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DIGESTION OF FIBRINOGEN BY TRYPSIN

I. KINETIC STUDIES OF THE REACTION

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SUMMARY

The digestion of fibrinogen by trypsin, as followed in the pH-stat, can be described in terms of three distinct reaction classes: (a) A fast reaction involving the splitting of 12 peptide bonds during which clottability is lost, (b) a slower reaction involving 80 peptide bonds, associated with the appearance of a slower sedimenting component in the ultracentrifuge and indicating the production of large fragments, and (c) a slow reaction which covers the remaining bonds susceptible to trypsin and which results in the fragmentation of the molecule into small peptides.

The digestion was also followed in the viscosimeter, ultracentrifuge and polarimeter, and the liberation of non-protein nitrogen measured. The disappearance of clotting ability associated with the first phases of the reaction was studied, and certain conclusions were drawn concerning the mechanism of this inactivation process.

INTRODUCTION

In investigations of the breakdown of proteins by endopeptidases, far more attention has been paid thus far to the isolation and characterization of low molecular weight peptides than to the early alterations during digestion of the protein molecule. The nature of such alterations, however, when monitored closely, often yield significant clues as to the structure and sometimes the mechanism of biological activity of the protein undergoing degradation[1,2].

The general pattern of protein degradation is determined by the relative rates of splitting of the peptide bonds in the various segments of the molecule[3]. These rates, in their turn, will be determined partly by the primary structure, but also to a large extent by the secondary and tertiary structure of the polypeptide chains. Generally, the native configuration of a protein molecule is much more resistant to proteolytic attack than the unfolded structure of its denatured form[4-8]. We may assume, however, that some proteins have parts that are loose, approximating the random structure of the denatured form, and parts which are tightly coiled in some specific configuration. An endopeptidase, such as trypsin, will split rapidly through

Abbreviation: TAME, *N*-*p*-toluenesulfonyl-L-arginine methyl ester.

the loose parts and if the secondary and tertiary structure of the tight segments remain stable, the enzyme might leave these fragments relatively intact[9-11].

Some proteins, like myosin[10], γ-globulin[13], and thyroglobulin[12] are split very quickly into large fragments by endopeptidases with the production of very little non-protein nitrogen and the fragments then are relatively resistant to further degradation. It appears that in such cases the protein molecule has narrow accessible regions through which the enzyme cuts like a knife, thus separating the large fragments. The mechanism of formation of the so-called "cores", on the other hand, is somewhat different[14-19]. Here the parent protein molecule possesses a large segment in which the peptide bonds are inaccessible to the proteolytic enzyme. This core is "surrounded" by a number of digestible segments which the enzyme removes as smaller or larger peptides. Therefore, the core appears late in digestion and is accompanied by a large amount of peptide material. On the basis of the above considerations it can be expected that the fast fragmentation will yield products which are more homogeneous than the "cores" and which retain most of their original secondary and tertiary structure. These expectations are borne out by the fact that such fragments often have the original or some modified form of the biological activity of the parent molecule*,[20-23].

The mechanism of protein fragmentation can be elucidated only by following closely the kinetics of peptide bond splitting (in the pH-stat), the formation of non-protein nitrogen, and the appearance of fragments (in the ultracentrifuge). Kinetic studies with other physiochemical (viscosity, optical rotation) or chemical methods (end-group analysis) may also yield valuable clues to the reconstruction of the molecular breakdown. These procedures can then be followed by the isolation and characterization of the fragments produced with respect to size, shape, and biological activity. With all these data at hand, a gross structural picture of the protein molecule can often be constructed.

Such a multilateral approach to the enzymic degradation of proteins has been applied to only a very few proteins. In the present paper we want to report the application of these principles to the digestion of fibrinogen by trypsin. It will be shown that trypsin splits fibrinogen very rapidly into fragments with concomitant production of only small amounts of non-protein nitrogen. The reaction has been followed by a number of techniques and the results will be discussed in terms of the structure of the fibrinogen molecule. A preliminary account of this work has been already published[24].

It may be mentioned here that several authors[25-27] have investigated the fragmentation of fibrinogen by plasmin; however, the process appears to be more complex than with trypsin and has not been studied as yet in sufficient detail.

MATERIALS

Bovine fibrinogen was purified from Armour's Bovine Fraction I, Lot T4105 according to LAKI's method[28]. The fibrinogen solutions were dialyzed extensively in

* Large fragments are also obtained by one-by-one removal of amino acids from the ends of the polypeptide chains by exopeptidases. Their mechanism of formation, however, is entirely different from those described above and discussion of them is omitted.

the cold against 0.3 *M* KCl adjusted to pH 7.0 with KOH and clarified by centrifugation for 30 min at 13 000 rev./min in the Servall SS 1 centrifuge. The purity of the preparations, in terms of percentage of protein material clottable by thrombin, determined by the method of LAKI[28], ranged from 92 to 95%. Protein concentrations were determined by absorbancy measurements in the Beckman DU spectrophotometer at 280 mμ. The specific absorption of fibrinogen at this wavelength, corrected for turbidity, is 1.51 per mg/ml. The intrinsic viscosity of our preparation was 0.24 dl/g a value in good agreement with those found in the literature[29]. Our sedimentation data were, however, somewhat different from those published by other workers[29-31]; therefore, a study of the concentration dependence of the sedimentation coefficient of fibrinogen was undertaken. Fig. 1 shows $s_{20,w}$ plotted against concentration in

