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DIGESTION OF FIBRINOGEN BY TRYPSIN

II. CHARACTERIZATION OF THE LARGE FRAGMENT OBTAINED

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SUMMARY

The large fragment of the fibrinogen molecule produced by short tryptic digestion has been isolated in a homogeneous state. The physico-chemical parameters of the fragment were determined: $s = 5.22$, $D = 5.12 \cdot 10^{-7}$, $\bar{v} = 0.735$. From these values and from the results of approach to sedimentation-equilibrium studies an average molecular weight of 95 000 was calculated. The complete amino acid and hexose content of the fragment was also determined. The relationship of the fragments to the parent fibrinogen molecule are discussed.

INTRODUCTION

In the preceding paper[1] the fragmentation of fibrinogen by trypsin has been reported. The fragments were subsequently isolated in a reasonably homogeneous state and their molecular parameters determined with the usual physico-chemical techniques. The details of these investigations are described in the present paper, and the relationship of the fragments to the parent fibrinogen molecule are discussed.

MATERIALS AND METHODS

The materials used in these studies, fibrinogen, trypsin and soybean trypsin inhibitor, were the same as those described in detail in the previous communication[1].

Isolation of the large fragment

Digestion procedure: Guided by the kinetic studies already reported, digestion conditions were selected which assured nearly complete fragmentation of the fibrinogen molecules, without significant secondary degradation also occurring. 20 ml of a fibrinogen solution, approx. 25 mg/ml in 0.3 *M* KCl, was mixed with 2.7 ml of 1 *M* potassium phosphate buffer (3 volumes primary to 7 volumes secondary phosphate) of pH 7.12 and placed in a water bath at 24.5°. 1 ml of a trypsin solution, 3.4 mg/ml, was added and the digestion allowed to proceed for 19 min, at which time it was stopped by the addition of 2 ml of a soybean trypsin-inhibitor solution (3.4 mg/ml). The digestion mixture was then cooled in an ice-bath. The fibrinogen-

to-trypsin ratio in the mixture was 155 : 1. Concentrations of both fibrinogen and trypsin solutions were estimated by absorbancy measurements as described in the first paper.

Ammonium sulfate fractionation: Increasing amounts of $(NH_4)_2SO_4$ solution were added to the above digest, the mixtures allowed to stand for 20 min at 0° and then centrifuged. The absorbancies of the supernatants were measured at 280 mμ in the Beckman DU spectrophotometer. Fig. 1 shows the precipitation curve obtained in this way, and, for comparison, the curve of native fibrinogen is also included. The

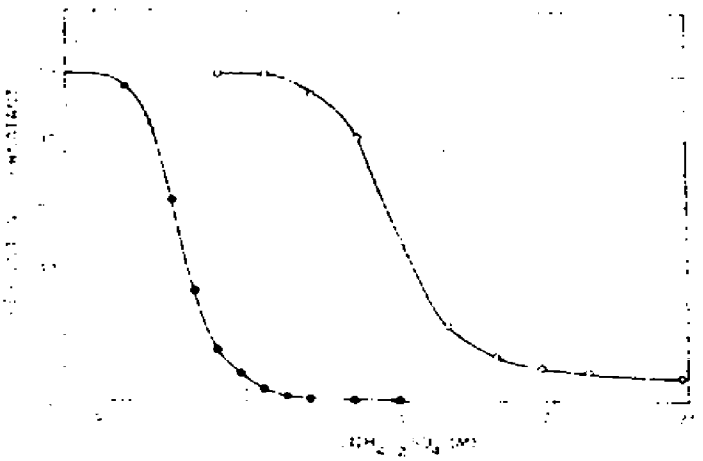

Fig. 1. Ammonium sulfate precipitation curve of native fibrinogen (● ●) and of the digestion mixture (○ ○): Ammonium sulfate concentration given in moles/l.

pH of the digest supernantants was approx. 6.3 and of the native fibrinogen supernatants, 5.5. It is apparent that the digestion shifted the precipitation curve by about 0.74 *M* toward higher $(NH_4)_2SO_4$ concentrations. Some 7.5% of the digest did not precipitate, even at the highest $(NH_4)_2SO_4$ concentration used. On the basis of this curve it was decided to isolate the fraction precipitating between 1.18 *M* and 1.61 *M* $(NH_4)_2SO_4$ concentration: 13.3 ml of 3 *M* $(NH_4)_2SO_4$ solution was added in the cold to 20 ml of digestion mixture; the precipitate was centrifuged and discarded. An additional 10.7 ml of 3 *M* $(NH_4)_2SO_4$ solution was added to the supernatant solution; the precipitate was separated by centrifugation, dissolved in 0.3 *M* KCl solution and dialyzed exhaustively against the same solvent in the cold. In a typical experiment 14% of the total protein remained in the first precipitate, 59% in the second precipitate, and 27% in the last supernatant fraction.

