Amino Acid Composition of Human Fibrinogen and Anticoagulant Derivatives

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1. The amino acid compositions of human fibrinogen and three intermediate anticoagulant derivatives were determined by column chromatography. The derivatives were isolated by ammonium sulphate fractionation and column electrophoresis from solutions of fibrinogen undergoing spontaneous breakdown. One derivative, isolated as the large electrophoretic peak at the end of the clottable period (100% CP) of the parent fibrinogen solution, was labelled LP₁₀₀ and others obtained at twice this period (200% CP) were designated as LP₂₀₀ and SP₂₀₀ (LP, large peak; SP, small peak). 2. Maximal 'molecular' weights of approx. 294 000 for LP₁₀₀, 137 000 for LP₂₀₀ and 37 000 for SP₂₀₀ were calculated for the protein moieties. At least 265 amino acid residues must have been lost from each fibrinogen molecule during the formation of LP₁₀₀, and 1362 during the formation of the other two derivatives. 3. Only one derivative (LP₂₀₀) had a partial specific volume ($\bar{v}$ 0.725 ml./g.) different from that of fibrinogen ($\bar{v}$ 0.721 ml./g.). 4. No significant differences in refractive index at 589 m μ were detected. 5. Calculation of the total number of ionizable groups/10⁵ g. of each protein moiety showed a preponderance of the following numbers of negative charges: 22 in fibrinogen; 24 in LP₁₀₀; 26 in LP₂₀₀; 49 in SP₂₀₀. The isoionic points were estimated to be approx. +0.03 pH unit (for fibrinogen), -0.06 pH unit for (LP₁₀₀) and +0.28 pH unit (for LP₂₀₀) from the pK of imidazole, and 0.78 pH unit above the average pK of aspartyl and glutamyl ions (for SP₂₀₀). These figures agree closely with experimentally determined values of the isoelectric point of fibrinogen and its derivatives.

Numerous studies have been concerned with fibrinogen, because of the fundamental role of this protein in blood coagulation. The search for an effective way to treat thrombosis and prevent embolism has given impetus to the study of fibrinolysis and indirectly to that of fibrinogenolysis. The interest in these processes became even greater when it was discovered that the products of fibrinogenolysis have anticoagulant properties (Triantaphyllopoulos, 1958; Niewiarowski & Kowalski, 1958). Most of the studies of these anticoagulants have been concerned with their mode of action, their electrophoretic, ultracentrifugal or immunological properties, their detection or their isolation from blood (Triantaphyllopoulos, 1958, 1959, 1960; Kowalski, Budzynski, Kopec & Murawski, 1960; Nüssenzweig, Seligmann, Pelmont & Grabar, 1961a; Nüssenzweig, Seligmann & Grabar, 1961b; Fletcher, Alkjaersig & Sherry, 1962; Alkjaersig, Fletcher & Sherry, 1962; Triantaphyllopoulos & Triantaphyllopoulos, 1962, 1965a,b, 1966a,b; Schwick, 1963). We have shown that the anti-

thrombic activity, electrophoretic mobility and thermosensitivity of the main anticoagulant fraction vary greatly with the stage of incubation of the parent fibrinogen solution (Triantaphyllopoulos & Triantaphyllopoulos, 1965a,b, 1966b). The greatest inhibitory effect is exhibited by preparations that are isolated at the time when the parent fibrinogen becomes completely unclottable by thrombin. This variability reflects a continuous change in the composition or tertiary structure or both of the anticoagulant. Information on structural changes is almost non-existent; there is only one report on the amino acid composition of a fibrinogen derivative, which was obtained from bovine fibrinogen after complete fragmentation by trypsin (Mihalyi & Godfrey, 1963).

We are primarily interested in the study of fibrinogen-derived anticoagulants that are produced under conditions resembling as closely as possible the ones prevailing in human blood. There, fibrinogen is believed to be broken down by fibrinolysin (plasmin) after activation of the endogenous

profibrinolysin (plasminogen). In the present studies, human fibrinogen was allowed to lyse spontaneously under sterile conditions; the main anticoagulant products were isolated at two different stages of incubation, and the amino acid compositions of these fractions were determined under the same conditions as those used to find the composition of the parent intact fibrinogen. From the amino acid content and the yield of each anticoagulant, and from accepted values for the molecular weight and carbohydrate content of human fibrinogen, the maximal 'molecular' weight and the molar amino acid composition of the protein moiety of each anticoagulant derivative was estimated. Knowledge of the amino acid composition of these fractions further allowed the calculation of apparent specific volumes, refractive indices and the nature and number of charged groups; from the last-named data, approximate isoionic points could be found.

