding of DNS-amino acid to silica gel as the solvent evaporates.

TABLE I: Fractionation Yields.

Fraction	Mean Recovery from Preceding Step (Range) in %	Mean Clottability (Range) in %	Over-all Yield Mean (Range)[a]
Total digest		88.0 (84.0–92.1)	
I-5D	22 (18–26)	87.9 (84.1–91.9)	100
I-7D	88 (80–97)	87.7 (83.2–90.8)	88 (80–97)
I-8D_0	57 (45–69)	89.5 (87.0–92.0)	51 (42–61)
I-8D_1	88 (80–91)	90.7 (89.0–92.4)	45 (36–55)
I-8D_2	69 (64–75)	91.5 (89.8–92.9)	31 (27–35)
I-8D_3	91 (87–94)	92.3 (90.5–93.8)	29 (19–38)
I-8D_4	79 (75–85)	94.6 (93.5–96.6)	23 (18–27)
I-8D_5	90 (86–94)	95.8 (95.1–97.4)	21 (16–24)

[a] Values obtained from preparation of five lots of I-8D_5 by means of the procedure outlined in Figure 2. Based upon I-5D = 100%.

tion steps were devised to obtain highly clottable ($\geq$95%) samples (Figure 2), using only those digests whose over-all clottabilities were $\geq$84%. Material from digests of lower clottability could not be purified to as high a level of clottability because of heavy contamination with incoagulable fragments with similar physical properties to the clottable protein. Analytical procedures (with the exception of DEAE chromatography) were performed only on fractions prepared from digests which were $\geq$84% clottable.

Preparation of fractions of increased clottability was achieved as follows (Figure 2, and Table I), with the average clottabilities and yields.

Step 1. Precipitation of I-8D_0 at 24% saturation with $(NH_4)_2SO_4$. The precipitate was termed I-8D_1. Clottability was increased 1.2% with an 88% yield.

Step 2. On gel filtration column chromatography I-8D_1 emerged with the void volume on all gel media studied (G-200, P-300, and Sagarose 6) with the exception of Sepharose 4B which was subsequently employed for this purification step. On the latter media, a single symmetrical peak resulted, which emerged later than the void volume. The first three-fourths of the protein to emerge had its clottability raised by 0.8% and was retained (I-8D_2).

Step 3. Fraction I-8D_2 (90% of it) at a concentration of 0.2% was precipitated in the presence of 13% ethanol and 0.1 M ϵ-aminocaproic acid (pH 7.0, $\Gamma/2$ = 0.154, $-2°$) and this raised clottability another 0.8%.[11] The precipitate was termed I-8D_3.

Step 4. At 2.0 M β-alanine concentration (modified from Straughn and Wagner, 1966), 90–95% of parent fraction I-4 (1% protein, pH 6.4, $\Gamma/2$ = 0.12) was precipitable. In contrast, under the same conditions, only 50–55% of I-8D_3 was precipitated. By raising the β-alanine concentration to 2.8 M, a higher yield (80%) was obtained and clottability increased by 2.3% (I-8D_4). Reprecipitation of I-8D_4 with 2.8 M β-alanine at pH 7.0 ($\Gamma/2$ = 0.24) increased clottability by 1.2% and gave a 90% yield. The final precipitate was termed I-8D_5.[12]

Five lots of I-8D_5 were prepared with clottabilities ranging from 95.1 to 97.4%. The final yield of I-8D_5 represented 35–50% of I-8D_0 and 21% of I-5D (Table I).