The fraction thus isolated proved to be homogeneous in the ultracentrifuge and no further purification was considered necessary.

Physicochemical techniques

Ultracentrifugation: Sedimentation runs were performed in the Spinco, Model E Ultracentrifuge, equipped with a Schlieren optical system, at room temperature

(22–24°), maintained constant in each run with the Rotary Temperature Indicator and Control unit of this instrument. In sedimentation velocity experiments the samples were centrifuged at 59 780 rev./min in a cell with a standard 4° Kel-F centerpiece. The plates were measured in a Bausch and Lomb Bench Microcomparator, Type 33-12-11.

In runs performed to calculate diffusion coefficients from boundary spreading during sedimentation, samples were spun at 42 040 rev./min in a cell with an aluminum filled-Epon double sector centerpiece. The cell was filled with an Agla, Micrometer Syringe mounted in a Radiometer Syringe Burette, Type SBU1a. Accurate filling of the two cell sectors to the same height was thus ensured. One sector was filled with the protein solution, the other with the solvent against which the protein had been dialyzed. The areas under the Schlieren curves were measured by tracing, cutting out and weighing the patterns projected on a thin, uniform cardboard (Strathmore, single ply). An Omega, Type 2 D photographic enlarger, free of distortions, was used for this purpose. The heights of the curves were measured with an accurate ruler on the same enlargements before they were cut out. The areas were then corrected for radial dilution by the relationship:

$$A_0 = A_t \left(\frac{x_t}{x_0}\right)^6 \qquad (1)$$

where A_t is the area of the boundary at time t, situated at a distance x_t from the center of rotation, A_0 is the area corresponding to the initial concentration and x_0 is the distance of the meniscus from the center of rotation. These distances were obtained by microcomparator measurements on the plates. The A_0 values showed a standard deviation of $\pm$ 2.8% and no detectable trend. To eliminate the effect of area fluctuations in the subsequent calculations, the mean of the A_0 values was used to recalculate the A_t values (by rearrangement of Eqn. 1) and these values were then employed in calculating the height-area ratios, as recommended by BALDWIN[2]. The height-area ratios were then used in a manner prescribed by FUJITA for evaluating diffusion coefficients from sedimentation patterns of systems in which the sedimentation rates are concentration dependent[3].

In approach to sedimentation equilibrium runs the technique of EHRENBERG was followed[4]: For the equilibrium run the centrifuge was operated at 8766 rev./min, at which speed partial separation of the boundary from the meniscus occurred; the synthetic boundary run duplicated exactly the equilibrium run in acceleration, speed and exposure intervals. The areas and the height of the refractive index gradient at the meniscus were measured on projected enlargements as described in the previous paragraph. Molecular weights were then calculated from these data with the equation given by EHRENBERG[4]. This procedure might be slightly less accurate than the one employing the microcomparator, but its simplicity and rapidity more than compensate for the sacrifice in accuracy. The reproducibility of the molecular weight determinations was excellent; however, the molecular weights tended to be about 5% higher than the accepted values when RNAase and bovine serum albumin were run.

The viscosity and density of the solvent at each temperature used in the ultracentrifuge runs were determined with a 50-ml pycnometer and an Ubbelohde

type viscosimeter with an outflow time of 428.3 sec with water at 20°. With these data the sedimentation coefficients were converted to standard conditions of water at 20°.

Free diffusion: The diffusion experiments were performed in the Spinco, Model H electrophoresis apparatus at 20° in the standard 11-ml cell, following largely the technique suggested by SCHACHMAN[5]. The Rayleigh interferometric optical system was used for recording the boundary spreading, and the plates were read on the Bausch and Lomb Bench Microcomparator. Two methods were used in calculating the diffusion coefficients; first, the one give by LONGSWORTH[6], based on the assumption of a Gaussian concentration distribution, and second, SVENSSON's[7] adaptation of the height–area method to interference patterns.