It has been reported (Finlayson & Mosesson, 1963; Mosesson & Finlayson, 1963) that human fibrinogen of high purity [fraction I-4 (Blombäck & Blombäck, 1956); lysine-treated fraction I-4 (Mosesson, 1962)] can be separated by chromatography on DEAE-cellulose into two fractions. Both of these fractions can be clotted by thrombin. They show the same sedimentation rate, u.v. absorption spectrum, *N*-terminal amino acids, phosphorus content and sialic acid content, and seem identical immunologically. We have similarly detected two components in preparations of human and bovine fibrinogen of high clottability, and in preparations of the main anticoagulant obtained from them (Triantaphyllopoulos & Triantaphyllopoulos, 1965a,b). Since these fibrinogen fractions are nearly identical, some believe that they are artifacts (Godal & Lüscher, 1960). No attempt was made in these studies to separate them, in the starting material or later in the main anticoagulant fraction.

MATERIALS AND METHODS

Human fibrinogen. This protein was obtained from pooled human plasma by double salt precipitation with $(\text{NH}_4)_2\text{SO}_4$ and NaCl (Triantaphyllopoulos & Triantaphyllopoulos, 1965a). The entire isolation procedure, including the separation of plasma, was completed within 2 days. The final product, tested spectrophotometrically, was 96–98% clottable. It was either used immediately or stored at -20° after the addition of sodium citrate to a final concentration of 0.02M. It was not stored for more than 15 days.

Pancreatic trypsin inhibitor. A purified preparation, containing 200,000 units of this inhibitor/ml., was purchased from Choay Laboratories, Paris, France, under the trade name Iniprol.

Clottable period. The clottable period (CP) was defined as the initial stage of incubation during which the fibrinogen could still be clotted by thrombin. The end of this period was indicated by the formation of one or two thin fibrin

strands after the addition of thrombin to samples of the incubated solution and dilution of the mixtures 1:2 with water. The duration of this period depends on the fibrinolytic activity of each preparation. In the experiments described here, it lasted for 4–5 days.

Anticoagulant fractions LP₁₀₀, LP₂₀₀ and SP₂₀₀. The fibrinogen was incubated at 37° , under sterile conditions, in solutions of 10 mg./ml., buffered with imidazole to pH 7.3, as described in detail earlier (Triantaphyllopoulos & Triantaphyllopoulos, 1965a). Fractionations were performed: (a) at the end of the clottable period (100% CP), when the antithrombic activity of the produced anticoagulants is always at its maximum; (b) at twice this period (200% CP), when the antithrombic activity is considerably lower but still higher than the activity of the final products (Triantaphyllopoulos & Triantaphyllopoulos, 1965a). The incubation period in each experiment was expressed as a percentage of the clottable period (CP) to eliminate differences in the rate of fibrinogenolysis (Triantaphyllopoulos & Triantaphyllopoulos, 1965a). This facilitated the comparison of results obtained from various experiments. The anticoagulant products were first isolated by precipitation at 25–50% saturation with $(\text{NH}_4)_2\text{SO}_4$ after the addition of pancreatic trypsin inhibitor (Iniprol) at 200 units/ml. of fibrinogen solution to stop further breakdown. This 25–50% saturation fraction, called previously by us the 'anticoagulant fraction of incubated fibrinogen' or 'AFIF', was further purified by column electrophoresis (Triantaphyllopoulos & Triantaphyllopoulos, 1966b), in 0.02M-veronal buffer, pH 8.6. The main subfractions from each preparation, emerging from the column as a large and a small peak, were collected and concentrated by freeze-drying. They were designated: LP₁₀₀, large peak obtained from preparations that were isolated at 100% CP; LP₂₀₀, large peak from preparations isolated at 200% CP; SP₂₀₀, small peak from preparations isolated at 200% CP. It is not possible to separate small-peak material at 100% CP. The large-peak fractions correspond to the α -derivative described by Seegers, Niefert & Vandenbelt (1945), and the small peak component is the β -derivative described by these authors. The subfractions were kept as dry powders at -20° . Before hydrolysis a portion was dissolved in a small volume of water and dialysed against a large volume of 0.85% NaCl with continuous stirring, to remove veronal. Dialysis was completed within 4–8 hr. after three or four changes of 0.85% NaCl. The protein content of each solution was determined by the biuret method of Kingsley for total protein (Hawk, Oser & Summerson, 1954).