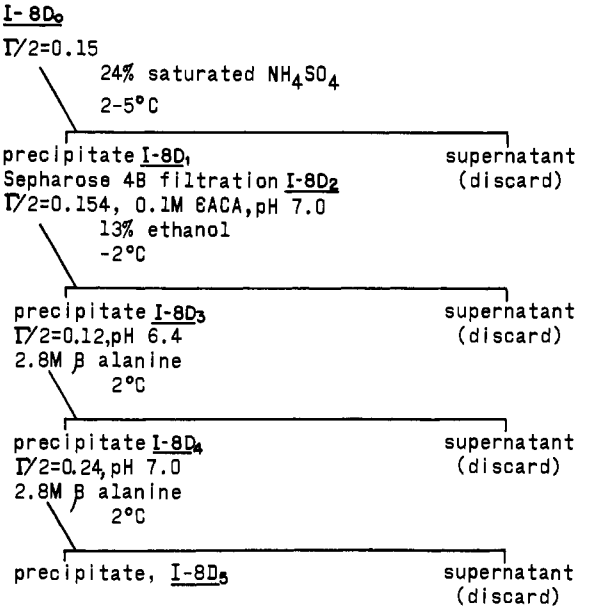

FIGURE 2: Outline of procedures employed to purify fraction I-8D_0.

[11] As indicated in Table I, in spite of the minimal increase in clottability resulting from steps 2 and 3, some preparations were distinctly improved. The chief reason for retaining these steps in the final procedure was that a gel filtration step was desirable to ensure removal of any low molecular weight contaminants and step 3 served to reconcentrate the dilute Sepharose 4B column eluate.

[12] Referred to as I-8D in abstract (Sherman *et al.*, 1967).

TABLE II: Comparative Characteristics of Fractions I-4, I-8, and I-8D_5.

Property	I-4	I-8	I-8D_5
$s^0_{20,w}$	7.89	7.52	7.44
$D_{20,w}$	2.00	2.42	2.50
Molecular weight	347,000	273,000	262,000
Sialic acid (g %)	0.61 ± 0.03	0.70 ± 0.04	0.74 ± 0.04
Hexoseamine (g %)	1.2 ± 0.2	1.3 ± 0.1	1.3 ± 0.1
Total hexose content (g %)	1.3 ± 0.2	1.8 ± 0.3	1.7 ± 0.2
Tyrosine:tryptophan ratio	1.32:1	1.37:1	1.34:1
Thrombin time (sec)			
With H_2O	13.0 (12.3–13.4)	25.0 (23.8–26.2)	19.0 (15.4–24.1)
With 0.025 M CaCl	9.1 (8.1–9.9)	15.3 (13.6–17.2)	13.7 (11.2–15.3)

II. Biophysical, Biochemical, and Biological Properties of I-8D_5; Comparison with I-4 and I-8.

Molecular Size (*Table II*). Sedimentation velocity studies yielded extrapolated $s^0_{20,w}$ values of 7.89 S for I-4, 7.44 S for I-8D_5, and 7.52 S for I-8. The slope of the concentration dependence of all three fractions was between −1.2 and −1.3. Only one ultracentrifugal peak was observed for each of the preparations. $D_{20,w}$ was 2.00 ± 0.11, 2.42 ± 0.08, and 2.50 ± 0.14 for I-4, I-8, and I-8D_5, respectively (for three preparations each of I-4 and I-8D_5 and two of I-8). These results compare with values of 2.18 and 2.54 for I-4 and I-8, respectively, when determined in the ultracentrifuge (Mosesson *et al.*, 1967).

Calculation of molecular weight by the Svedberg equation yielded values of 347,000 for I-4, 273,000 for I-8, and 262,000 for I-8D_5.

Three preparations each of I-4 and I-8D_5 and two of I-8 were studied by Sepharose 4B gel filtration. No significant difference was found between the $V_{elution}/V_{void}$ ratios for I-8 and I-8D_5, being 2.20 ± 0.08 and 2.21 ± 0.11. There was a marginal difference between these values and that of I-4 (2.03 ± 0.09) which was consistent with the results of acrylamide gel electrophoresis (*vide infra*).

Electrophoresis. Agarose gel electrophoresis (1%) and immunoelectrophoresis did not reveal any differences between the three fractions. Previous studies with acrylamide gel electrophoresis (Mosesson *et al.*, 1967) demonstrated that I-8 migrated faster than I-4 in this medium (Figure 3). In the present experiment, I-8D_5 did also and moved slightly faster than I-8. In contrast to the two, more or less distinct bands of the other preparations, I-8D_5 had at least three bands.