Partial specific volume determinations: A solution of the fragment, approx. 20 mg/ml, was dialyzed against 0.3 *M* KCl. The protein concentration in the solution was obtained by drying samples of both the protein solution and solvent to constant weight in a vacuum oven at 105° over P_2O_5. The density of the solution and solvent were determined in a 25-ml pycnometer at 25°, and the partial specific volume calculated with the densities and concentration, using the standard formula.

The absorbancy of the protein solution at several dilutions was also measured in the Beckman DU spectrophotometer. To correct for turbidity the absorbancy at 320 mμ was subtracted from that at 280 mμ (a correction that amounted to less than 1%). The corrected optical density of a 1% solution at 280 mμ was 1.57. Subsequently all the protein concentrations were determined by absorbancy measurements using the above figure as a conversion factor.

Viscosity measurements: An Ubbelohde type suspended level viscosimeter (Cannon 50 K, Cannon Instrument Co., Pa.) was used at 24.5 ± 0.01°. Viscosities were calculated relative to the solvent, 0.3 *M* KCl, without density correction. The kinetic energy correction was negligibly small for the viscosimeter used.

Optical rotatory dispersion determinations were made at 25° with the equipment described in the first paper of this series. Ultraviolet absorption spectra were recorded with the Cary, Model 14 M, recording spectrophotometer. From the alkaline spectrum of the protein the tyrosine and tryptophane content was calculated with the formulae given by GOODWIN AND MORTON[8].

Chemical techniques

Hexose determination with the orcinol method: The orcinol method was used essentially as described by HEWITT[9]. Since fibrinogen contains galactose and mannose[10,11] standard curves were run with solutions of these two hexoses, singly and in mixtures of varying proportions. It was found that the sum of the absorbancies at 475 mμ and 516 mμ is independent of the proportion of the two sugar components, but that their ratio is a linear function of this proportion. Therefore, the orcinol-treated fragment solutions were read at these two wavelengths and the total hexose content and ratio of galactose to mannose calculated from the absorbancies.

Amino acid analysis: Approx. 100 mg of protein in 10 ml of 6 *N* HCl was sealed in a vial with very little air and maintained at 116° for 18 h. The resulting hydrolysate was filtered and freed of HCl by repeated evaporations in vacuum. Aliquots were

used for total nitrogen estimations and for amino acid analysis in the Spinco, Model MS, automatic amino acid analyzer following the procedure of SPACKMAN, STEIN AND MOORE[12]. The amount of protein analyzed was calculated from the nitrogen content of the hydrolysate; it was assumed that the fragment has a nitrogen content equal to that of fibrinogen (16.77%).

RESULTS

Molecular weight of the fragments

In sedimentation velocity experiments the fibrinogen fragment sedimented as a single symmetrical boundary. Fig. 2 shows a typical sedimentation run in a double sector cell. The superimposed base line facilitates detection of small amounts of material sedimenting outside of the main boundary. No evidence of the presence of

Fig. 2. Sedimentation of the isolated fragment in the ultracentrifuge at 42 040 rev./min in a double sector cell with superimposed base line.

either lighter or heavier material than the main component was found. The sedimentation coefficients showed a slight dependence on initial protein concentration. Fig. 3 shows this dependence (obtained with two different preparations of the fragment) at 0.3 ionic strength, pH 6.10, and with the initial concentration varied between 0.177 and 1.77%. The points fall reasonably close to a straight line. Least squares analysis of the data resulted in the following equation for the best fitting line:

$$s_{20,w} = 5.22 - 0.227\,C_0 \qquad (2)$$

where C_0 is the initial protein concentration in gram/100 ml and 5.22 is the extrapolated value of the sedimentation coefficient at zero concentration.