Amino acid analysis. Duplicate samples of 4–6 mg. of the dialysed protein were hydrolysed by 6N-HCl in sealed evacuated tubes, which were immersed in boiling toluene (110°) for 22 hr. The hydrolysates were dried *in vacuo* at room temperature and the residues were dissolved in 3–5 ml. of 0.20M-citrate buffer, pH 2.2. (This was prepared as described in the Instruction Manual AIM-2 of Beckman Instruments Inc., Palo Alto, Calif., U.S.A., and contained in 11.: sodium citrate dihydrate, 19.6 g.; conc. HCl, 16.5 ml.; thiodiglycol, 20 ml.; polyoxyethylene (23) lauryl ether (50%, v/v), 2 ml.; octanoic acid, 0.1 ml.). The buffered hydrolysates were centrifuged in a clinical centrifuge at 2000 rev./min. (1200 $\times$ g) for 20 min. to remove precipitated humin. The clear supernatants were analysed in a Beckman model 120 amino acid analyser by the procedure

of Spackman, Stein & Moore (1958). Runs with a standard solution of amino acids purchased from Beckman Instruments Inc. (calibration mixture type 1) were made frequently and the values obtained for each amino acid in the different runs were averaged and used as constants. The ammonia constant was determined with a calibration mixture prepared by Bio-Rad Laboratories, Richmond, Calif., U.S.A. Tryptophan, which is destroyed by acid hydrolysis, was determined spectrophotometrically (Bencze & Schmid, 1957).

Partial specific volumes ($\bar{v}$) and refractive indices at 589 m μ . These were calculated from the amino acid composition by the methods of Cohn & Edsall (1965) and McMeekin, Wilensky & Groves (1962) respectively.

RESULTS

Amino acid analysis. Analyses were performed on five samples of human fibrinogen and on 16 specimens of anticoagulant derivatives; four of the large peak isolated at 100% CP (LP₁₀₀), seven of the large peak isolated at 200% CP (LP₂₀₀) and five of the small peak obtained at 200% CP (SP₂₀₀). The mean number of residues and the standard deviation of each amino acid/10⁵ g. of protein in each group is shown in Table 1. The carbohydrate content of the proteins was not considered in these studies and the values in the Table refer to 10⁵ g. of the protein

Table 1. *Amino acid composition of human fibrinogen and its main anticoagulant derivatives*

Human fibrinogen, 96–98% clottable, was incubated at 37° and pH 7.3 under sterile conditions. Anticoagulant fractions were isolated by 25–50% saturation with (NH₄)₂SO₄ and column electrophoresis at the following times: (a) the end of the clottable period (100% CP); (b) twice the length of this period (200% CP). The electrophoretic fractions, large peak from 100% CP (LP₁₀₀), large peak from 200% CP (LP₂₀₀) and small peak from 200% CP (SP₂₀₀), were hydrolysed with 6N-HCl *in vacuo* at 110°. Amino acid analyses were performed in a Beckman model 120 amino acid analyser by the method of Spackman *et al.* (1958). Tryptophan was determined spectrophotometrically (Bencze & Schmid, 1957). Results are given as mean $\pm$ s.d. Percentages were calculated from the number of residues in 100 g. of protein multiplied by residue weight. The number of atoms of N contributed by each amino acid were added and multiplied by 14 $\times$ 100/sum of residue weights, to obtain the percentage of amino acid N. The total percentages are not exactly 100 because of experimental error. For the percentage amino acid composition, corrected to 100% total, see Table 4.