DEAE Chromatography (*Figure 4*). Fraction I-8D_5 preparations which had been purified from incubation mixtures with "digest clottabilities" of 88–92%, had elution profiles which were indistinguishable from that of naturally occurring I-8 and demonstrated the same slight but consistent differences from I-4 as previously noted for I-8 (Mosesson *et al.*, 1967), *i.e.*, the first peak contained a slightly higher proportion of the total protein and was eluted slightly earlier from the column than was the first peak of "parent" I-4 preparations run in the same system. When "digest clottability" was from 84 to 86%, chromatography of the resultant I-8D_5 demonstrated a second component in the first peak. In two experiments with 90% clottable material from digests of 67–70% clottability, the first peak appeared even earlier than that of the more clottable preparations, and for the first time, peak 2 was eluted earlier than that of fraction I-4.

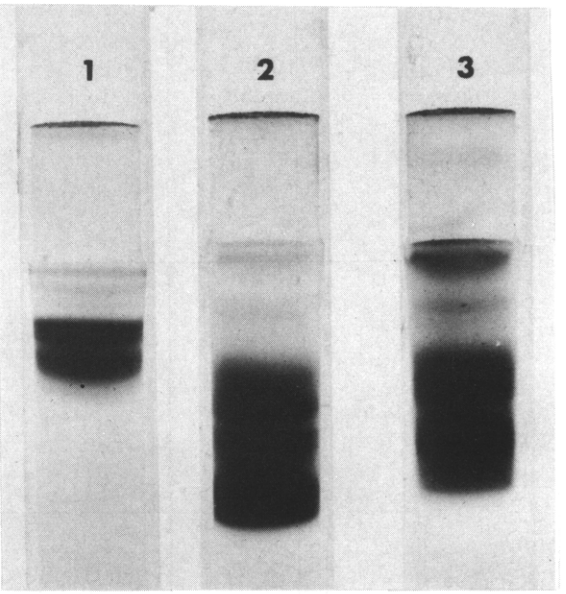

FIGURE 3: Acrylamide gel electrophoresis. Tubes 1, 2, and 3 are I-4, I-8D_5, and I-8, run on a 9% gel concentration.

Ultraviolet Spectra and Related Data. The ultraviolet spectra between 254 and 330 mμ were the same for I-4, I-8, and I-8D_5 in alkaline urea (5 M urea–0.1 N NaOH).

The tyrosine:typtophan ratios calculated from these data were I-4, 1.32:1; I-8, 1.37:1; and I-8D_5, 1.34:1 (Table II) for two preparations each. These values were not significantly different. The mean absorbancy coefficients ($A^{1\%}_{1cm}$) in neutral salt solution, at 280 mμ, for five preparations each of I-4 and I-8D_5, were 15.2 ± 0.3 and 15.4 ± 0.4, respectively. The difference was not significant. Previously published data (Mosesson *et al.*, 1966) showed virtually identical absorbancy

TABLE III: N-Terminal Amino Acids.[a]

Amino Acid	Residues per Mole			
	Fraction I-4		Fraction I-8D$_5$	
	Fibrinogen	Fibrin	Fibrinogen	Fibrin
Glycine		3.4 (3.0–3.6)		3.3 (3.0–3.6)
Tyrosine	1.9 (1.6–2.1)	1.5 (1.2–1.8)	1.8 (1.5–2.1)	1.6 (1.1–2.0)
Alanine	1.5 (1.2–1.7)		1.4 (1.3–1.6)	
Aspartic	0.3 (0.2–0.4)		0.2 (0.0–0.3)	

[a] Based on molecular weights of 347,000 for I-4 and 262,000 for I-8D$_5$.