The free-diffusion experiments were conducted with samples from two different preparations: one, a fresh preparation of 0.22% protein concentration at pH 5.1 and 0.3 ionic strength, and the other, a preparation which had been stored for 6 months at −15°, of 0.27% protein concentration at pH 6.6 and 0.3 ionic strength. The latter showed a slight degree of heterogeneity, with the extremes of the boundary giving slightly higher diffusion coefficients. Apparently some aggregation occurred during the long storage, which probably was the cause also of the slightly lower calculated diffusion coefficient. The concentration dependence of the diffusion coefficient was not investigated because it was felt that the concentrations employed were low enough for the determined diffusion coefficient to approximate the zero concentration value. This assumption is supported by the very slight difference, of the order of 1%, between the sedimentation coefficients at the concentration used in the diffusion

runs and at zero concentration; furthermore, the concentration dependence of diffusion is usually less than that of sedimentation rate because backflow and density effects are absent. Therefore, in our calculations the mean of the two determinations, $5.12 \cdot 10^{-7}$ was used as an approximation of the zero concentration value.

The partial specific volume, determined at 25° in 0.3 *M* KCl, was 0.735 ml/g. With $s_{20,w} = 5.22$, $D = 5.12 \cdot 10^{-7}$ and $\bar{v} = 0.735$, the Svedberg equation yields a molecular weight of 93 700.

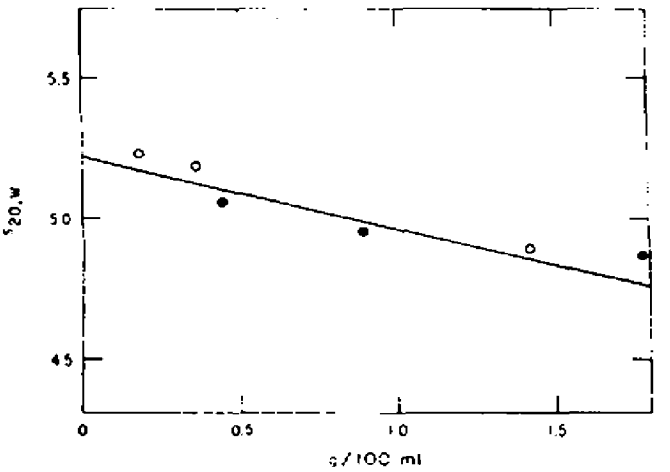

Fig. 3. Concentration dependence of the sedimentation coefficient. The symbols refer to two different preparations.

The approach to sedimentation equilibrium experiments were performed at three different protein concentrations, 0.68, 1.37 and 2.05%, each at 0.3 ionic strength and pH 6.5. Several frames were analyzed in each run for molecular weight values with results agreeing to within ± 1000. The calculated molecular weights at the three concentrations were: 103 500, 103 700 and 96 900. These data were considered as preliminary evidence of the absence of concentration dependence of the apparent molecular weight. The mean of the three determinations, 101 300, is in fair agreement with the molecular weight calculated from sedimentation and diffusion data. It may be recalled that the EHRENBERG procedure gave in our hands molecular weights of approx. 5% in excess of the accepted values of standard substances. Taking this error into account, an average of the molecular weights obtained by the two methods yields a value of 95 000.

Molecular shape of the fragments

The molecular asymmetry of the particles can be evaluated in three different ways from our data: (a) from the intrinsic viscosity with SIMHA's relation*,[13] (b) from

* As is seen in Fig. 4, the reduced viscosity plotted against concentration fits a straight line, whose intercept, the intrinsic viscosity, calculated with the method of least squares is 0.0718. The SIMHA equation requires that this value be converted into the viscosity increment, ν, *i.e.*, it must be multiplied by $100/\bar{v}$, where $\bar{v}$ is the partial specific volume of the protein.

the frictional ratio using PERRIN's equation[14], and (c) from SCHERAGA AND MANDELKERN's β-function[15]. These equations were derived on the assumption of a rigid, impermeable ellipsoid of revolution as the molecular model. Therefore, they give results consistent with each other only when applied to a molecule approximating this shape. Furthermore, when the anhydrous partial specific volume is used, the first two equations give the axial ratio of an ellipsoid of revolution with the same hydrodynamic properties as the particle but an assumed effective volume equal to that of the anhydrous material. The β-value, on the other hand, gives the axial ratio of an equivalent ellipsoid with the actual hydrated effective volume. Thus, in the absence of hydration and deviation from ellipsoidal shape, all three methods yield identical axial ratios, whereas with hydrated particles the SCHERAGA AND MANDELKERN analysis yields a much lower axial ratio than the first two methods. Therefore, comparison of the axial ratios obtained by the three equations may give information on the hydration and shape of the particles.