Amino acid	Amino acid composition (moles/10 ⁵ g. of protein moiety)				Amino acid composition (% of protein moiety, w/w)			
	Fibrinogen (5*)	LP ₁₀₀ (4*)	LP ₂₀₀ (7*)	SP ₂₀₀ (5*)	Fibrinogen	LP ₁₀₀	LP ₂₀₀	SP ₂₀₀
Lys	60.7 $\pm$ 2.5	61.2 $\pm$ 4.0	61.8 $\pm$ 3.6	56.2 $\pm$ 3.7	7.77	7.84	7.92	7.20
His	18.2 $\pm$ 0.7	17.5 $\pm$ 0.6	16.1 $\pm$ 0.8	16.5 $\pm$ 0.8	2.49	2.40	2.21	2.26
NH ₃ †	96.9 $\pm$ 4.1	99.4 $\pm$ 6.8	100.9 $\pm$ 6.5	130.4 $\pm$ 7.5				
Arg	45.0 $\pm$ 2.2	43.4 $\pm$ 0.4	45.4 $\pm$ 2.9	36.3 $\pm$ 2.4	7.02	6.77	7.09	5.67
Trp	24.0 $\pm$ 3.1	25.1 $\pm$ 1.1	26.9 $\pm$ 2.1	17.6 $\pm$ 0.8	4.47	4.67	5.01	3.28
Asp	106.8 $\pm$ 3.7	109.5 $\pm$ 2.6	105.8 $\pm$ 7.1	138.9 $\pm$ 10	12.19‡	12.50‡	12.07‡	15.85‡
Thr	59.2 $\pm$ 2.7	59.6 $\pm$ 3.1	46.8 $\pm$ 2.4	41.2 $\pm$ 3.1	5.99	6.03	4.73	4.16
Ser	77.3 $\pm$ 4.3	78.5 $\pm$ 4.2	62.7 $\pm$ 6.2	62.0 $\pm$ 1.8	6.36	6.84	5.46	5.40
Glu	104.6 $\pm$ 2.4	103.9 $\pm$ 2.5	107.8 $\pm$ 7.1	119.4 $\pm$ 6.6	13.50	13.41	13.91	15.41
Pro	46.2 $\pm$ 2.6	42.0 $\pm$ 2.7	34.7 $\pm$ 5.1	33.6 $\pm$ 3.3	4.49	4.08	3.37	3.26
Gly	86.8 $\pm$ 3.6	90.7 $\pm$ 1.0	79.8 $\pm$ 6.6	48.2 $\pm$ 4.2	4.95	5.17	4.55	2.75
Ala	43.7 $\pm$ 1.6	42.0 $\pm$ 1.4	36.5 $\pm$ 2.7	40.6 $\pm$ 4.2	3.10	2.98	2.59	2.88
Cys	6.0 $\pm$ 1.3	4.9 $\pm$ 0.4	4.3 $\pm$ 1.0	22.8 $\pm$ 3.6	1.33	1.09	0.96	5.07
Val	41.4 $\pm$ 1.4	41.4 $\pm$ 1.6	43.3 $\pm$ 3.7	48.6 $\pm$ 2.4	4.10	4.10	4.29	4.82
Met	19.8 $\pm$ 0.8	20.5 $\pm$ 0.3	21.3 $\pm$ 1.4	9.4 $\pm$ 2.0	2.60	2.69	2.79	1.23
Ile	32.3 $\pm$ 1.5	35.6 $\pm$ 1.7	42.8 $\pm$ 3.7	29.6 $\pm$ 3.3	3.65	4.03	4.84	3.35
Leu	54.5 $\pm$ 1.7	54.0 $\pm$ 2.2	54.6 $\pm$ 4.4	78.0 $\pm$ 4.8	6.16	6.11	6.17	8.82
Tyr	31.8 $\pm$ 1.6	32.7 $\pm$ 0.8	36.0 $\pm$ 2.6	30.6 $\pm$ 2.0	5.19	5.33	5.87	4.99
Phe	28.8 $\pm$ 1.7	28.1 $\pm$ 0.9	22.2 $\pm$ 1.2	30.3 $\pm$ 1.6	4.24	4.13	3.27	4.46
Total percentage					99.60	100.17	97.10	100.86
Mean total	887 $\pm$ 18	891 $\pm$ 14	849 $\pm$ 40	860 $\pm$ 26				
no. of residues								
Amino acid N					17.51	17.43	17.46	17.06

* Figures in parentheses represent numbers of samples (determinations in duplicate).

† Ammonia figures were not added into the totals.

‡ Asparagine and aspartic acid.

moiety only. The ammonia values were extrapolated to zero hydrolysis in order to measure amide nitrogen (Mihalyi, Small & Cooke, 1964), and they were not added into the totals. There were obvious differences in the concentrations of several amino acids between fibrinogen and LP₂₀₀ and even more pronounced differences between SP₂₀₀ and fibrinogen. For this reason statistical analysis of these results was not considered necessary. Analysis of the results for LP₁₀₀ showed that only the concentrations of histidine ($P < 0.01$), isoleucine ($P = 0.014$) and proline ($P = 0.05$) were significantly different from those for fibrinogen. It is pertinent to add here that thrombin cannot split trichloroacetic acid-soluble protein fragments from LP₂₀₀ and SP₂₀₀, but that the evidence indicates that it can split such fragments from LP₁₀₀ (Triantaphyllopoulos & Triantaphyllopoulos, 1966a).

The specific activity of purified preparations of profibrinolysin has been reported to reach 90–100 caseinolytic units/mg. of nitrogen, the protein containing 16% of nitrogen (Sgouris, Inman, McCall, Hyndman & Anderson, 1960), and there is always the possibility of loss in activity during purification. We find that addition of fresh solutions of purified fibrinolysin to fibrinogen preparations that are almost free of profibrinolysin, at 0.0008 caseinolytic units of fibrinolysin/mg. of fibrinogen, renders the latter uncoagulable in 3–4 days. The parent fibrinogens in the present experiments reached this stage in 4–5 days. The maximum amount of profibrinolysin that was present in our preparations should therefore be less than 0.05 μ g. of protein/mg. of fibrinogen. This is well below the limit that can be determined by the ninhydrin method. It is safe therefore to conclude that all the amino acid residues that were detected in these analyses belong to fibrinogen and the fibrinogen fractions.