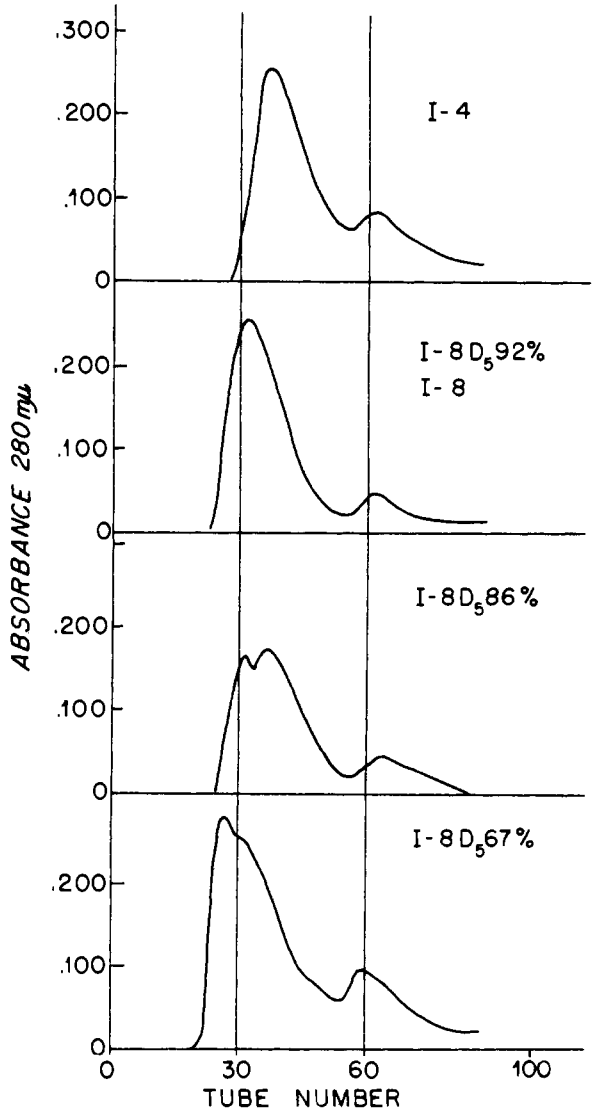

FIGURE 4: DEAE-cellulose gradient chromatography elution patterns. Percentage figures refer to the starting "digest clottability" of the I-8D$_5$ lots depicted; 92% represents three lots with "digest clottabilities" of 88–92%, 86% represents two lots from 84 to 86%, and 67% represents two lots from 67 to 70%. Fraction I-4 is depicted in the top pattern and I-8 was identical with the 92% pattern. Total gradient volume was 350 ml, collected in aliquots of 3.5 ml (±0.1 ml).

coefficients for I-4 and I-8.

Carbohydrate Content and N-Terminal Amino Acids. The number of preparations analyzed for sialic acid, hexosamine, and total hexose, respectively, was I-4, 16, 12, 18; I-8, 11, 7, 13; and I-8D$_5$, 5, 5, 5. Both I-8 and I-8D$_5$ had significantly higher carbohydrate contents than I-4 on a weight basis (Table II). For I-4 compared with I-8 and I-8D$_5$, the differences in sialic acid and total hexose content (w/w) were highly significant at the $P \leq 0.001$ level. The differences in hexosamine levels were not significant ($0.05 < P < 0.10$). There were no significant differences in carbohydrate content when calculated on a molar basis nor were there any differences between I-8 and I-8D$_5$.

The N-terminal amino acids of I-8D$_5$, fibrinogen, and fibrin, were the same as those of the parent I-4 for duplicate determinations on three preparations each of I-8D$_5$ and I-4. No quantitative differences could be detected (Table III). Previous dinitrofluorobenzene studies (Mosesson *et al.*, 1967) comparing I-4 and I-8 had demonstrated the same end groups in these fractions and found essentially the same quantitative values. The values for I-4 are also in agreement with the results of others for this preparation (Blombäck *et al.*, 1966; von Korff *et al.*, 1963).

Biological Activity. Thrombin times were performed on 8, 6, and 5 preparations, respectively, of I-4, I-8, and I-8D$_5$. The thrombin times of I-8D$_5$ preparations showed a wider variation than I-8 preparations, although both I-8 and I-8D$_5$ had distinctly longer times than did I-4, both with and without calcium (Table II). Fraction I-8 thrombin times were somewhat longer than those of I-8D$_5$. The thrombin times of I-8D$_5$ were inversely related to the digest clottability; *i.e.*, the lower the over-all clottability when the reaction was halted, the longer the thrombin times of the resultant I-8D$_5$.