Table I gives the three hydrodynamic parameters and the axial ratios of prolate ellipsoids corresponding to them, interpolated from the tabulated functions of

TABLE I

HYDRODYNAMIC PARAMETERS AND AXIAL RATIOS OF THE FRAGMENT AND NATIVE FIBRINOGEN

	Hydrodynamic parameters		*Derived axial ratios*	
	Fragment	*Fibrinogen*[13,11]	*Fragment*	*Fibrinogen*[13,15]
Viscosity increment (ν)	9.76	35.2	7.8	18.9
Frictional ratio (f/f_0)	1.38	2.34	7.1	29.5
β-function, β	$2.39 \cdot 10^6$	$2.23 \cdot 10^6$	9.3	5

SIMHA, PERRIN, and SCHERAGA AND MANDELKERN[16]. It must be remembered that all three of these functions are fairly insensitive at the low dissymmetry end. Therefore, no significance can be attributed to the slight variations in axial ratios seen in Table I. It can be assumed fairly safely, however, that the particles conform to an ellipsoid of revolution, with a very low degree of hydration and a moderate dissymmetry. In fact, the β-function gives an effective volume smaller than the anhydrous volume, but this anomaly is, no doubt, a result of the inaccuracy of the experimental data. Thus, when a molecular weight of 101 000 is assumed, as given by the EHRENBERG determinations, β is $2.27 \cdot 10^6$ and the axial ratio, 5.85.

Even accepting large experimental errors, it is clear from the data that the fragments and native fibrinogen do not differ markedly in asymmetry in the hydrated state, but the effective hydrodynamic volume of the fragments is much lower than that of fibrinogen.

Homogeneity of the fragments

The preparation proved homogeneous by several criteria: (a) It showed a symmetrical, single peak in the sedimentation velocity experiments and the area of

the peak, when corrected for radial dilution, remained reasonably constant over the whole sedimentation process. (b) The analysis of the free diffusion boundary, assuming Gaussian distribution, yielded diffusion coefficients across the whole boundary which agreed to within 2% (Table II). This amply demonstrates the conformation of the boundary to an ideal Gaussian distribution curve typical of homogeneous substances. (c) Perhaps the best criterion of homogeneity is the identity of the diffusion coefficient calculated from boundary spreading during sedimentation experi-

TABLE II

ANALYSIS OF THE DIFFUSION PATTERN AT TWO DIFFERENT TIMES

Total fringe number: 33.73; temperature, 20°; ionic strength, 0.3 (pH 5.10); protein concentration 0.22%.

Diffusion time (sec.)	Fringe pair	Fringe separation (cm)	Diffusion coefficient	
			D apparent × 10⁷	Mean D apparent × 10⁷
45 420	2–18	0.3646	5.40	5.30 ± 0.076
	4–20	0.3449	5.43	
	6–22	0.2876	5.26	
	8–24	0.2785	5.26	
	10–26	0.2779	5.22	
	12–28	0.2870	5.17	
	14–30	0.3171	5.34	
	16–32	0.3743	5.35	
165 300	2–18	0.6802	5.17	5.28 ± 0.062
	4–20	0.5895	5.22	
	6–22	0.5469	5.23	
	8–24	0.5302	5.24	
	10–26	0.5341	5.29	
	12–28	0.5586	5.37	
	14–30	0.6127	5.49	
	16–32	0.7070	5.25	

ments with the one calculated from free diffusion. Heterogeneity will cause much faster boundary spreading during sedimentation than during free diffusion alone and consequently a discrepancy between the two calculated diffusion coefficients. Unfortunately, the self-sharpening of the boundary in the sedimentation of substances with concentration dependent sedimentation rates works to counteract this spreading effect and can completely mask it. But calculation of the true diffusion coefficient from a self-sharpening sedimenting boundary became possible after corrective equations were worked out by FUJITA[3].