The percentage (w/w) of each amino acid residue in human fibrinogen and its derivatives is shown on the right half of Table 1. Ammonia was calculated with aspartic acid as asparagine. The final result is independent of whether this substance is counted as asparagine or glutamine (Cohn, 1965). The amounts of nitrogen contributed by the individual amino acids were added and the sum was divided by the total weight of the residues in each group and multiplied by 100. This gave the concentration of nitrogen in exactly 100g. of each protein and this value is recorded at the bottom. If allowances are made for the carbohydrate content of fibrinogen (3.2% of hexoses, 1.0% of glucosamine and about 0.8% of sialic acid; Blombäck, 1958), the value for the nitrogen concentration in this glycoprotein is 16.74% (w/w). This is nearly identical with values obtained by Kjeldahl analyses [16.73% (Blombäck, 1958); 16.72% (Mihalyi *et al.* 1964)].

Table 2. Yields of 0–25%- and 25–50%-saturated ammonium sulphate fractions from lysed human fibrinogen at 200% CP

The yields are expressed as percentages of the weight of the original protein taken.

Expt. no.	0–25%-satd. (NH ₄) ₂ SO ₄ fraction	25–50%-satd. (NH ₄) ₂ SO ₄ fraction
1	3.8	64
2	1.9	60
3	2.3	52
4	3.3	56
5	1.2	55
Mean $\pm$ S.D.	2.5 $\pm$ 1.0	57 $\pm$ 5

Yield of 0–25%- and 25–50%-saturated ammonium sulphate fractions at 200% CP and distribution of protein in the electrophoretic subfractions. Fractionation of lysed fibrinogen solutions (200% CP) into 0–25% and 25–50%-saturated ammonium sulphate fractions was performed quantitatively and the yields were calculated as a percentage of the weight of the original fibrinogen from which the fractions were obtained (Table 2). The 25–50%-saturated ammonium sulphate fractions were then subjected to electrophoresis, as described in the Materials and Methods section. As earlier experiments have shown (Triantaphyllopoulos & Triantaphyllopoulos, 1965a), fractions taken at this period (200% CP) separate under these conditions into three sub-fractions. Besides LP₂₀₀ and SP₂₀₀ there is also a third smaller peak due to a positively charged component. The percentage distribution of the protein into these subfractions was estimated by measuring the area under each electrophoretic peak, or by totalling the amounts of protein that were detected from absorption measurements at 280m μ in the tubes belonging to each peak. Table 3 shows the results obtained from eight such experiments.

Partial specific volumes ($\bar{v}$). One of the derivatives, LP₁₀₀, was found to have the same partial specific volume (0.721 ml./g.) as fibrinogen. The $\bar{v}$ of LP₂₀₀ was 0.725 ml./g. and that of SP₂₀₀ was 0.720 ml./g. If the value obtained for fibrinogen is adjusted for carbohydrate content, the calculated $\bar{v}$ for the glycoprotein is 0.718 ml./g. The same value has been found experimentally by Scheraga *et al.* (1954) for native bovine fibrinogen, whereas denatured preparations gave a higher figure.

Refractive indices at 589m μ . The calculated refractive indices of the proteins at 589m μ were all practically the same; values of 1.612 were obtained for fibrinogen, LP₁₀₀ and LP₂₀₀, and one of 1.613 was obtained for SP₂₀₀. Adjustment of the re-

Table 3. *Protein distribution in the 25–50% saturated ammonium sulphate fractions from lysed fibrinogen solutions at 200% CP*

For definition of the three electrophoretic fractions, see the text.

Expt. no.	% of total protein		
	LP ₂₀₀	SP ₂₀₀	Positively charged peak
1	73	19	8
2	78	18	4
3	77	18	5
4	74	18	8
5	75	17	8
6	72	22	6
7	70	23	7
8	68	24	8
Mean $\pm$ S.D.	73 $\pm$ 3	20 $\pm$ 3	7 $\pm$ 2

fractive index of fibrinogen for carbohydrate content gave a value of 1.610. The value (1.603) found by Armstrong, Budka, Morrison & Hasson (1947) was obtained with Cohn's fraction I-1, which had a clottability of about 90%.

DISCUSSION

The amino acid composition of human fibrinogen presented by Tristram (1949) was obtained from the data of Bailey (1944) and Brand & Edsall (1947). These analyses were performed at a time when neither were sufficiently purified preparations of fibrinogen available [Brand & Edsall (1947) worked with preparations that were 87% clottable] nor had accurate chromatographic methods of amino acid analysis been developed.