Comparative studies of the polymerization of fibrin monomer (Figure 5) were carried out on I-4, I-8, and I-8D$_5$ fractions, all prepared from the same lot of plasma. The polymerization curve for I-8D$_5$ fell between those of I-4 and I-8 and the data were consistent with the thrombin time observations (Table II). Mixing any two of the three monomer preparations resulted in a curve intermediate between those of the two pure preparations.

Discussion

The experiments described in this paper were designed to provide data to test the possibility that the low molecular weight fibrinogen known to be present in normal human plasma was derived from higher molecular weight parent species by the degradative action of plasmin. The high solubility material produced early during plasmin degradation of fraction I-4 was isolated by adapting the technique used for isolation of the high solubility plasma fibrinogen fractions, (Mosesson and Sherry, 1966). Further separation from unclottable derivatives was achieved by a combination of physicochemical techniques (*vide supra*). The final material was $\geq 95\%$ clottable, if prepared from digests of $\geq 84\%$ clottability. This material, designated I-8D_5, was then compared with its parent fibrinogen (I-4) and naturally occurring fraction I-8, isolated from plasma.

Differences, if any, between I-8 and I-8D_5, were marginal with regard to $s^0_{20,w}$, $D_{20,w}$, the molecular weights calculated from these data, and tyrosine/tryptophan ratios (Table II). There were no differences in electrophoretic behavior on agarose gels and carbohydrate content. However, while both I-8 and I-8D_5 had greater mobility than I-4 on acrylamide gel electrophoresis, I-8D_5 was more heterogeneous than I-8, and the gel pattern suggested that part of I-8D_5 had a somewhat smaller molecular size than I-8 (Figure 3). All three fractions had the same N-terminal amino acid contents for fibrinogen and corresponding fibrin. Both I-8 and I-8D_5 were distinctly different from I-4 in $s^0_{20,w}$, $D_{20,w}$, molecular weight, sialic acid and hexose contents (w/w), and thrombin times.

Reproducible differences existed between I-4 and the other two fractions on DEAE gradient chromatography, I-8D_5 prepared from digests with over-all clottabilities $>86\%$ and fraction I-8 had identical DEAE patterns. Reduction of digest clottability to 86% or less resulted in reproducible alterations in the DEAE patterns of the resultant I-8D_5, suggesting that DEAE gradient chromatography was an appropriate means to study the subtle changes occurring during plasmin degradation of fibrinogen.

Retarded polymerization of fibrin monomer preparations accounted for longer thrombin times in I-8 and I-8D_5 compared with I-4 (Figure 5). The retarded polymerization was more pronounced in I-8 than I-8D_5, whether or not calcium ions were present. The variation of I-8D_5 thrombin times in relation to digest clottability suggested that there were several bonds in the parent molecule whose cleavage impaired polymerization, but did not prevent ultimate clot formation and that a range of polymerization potential could exist within fibrinogen preparations of high clottability.

If plasma fraction I-8 is derived from a higher molecular weight molecule, such as I-4, by plasmin degradation, then differences between I-8 and I-8D_5 in polymerization rates and behavior on acrylamide gel electrophoresis may be related to the modifying effects on plasmin proteolysis which occurs in a plasma mileau (*e.g.*, I-8) as compared with our *in vitro* model. Thus, the significance of the acrylamide gel and polymerization differences between I-8 and I-8D_5 is not clear. Over-all, these studies do provide evidence that a fibrinogen very similar to plasma fraction I-8 may be produced *in vitro* by plasmin.

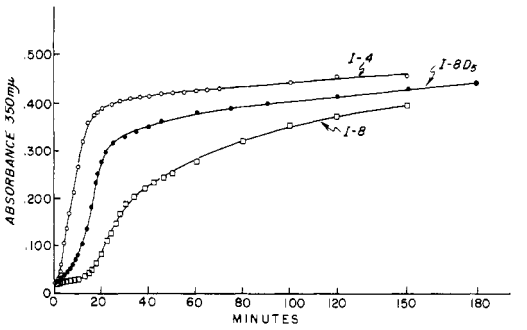

FIGURE 5: Polymerization of fibrin monomer solutions. One part of a 0.53% solution of monomer in 1 M NaBr (pH 5.3) was diluted with ten parts of 0.1 M sodium phosphate buffer (pH 6.0) and the absorbance at 350 mμ was followed.