Our data, with the equation given by LAMM[17], which corrects only for the inhomogeneity of the centrifugal field, yielded an apparent diffusion coefficient of $4.19 \cdot 10^{-7}$, a value much lower than the true one, obviously, because of the pronounced sharpening effect. Applying the simplified calculations of FUJITA[18] resulted in the plot shown in Fig. 5. A corrected function $\frac{1}{t}$ is plotted against a function

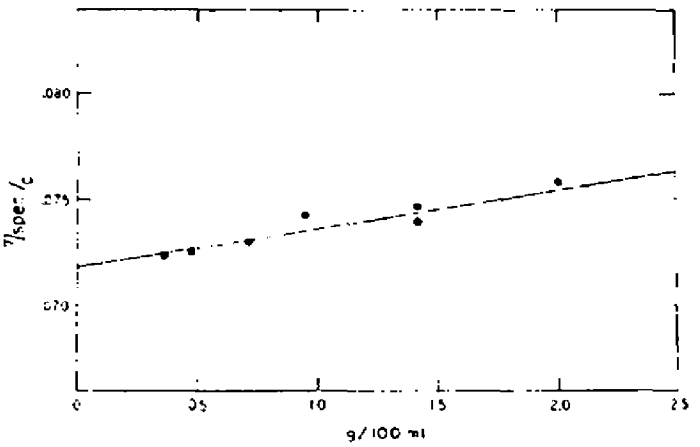

Fig. 4. Reduced viscosity of the fragment plotted against concentration.

of the height–area ratio obtained graphically by the use of a table published in FUJITA's paper. The plot shows the straight line relationship as required of a homogeneous substance, and the line passes through the origin as demanded by the theory. The diffusion coefficient calculated from the slope was $5.49 \cdot 10^{-7}$, which is slightly higher than the free diffusion value. However, a very slight adjustment of the coefficient

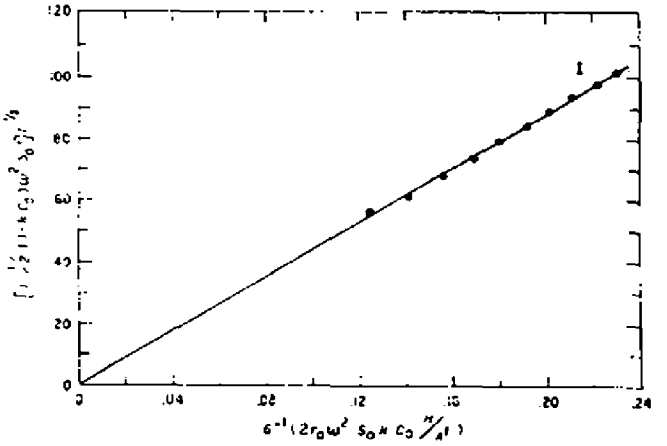

Fig. 5. FUJITA-plot of the boundary spreading in the sedimentation velocity experiment.

of the concentration dependence would bring the two figures to coincidence. Our sedimentation data are not numerous enough to discount such an adjustment.

Optical rotatory properties

The optical rotatory power of the fragment was measured in the wave-length

range of 325 to 750 mμ, in 0.3 *M* KCl in 0.15 *M* potassium phosphate buffer (pH 6.5), at 25°. As shown by the YANG AND DOTY plot[19] of Fig. 6, the data conformed closely to a one term Drude-equation, except at the longer wave-length end. The slope of the line gave a λ_c of 257 mμ. The specific rotation of the fragment at the D line was −34.0°. For comparison the optical rotatory properties of native fibrinogen were

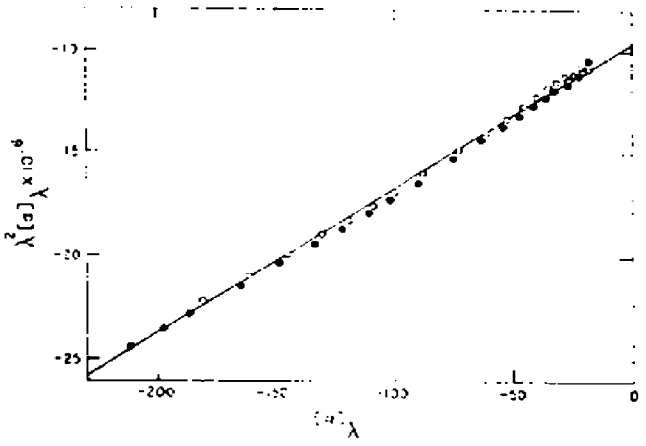

Fig. 6. YANG AND DOTY-plot of the optical rotatory dispersion of the fragment. The symbols refer to two different preparations.