If allowances are made for 5% carbohydrate content (Blombäck, 1958), there is a very good agreement between most of our values and the values obtained by Henschen & Blombäck (1964) for human fibrinogen, fraction I-4 (Blombäck & Blombäck, 1956). Differences were observed in serine (six residues), glutamic acid (12), arginine (five) and lysine (four), for which our values were higher, and in isoleucine (seven) and aspartic acid (four), where our values were lower (the numbers in parentheses indicate differences in numbers of residues/10⁵g.). The disagreement in these amino acids may be due to the following: (a) differences in the methods of isolation of human fibrinogen used in each Laboratory; (b) differences in the determination procedures; Henschen & Blombäck (1964) used a common calibration standard (β -2-thienyl-alanine) for all the amino acids.

Table 4. *Amino acid composition of fibrinogen and its main anticoagulant derivatives, corrected for experimental error*

The numbers of residues from the last four columns of Table 1 were divided by the total percentages of each protein moiety (Table 1), and multiplied by 100. The corrected values below were used in the calculations of charge and maximal molecular weights.

Amino acid	Residues/10 ⁵ g. of protein moiety exactly			
	Fibrinogen	LP ₁₀₀	LP ₂₀₀	SP ₂₀₀
Lys	60.9	61.1	63.6	55.7
His	18.3	17.5	16.6	16.4
NH ₃ *	97.3	99.2	103.9	129.3
Arg	45.2	43.3	46.8	36.0
Trp	24.1	25.1	27.7	17.4
Asp	107.2	109.3	109.0	137.7
Thr	59.4	59.5	48.2	41.2
Ser	77.6	78.4	64.6	61.5
Glu	105.0	103.7	111.0	118.4
Pro	46.4	41.9	35.7	33.3
Gly	87.1	90.5	82.2	47.8
Ala	43.9	41.9	37.6	40.3
CyS	6.0	4.9	4.4	22.6
Val	41.6	41.3	44.6	48.2
Met	19.9	20.5	21.9	9.3
Ile	32.4	35.5	44.1	29.3
Leu	54.7	53.9	56.2	77.3
Tyr	31.3	32.6	37.1	30.3
Phe	28.9	28.1	22.9	30.0
Total	889.9	889.0	874.2	852.7

* Ammonia values were not included in the totals.

Charges and isoelectric points. There are no free SH groups in fibrinogen, and, since S-S bonds can be detected only after denaturation (Carter & Warner, 1954; Henschen, 1964), all the cystine residues must be occupied in disulphide bridges and be unable to contribute any charges. The number of residues, from Table 1, that have ionizable groups in their side chains (lysine, histidine, arginine, tyrosine and dicarboxylic acids minus ammonia) were adjusted for 10⁵g. of each protein moiety exactly (Table 4) and the balance of charges was calculated. There is a total of about 271, 268, 280 and 265 charged groups in fibrinogen, LP₁₀₀, LP₂₀₀ and SP₂₀₀ with an excess of about 22, 24, 26 and 49 negative charges respectively. In very acidic solutions, when all the side chains of lysine, histidine and arginine will be charged and all the acidic groups will be uncharged, these proteins will carry about 124, 122, 127 and 108 positive charges respectively; SP₂₀₀ will be significantly less positive than fibrinogen, LP₁₀₀ or LP₂₀₀. From the number and nature of the charged groups, fibrinogen and its derivatives

are expected to show considerable buffering capacity in two pH regions, about 2-5 and 9-13. There should be slight buffering action at about pH 5-8, but none of the proteins can be expected to neutralize significant amounts of base or acid at pH 7-9.

It has been claimed that a great discrepancy exists between the experimentally determined values of the isoelectric point of fibrinogen and the values calculated from the amino acid composition (Mihalyi *et al.* 1964). Electrophoretic studies with purified preparations have given values of 5.5-5.95, depending on the nature of the buffer and the ionic strength of the solution (Seegers *et al.* 1945; Sheppard & Wright, 1954; Abe, Sheppard & Wright, 1955). From measurements of the ζ -potentials, Wright & associates calculated a value of 5.8 at zero ionic strength. Mihalyi *et al.* (1964), on the other hand, on the basis of their data on the amino acid composition of bovine fibrinogen, have suggested that the isoelectric point of this protein should be approx. 9. Calculation of the balance of charges in human fibrinogen from our corrected data (Table 4) shows that, after complete dissociation of the dicarboxylic acid residues that are not bound as glutamine or asparagine, there will be an excess of 9.5 positive charges in human fibrinogen. Equilibrium will be attained when an equal number of imidazolium ions have become uncharged. This will occur at about 0.03 pH unit from the pK value of this ion [$\text{pH} = \text{pK} + \log(9.5/8.8)$]. The pK value of the imidazolium ion in proteins has been found to vary between 5.6 and 7.0 (Edsall, 1965). Proteins bind anions and cations to a different extent, and their net charge in solution will be influenced by the nature of the buffer electrolytes and the ionic strength of the solution. Isoelectric points of 5.5 to 5.95 can all be compatible with the amino acid composition of fibrinogen presented in Table 1. The values of the isoelectric point suggest that the pK of the imidazolium ion in fibrinogen is close to the lower limits recorded for this ion. The value calculated by Abe *et al.* (1955) for zero ionic strength, considered together with the logarithm of the ratio of the uncharged to the charged residues of histidine (0.03), points to a pK value of about 5.8.