Previous studies (Iwanaga *et al.*, 1967; Wallén and Bergström, 1958) have indicated that the thrombin-susceptible bonds in fibrinogen are not cleaved by plasmin. The fact that the same amount of N-terminal glycine appeared in I-8D_5 fibrin as in I-4 fibrin (Table III) indicated not only that the thrombin-susceptible bonds remain intact, but that they remain with the core of the molecule during very early plasmin-induced fibrinogenolysis. This conclusion is not in agreement with the suggestion by Budzynski *et al.* (1967) that the first fragments cleaved from the core may include the thrombin-susceptible sites.

In contrast to plasmin degradation, studies of tryptic degradation of fibrinogen indicate not only that the thrombin-susceptible sites are cleaved early in the digestion (Alexander *et al.*, 1966; Mihalyi and Godfrey, 1963), but that the rapid loss of clottability could be accounted for by the cleavage of a single critical bond, which is not thrombin susceptible (Mihalyi and Godfrey, 1963).[13] Our data and that of Beck and Jackson (1966) indicate that this critical bond in plasmin digests remains intact or is not broken as early.

The close agreement in the respective N-terminal contents of fibrinogen and fibrin of I-4 and I-8D_5 suggests that the earliest plasmin action involves cleavage of peptide material from the C-terminal end of the molecule with resultant reduction in molecular weight. Furthermore, it does not appear as if there is any early intrachain peptide hydrolysis in the core molecule because no new N-terminal amino acids were found. It is possible that N-terminal arginine and/or histidine may have been formed but remained undetected by our analytical system.[9] However, this seems unlikely since neither Wallén and Bergström (1957) nor Miles *et al.* (1964) found either of these N-terminal amino acids during the course of their studies of plasmin

[13] Of note in the study of Alexander *et al.* (1966), using water-insoluble trypsin derivatives, was their suggestion that at the earliest stage of tryptic digestion (one to two peptide bonds cleaved), a slowly clotting product was present.

degradation of fibrinogen. Finally, the higher carbohydrate content of I-8D_5, compared with the parent material I-4, indicates that at this stage of proteolysis, the material hydrolyzed from the core contains little or no carbohydrate.

The early stages of plasmin-induced fibrinogenolysis have been the subject of several recent investigations. Fletcher *et al.* (1966) measured $s^0_{20,w}$ and $D_{20,w}$ of bovine fibrinogen digests and demonstrated an increase in $D_{20,w}$ with a molecular weight of 265,000 for their "first derivative." This reduction in molecular weight could not be demonstrated when human fibrinogen was used, because of the formation of polymers. However, a human fibrinogen derivative of comparable size with the bovine derivative could be seen on acrylamide gel electrophoresis. "First derivative" and the purified portion of a human fibrinogen digest, I-8D_5, appear to be representative of the same early stage of degradation. In addition to the similarity in molecular weight, they both display prolonged thrombin times and most importantly, retain virtually complete clottability. Finally, both studies were at approximately the same point in digest clottability (93% for Fletcher *et al.*, 84–92% in the present study).

Marder *et al.* (1967) isolated fragments termed X and Y from plasmin digests of human fibrinogen. The X fragment was described as having a molecular weight of 270,000 and both a slightly more cathodal electrophoretic mobility in agar gel and a slightly greater elution volume on G-200 Sephadex than intact fibrinogen. The X fragment preparations were only partly clottable and formed soluble unclottable complexes with fibrin monomer. Review of their data suggests that the X fragment was isolated when digest clottability was approximately 60–70%; *i.e.*, later than I-8D_5. Fraction I-8D_5 has essentially the same molecular weight as the X fragment, but does not have altered electrophoretic mobility (in 1% agar gel) nor can it be distinguished from intact fibrinogen (I-4) on G-200 Sephadex. A major distinguishing feature between fragment X and I-8D_5 is that I-8D_5 is virtually completely clottable by itself and does not form unclottable complexes with fibrin monomer from intact fibrinogen. These differences in properties support the conclusion from the digest data that I-8D_5 is representative of a somewhat earlier stage of degradation than fragment X.