also determined: λ_c was 260 mμ and $[\alpha]_D$, −48.2°. The fragmentation did not change λ_c to any appreciable extent but decreased $[\alpha]_D$. During the digestion (as reported in the first paper), the optical rotation changed in the same direction but to a much lesser extent; the kinetic measurements were performed at 370 mμ and showed a change of 6.3° at this wave-length and only 0.8° at 589 mμ. It should be remembered, however, that in the kinetic experiments the low molecular weight split products were also present and obviously compensated to a great extent for the lower rotatory power of the fragment. The data on the change of $[\alpha]_D$ support the idea that the enzymic process removes material from the more randomly disposed parts of the molecule[30], leaving intact a fragment with a higher helical content and a lower specific rotation. However, the behavior of λ_c is not consistent with this interpretation, since, instead of being shifted to a longer wave-length, it remains practically constant.

Chemical composition of the fragment

The amino acid composition of an 18-h hydrolysate is given in Table III. For comparison, the table also includes the data obtained on a similar hydrolysate of native fibrinogen. To facilitate their comparison, both sets of data are calculated as numbers of residues per molecule with an arbitrary molecular weight of 100 000. The calculations were based on total nitrogen estimations, and, as an approximation,

it was assumed that both materials have the same nitrogen content. This assumption is obviously not true, especially in view of the significant differences in the amounts of basic amino acids contained in the two proteins, but the error introduced in this way was estimated to be only about 2%. The third column in the table gives the differences between the amino acid contents of fibrinogen and the fragment. When the relative proportion of an amino acid is higher in the small fragments liberated by the enzyme than in the whole molecule, then the difference is shown as positive, and if it is lower, then the difference is shown as negative.

TABLE III

AMINO ACID COMPOSITION OF NATIVE FIBRINOGEN AND OF THE FIBRINOGEN FRAGMENT

The amino acid compositions are expressed as moles/10^6 g. For detailed explanation of headings see text.

	Fibrinogen	*Fragment*	*Difference*	*Residues removed*
Lysine	64	64	0	10
Histidine	17	13	− 4	6
Ammonia	110	114	+ 4	13
Arginine	46	42	− 4	10
Aspartic acid	103	120	+ 17	1
Threonine	56	53	− 3	11
Serine	59	59	0	9
Glutamic acid	95	102	+ 7	8
Proline	45	38	− 7	13
Glycine	83	76	− 7	18
Alanine	36	35	− 1	6
Cystine/2	21	23	+ 2	2
Valine	42	40	− 2	8
Methionine	16	19	+ 3	0
Isoleucine	36	45	+ 9	− 2
Leucine	51	56	+ 5	3
Phenylalanine	26	26	0	4
Tyrosine	29	39	+ 10	− 4
Tyrosine	30*	36*	+ 6	0
Tryptophan	20*	20*	0	3

* From ultraviolet absorption data.

It is possible to calculate the actual number of residues lost, if one knows the fraction of the molecule appearing as non-protein nitrogen. There are two ways of calculating this loss of non-protein nitrogen: (a) by comparison of the molecular weights of the fragment and of parent fibrinogen, which gives 16.2% non-protein nitrogen, (b) by analysis of the digestion kinetics, as reported in the first paper, which gives 14.2%. It can then be stated that for 100 000 g of fibrinogen there corresponds approx. 85 000 g of fragment. When the amino acid figures for the fragment are multiplied by 0.85 and subtracted from the fibrinogen figures, the difference, given in the fourth column of the table, corresponds to the actual number of residues removed from 100 000 g of fibrinogen during fragmentation.

Most of the data presented in Table III are self-explanatory, nevertheless, a discussion of the errors involved is necessary in order to establish the significance of the figures presented. The analysis of the fragment was performed in triplicate, with the results for individual amino acids not differing by more than two residues in 100 000 g of protein. In percentage, the largest variation of an individual amino acid was 3.2% and the average standard deviation was ± 1.7%. These data were compared with the results of a single analysis of an 18-h hydrolysate of native fibrinogen, however, this analysis was one of a series of four of hydrolysates of increasing hydrolysis time. In this series, those amino acids which did not show hydrolytic destruction agreed again to within two residues per 100 000 g of protein[24]. Consequently, a difference larger than 4 in the last column of the Table may be certainly considered as significant.