Similar calculations indicate that the isoionic points of LP₁₀₀ and LP₂₀₀ should be about pH 5.7 and 6.1 respectively, if the pK of the imidazolium ion in these derivatives is the same as in fibrinogen. Seegers *et al.* (1945), working with acetate, phosphate and borate buffers, found an isoelectric point of approx. 5.5 for their α -derivative, which corresponds to our LP from the final stages of incubation.

The amino acid composition of SP₂₀₀ suggests that the isoionic point of this anticoagulant must be approx. 0.76 pH unit above the average pK value

of the aspartyl and glutamyl ions. Seegers *et al.* (1945) have shown that the isoelectric point of the β -derivative (SP from the final stages of incubation) is approx. 4.2. The average pK value of the side carboxyl groups of the two carboxy amino acids in this protein must therefore be close to pH 3.4.

Maximal molecular weights. The 2½% of the original protein that was precipitated at 0-25% saturation with ammonium sulphate (Table 2) must be composed of fibrinogen molecules that were in a rather early stage of lysis since they were still precipitated at the same salt saturation as intact fibrinogen. The 57% that formed the 25-50%-saturated ammonium sulphate fraction must have come from the remaining 97.5% of the protein. This fraction must then represent $57 \times 100/97.5 = 58.5\%$ of the fibrinogen molecules. Since SP₂₀₀ and LP₂₀₀ make up 20% and 73% of the 25-50%-saturated ammonium sulphate fraction respectively (Table 3), SP₂₀₀ must represent $58.5 \times 20/100 = 11.7\%$ and LP₂₀₀ 42.7% of the parent molecule. The accepted value for the molecular weight of fibrinogen is 340 000 (Caspary & Kekwick, 1957), with a carbohydrate content of about 5% (Blombäck, 1958). The weight of the protein moiety must be approx. 323 000. One mole of this moiety must therefore have produced $323\,000 \times 11.7/100 = \text{approx. } 38\,000\text{g.}$ of SP₂₀₀ and $323\,000 \times 42.7/100 = 138\,000\text{g.}$ of LP₂₀₀.

Comparison of the amino acid composition of LP₁₀₀ with that of fibrinogen (values adjusted for 10⁵g. of protein exactly) showed that the concentration of isoleucine in the derivative increased more than the concentration of any other amino acid. This indicates that very few, if any, residues of isoleucine were removed from fibrinogen during the formation of LP₁₀₀. If we assume that no residues of isoleucine were removed at all, the 35.5 (adjusted value) residues of this amino acid that were detected in 10⁵g. of LP₁₀₀ must have originated from $35.5 \times 10^5/32.4 = 1.10 \times 10^5\text{g.}$ of fibrinogen. The parent protein must have lost at least 9% of its weight to become LP₁₀₀. This indicates that the molecular weight of this derivative cannot be greater than 294 000.

LP₂₀₀ and SP₂₀₀ were isolated during the same stage of incubation. It would therefore be rather unlikely that each of these came from separate molecules of fibrinogen. LP₂₀₀ and SP₂₀₀ have different compositions, and there seems no reason why some of the fibrinogen molecules could be broken down to only one of these derivatives and others to the other derivative exclusively. The possibility is greater that both derivatives were derived from the same parent molecule, and that as fibrinogenolysis progressed more peptides or free amino acids were removed from LP₁₀₀, and at a certain stage this derivative was split to a smaller