Thus, the first stage of plasmin-mediated fibrinogenolysis appears to involve considerable loss of mass with altered polymerization rates, but virtually no change in the ultimate clottability of the core molecule (*i.e.*, first derivative, I-8D_5). Then slightly later, there is progressive loss of clottability and a slight alteration in electrophoretic mobility without further significant change in mass (X fragment).[14] Later, clottability is completely lost and there is further loss of mass. This is approximately the point in the proteolysis of fibrinogen when the "antithrombin" effect of the digest is the greatest and where Marder *et al.* (1967) identified their Y fragment (mol wt 165,000). At this same stage, Latallo *et al.* (1964) and Triantaphyllopoulos (1966) have indicated an inhibition of the enzymatic action of thrombin using "early breakdown products" and "AFIF," respectively. While the nature of the "antithrombin" activity of these intermediate products has not been completely elucidated, their action should not be confused with the delayed polymerization inherent in the early clottable product (I-8D_5) or with the polymerization inhibition produced by the final D and E fragments (Latallo *et al.*, 1962b). Additionally, both the X, Y, and D fragments (Marder *et al.*, 1967) and the "early" and "late" degradation products (Lipinski *et al.*, 1967) have been shown to form soluble nonclottable complexes with fibrin monomer. The relative importance of these several different effects in the genesis of bleeding in clinical fibrinolytic disorders remains to be established.

The similarities between I-8 and I-8D_5 support the concept that I-8 is an *in vivo* degradation product of I-4. Indirect evidence for the I-4 to I-8 conversion by plasmin in the human is the observation that there is an increase in 8% ethanol-soluble clottable protein during the course of urokinase therapy (M. W. Mosesson, 1966, unpublished observations). This is correlated with studies by other methods which demonstrated "first derivative" in patients with abnormally increased fibrinolysis (Alkjaersig *et al.*, 1966; Fisher *et al.*, 1967). Additionally, recent studies (Sherman *et al.*, 1969) have indicated that such an *in vivo* change does occur physiologically in the normal rabbit. However, it was not established as to whether or not plasmin was the enzyme responsible. Other studies in the literature dealing with *in vivo* fibrinogen metabolism do not relate directly to this question. There have been studies of the influence of ϵ-aminocaproic acid on the half-life of radioactive fibrinogen in the normal mammal (Amris and Amris, 1964; Gajewski and Alexander, 1963; Hart 1964; Lewis, 1963; Mutschler, 1964) for the most part showing no effect. Since these experiments followed the radioctivity of the plasma or of the total clottable protein, they could not have yielded any information relating to a subtle transformation from higher to lower molecular weight *clottable* fibrinogen.

Other hypotheses for the origin of I-8 are not in accord with available data. Current evidence suggests that each fibrinogen molecule has three pairs of peptide chains, each of 47,000–63,500 molecular weight, (Henschen, 1963; McKee *et al.*, 1966). The quantitative agreement of the N-terminal amino acids of I-4 and I-8 weigh heavily against the possibility of I-8 being a fibrinogen with only five chains. Genetic variation is unlikely since all individual samples studied to date have had 8% ethanol-soluble fibrinogen (M. W. Mosesson and L. A. Sherman, 1967, unpublished observations). For I-8 to be a precursor of I-4 would require that peptide material be added to a preexisting extracellular molecule (I-8). This is also unlikely.

[14] The data of Iwanaga *et al.* (1967) raises the possibility that fragment X may be formed by plasmin cleavage at position 43 on the A chain, removing acidic peptide fragments containing peptide A and/or by cleavage at position 21 on the B chain and removing peptide B. Clottability would be adversely affected without a major reduction in molecular weight. However, there is no direct evidence presently available which relates to this speculation.

It is apparent that there are several types of fibrinogen of differing physicochemical characteristics found in normal plasma (*e.g.*, cryofibrinogen, I-4, I-8, etc.). The reasons for such wide variation in a protein with but one apparent function are still unclear. On the basis of the studies presented here, at least part of the variation may be the result of *in vivo* proteolysis as part of normal catabolism.

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