It appears that some amino acids, like tyrosine, methionine, aspartic acid, half cysteine, were not removed at all with the proteolytic debris. They must, therefore, be situated well within the molecule, inaccessible to trypsin during the early phases of digestion. Comparing the number of arginine and lysine residues removed with the number of peptide bonds split during the fragmentation (24 bonds per 100 000 g) reveals that a large fraction of the splits resulted in liberation of a basic amino acid. It is reasonable to assume, therefore, that these splits occurred close to the N-terminal ends of the chains. Had the splitting occurred at the other end of the polypeptide chain, the basic amino acid residue would have stayed with the large fragment, and of course the same would have been true of an internal split which did not result in the liberation of peptide material.

Table III also contains the tyrosine and tryptophan contents calculated from alkaline ultraviolet absorption spectra. There is a considerable difference between the chemical and spectroscopic determinations of tyrosine, the latter, probably, being the more accurate. Acid hydrolysis is notoriously destructive of aromatic amino acids and the large negative value found by this method for the number of tyrosine residues removed attests to this fact.

The hexose content of the fragment was found to be 1.36% as compared with 1.33% for native fibrinogen* and the galactose to mannose ratio (2 : 1) did not change. Thus, the hexose loss was proportional to the loss of proteolytic debris.

DISCUSSION

The fragment was isolated in high yields. There was no indication of fractionation during the isolation, or for that matter, no indication of more than one large species in the digestion mixture itself. Therefore, the conclusion seems justified that the digestion of fibrinogen by trypsin under the conditions used produces large fragments of only one species, at least as far as they can be defined by our observation methods.

The relationship of this fragment to the parent fibrinogen molecule can be reasonably established. A comparison of the molecular weights of the fragments and fibrinogen, 95 000 and 340 000, at once suggests that three fragments were

* Our hexose value for fibrinogen is considerably lower than that reported by BLOMBÄCK[21] and is closer to that of BAGDY AND SZÁRA[11].

produced from every fibrinogen molecule*. The difference between the weight of three fragments and of one molecule of fibrinogen of 340 000 molecular weight is 16.2% of the fibrinogen weight, and this amount corresponds well to the 14.2% of non-protein nitrogen produced during digestion and determined by trichloroacetic acid precipitation.

The electronmicrographs of fibrinogen, produced by several authors[25-27] invariably show a nodous structure, like a string of beads of unequal length. More recently HALL[28] proposed a more definite molecular model of three interconnected beads of slightly unequal size. Thus, the effect of trypsin can be easily envisaged as cutting these beads loose from one another and liberating, at the same time, the connecting pediculae as low molecular weight proteolytic debris. However, several discrepancies appear when one takes into account the size and shape of the fragments. First, HALL's beads are unequal in size, whereas we found only one fragment species. But this is not a very serious objection because the beads may actually have equal masses and hydrodynamic properties in solution and be of unequal sizes when in the dry state. The results of our investigations into the shape of the fragment, however, are more disturbing. The hydrodynamic shape of the fragment is an ellipsoid of revolution with an axial ratio of approx. 7. The beads in the electronmicroscope, on the other hand, appear to be spherical. This difference is probably too large to be explained by drying effects alone.

A comparison of the asymmetry of the fragments with that of native fibrinogen similarly leads to discrepancies. The axial ratio of the equivalent ellipsoid, calculated from the β-values, is 5 for fibrinogen[27] and 8 for the fragment. Thus paradoxically, the fragments are more asymmetrical than the parent molecule. It is possible that the digestion produces loose polypeptide chains attached to the compact fragment and these increase the frictional resistance of the fragments, but are too thin to be visible in the electronmicroscope. Clearly, before these questions can be resolved, the shape of both fibrinogen and its fragments must be more reliably established.

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* Naturally, this estimate depends to a large extent on the molecular weight assumed for fibrinogen. For the purposes of this discussion we have accepted the value of SHULMAN[25], 340 000, although recently SOWINSKI, OHARENKO AND KOENIG[29] reported a much lower value of 250 000. With the latter figure, one has to assume two fragments per molecule and 24% of low molecular weight split products, a proportion of non-protein nitrogen much higher than was actually found.

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