LP and an SP. Comparison of the amino acid composition (corrected values) of LP₁₀₀ with those of LP₂₀₀ and SP₂₀₀ showed that isoleucine (in LP₂₀₀) and cystine (in SP₂₀₀) were the amino acids the fewest residues of which were lost during the formation of these derivatives, since the concentration of these amino acids increased the most in the respective anticoagulants. The 44.1 residues of isoleucine that were found in 10⁵g. of LP₂₀₀ must have come from at least $44.1 \times 10^5 / 35.5 = 1.24 \times 10^5$ g. of LP₁₀₀. The 22.6 residues of cystine that were found in 10⁵g. of SP₂₀₀ must have come from at least $22.6 \times 10^5 / 4.9 = 4.61 \times 10^5$ g. of LP₁₀₀. There seems to be a 4.61:1.24, or 3.7:1, ratio in the requirements for the parent protein to give equal

amounts of SP₂₀₀ and LP₂₀₀. Since both are presumed to come from the same molecule of LP₁₀₀, in any mixture of these particular derivatives 78.7% of the protein will exist as LP₂₀₀ and 21.3% as SP₂₀₀. If we assume that none of the cystine residues of LP₁₀₀ was removed as free amino acid or in other small fragments during this period, 10⁵g. of a mixture of LP₂₀₀ and SP₂₀₀ will have $22.6 \times 0.213 = 4.8$ cystine residues from its content of SP₂₀₀ and $4.4 \times 0.787 = 3.5$ cystine residues from LP₂₀₀. In order to get 8.3 cystine residues, $8.3 \times 10^5 / 4.9 = 1.69 \times 10^5$ g. of LP₁₀₀ ought to be degraded. By analogy, 1 mole of LP₁₀₀ (294 000g.) should give 174 000g. of mixture, of which 21.3% (about 37 000g.) should be SP₂₀₀ and 78.7% (about 137 000g.) LP₂₀₀. These values are almost identical with those calculated previously from the yields and distributions of the proteins in the 25–50%-saturated ammonium sulphate fraction, and represent maximal molecular weights. The calculated amounts of protein in each derivative may exist as one fragment or as many smaller but identical fragments. The molecular weight in the latter instance should be a fraction of the calculated values.

All the derivatives studied here, even the fragments that were isolated at 200% CP, are intermediate, not final, products. We have shown that both the quantity and the quality of the large-peak fraction obtained from spontaneously lysed human fibrinogen changes continuously during 110–400% CP (Triantaphyllopoulos & Triantaphyllopoulos, 1965*a*, 1966*b*), but the small-peak component remains more or less constant during this period. Nüssenzweig *et al.* (1961*a*) have determined the molecular weights of the final products by sedimentation and diffusion studies. They have found that the molecular weight of constituent D, corresponding to an LP isolated during very advanced stages of fibrinogenolysis, was about 83 000, and that of constituent E, analogous to an SP of the same period, was approx. 35 000. The values calculated in the present paper for the intermediate derivatives are in a range compatible with these values.

It is extremely difficult to apprehend the transformations that the fibrinogen molecule underwent during fibrinogenolysis from Table 1. Since removal of some amino acids increased the concentrations of others, the data represent the combined result of the changes that each amino acid suffered in relation to the changes of all others. Presentation of the data on a molecular basis is the only way to show which and how many residues were removed during the formation of each derivative (Table 5).

The composition of the fragment described by Mihalyi & Godfrey (1963) seems somewhat similar to the composition of LP₂₀₀. This fragment was

Table 5. Molar amino acid composition of human fibrinogen and its main anticoagulant derivatives

The weight of the protein moiety of human fibrinogen was calculated from the molecular weight of fibrinogen (340 000) reported by Caspary & Kekwick (1957) by subtracting 5% for its carbohydrate content (Blombäck, 1958). Maximum molecular weights for the derivatives were computed from the minimum amount of fibrinogen needed to fulfil all the amino acid requirements of LP₁₀₀, and the amount of LP₁₀₀ needed for the requirements of LP₂₀₀ and SP₂₀₀; see the text for these calculations.

Amino acid	Amino acid composition (moles/mole of protein moiety)			
	Human fibrinogen (mol.wt. 323 000)	LP ₁₀₀ (mol.wt. 294 000)	LP ₂₀₀ (mol.wt. 137 000)	SP ₂₀₀ (mol.wt. 37 000)
Lys	197	178	87	21
His	59	51	23	6
NH ₃ *	314	292	142	48
Arg	146	127	64	13
Trp	78	74	38	6
Asp	346	321	149	51
Thr	192	175	66	15
Ser	251	230	89	23
Glu	339	305	152	44
Pro	150	123	49	12
Gly	281	266	113	18
Ala	142	123	52	15
Cys	20	14	6	8
Val	134	121	61	18
Met	64	60	30	3
Ile	105	105	60	11
Leu	177	158	77	29
Tyr	101	96	51	11
Phe	93	83	31	11
Total no. of residues	2875	2610	1198	315

* Ammonia values were not included in the totals.

isolated after complete fragmentation of bovine fibrinogen by trypsin and was a final product.

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