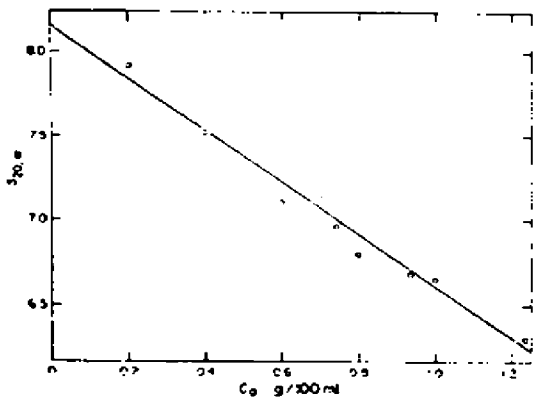

Fig. 1. Concentration dependence of $s_{20,w}$ of fibrinogen. Sedimentation at 59 780 rev./min, at 22°, in 0.26 *M* KCl, 0.133 *M* potassium phosphate buffer (pH 7.1). The equation of the line, calculated by the method of least squares is: $s_{20,w} = 8.14 - 1.53 \cdot C_0$.

0.26 *M* KCl, 0.133 *M* potassium phosphate buffer (pH 7.12). The concentration dependence was found to be more pronounced than any reported by other workers, and the 8.14 value of $s_{20,w}$, though in the range of values found in the literature, is slightly higher than the currently accepted value of 7.9 (see ref. 32).

The trypsin used was a crystallized, lyophilized preparation purchased from Worthington Biochemical Co., Freehold, N.J., Lot TL725. It was dissolved in 0.005 *N* HCl and its concentration estimated on the basis of its absorbancy at 280 mμ using a previously determined conversion factor: absorbancy of a 1.0 mg/ml solution at 280 mμ equal to 1.660 (see ref. 10). The concentration determined this way amounted to 74–75% of the concentration calculated on the basis of the weight of the moist lyophilized enzyme powder. The activity of the enzyme was determined in the pH-stat under the conditions recommended by EHRENPREIS AND SCHERAGA[33] (10 ml 0.01 *M* TAME in 0.15 *M* KCl (pH 8) at 25°), defining one unit equal to that amount which would hydrolyze 0.1 μmole of TAME/min. The activity of the enzyme remained unchanged for weeks at 4° in the 0.005 *N* HCl. 1 mg of enzyme (concentration determined by absorbancy) had an activity of 2450 units and the specific activity of the enzyme powder did not decrease over a period of one and one-half years of storage at −15°. In some experiments a trypsin preparation was used in

which the chymotryptic activity was destroyed preferentially by partial inhibition of the enzyme with diisopropylthiorophosphate (kindly supplied by Dr. W. J. DRYER).

Soybean inhibitor, crystallized from ethanol and obtained from Worthington Biochemical Co., was dissolved in distilled water by adjusting the pH to approx. 7 with dilute alkali. Thrombin was purified by RASMUSSEN's method[34] from Parke, Davis Thrombin, Topical. Its activity was determined on TAME under the same conditions used for trypsin and the activity expressed in the same way. The purified enzyme had an activity of 290 units/mg.

All the other reagents used were commercial preparations of reagent grade.

METHODS

pH-stat runs

A Titrator, Type TTT 1a, from Radiometer Corp., Copenhagen, was used in connection with a Titrigraph, Type SBR 2b, from the same company. In most of the determinations a "miniature pH electrode", Leeds and Northrup, cat. No. 124138, was used. The glass electrode was coated with Beckman Desicote 18772 as described in the Beckman Bulletin 262-C. The Titrigraph activated a Radiometer Syringe Burette, Type SBU 1a, equipped with an "Agla" Micrometer Syringe from Burroughs Wellcome and Co. (Great Britain), of 0.5 ml delivered volume. The reaction was carried out in a beaker surrounded by a jacket through which water of 24.5° was circulated. Stirring was achieved by a magnetic stirrer, and a vigorous current of nitrogen, washed through 40% NaOH and water, excluded CO_2 from the reaction vessel. The pH scale was standardized with standard buffers of 6.86 and 9.19 pH before and after each series of runs; no drift in the pH was ever observed.

A typical run was made as follows: 10 ml of fibrinogen solution in 0.3 *M* KCl (15.3 mg/ml) was pipetted into the reaction vessel. After temperature equilibration and with the stirrer and nitrogen stream on, the pH-stat was allowed to bring the pH of the solution to the desired value. In a few minutes a stable base line was obtained (at all pH values used); 0.5 ml of trypsin solution (4940 units/ml) was added with a blow-out pipette and the reaction allowed to proceed for 20 min. 0.166 *N* KOH solution was used as titrant, diluted from Fisher Certified Reagent, CO_2-free 1 *N* KOH.

Physicochemical and chemical studies of samples inhibited at various stages of the digestion

The general procedure for obtaining samples inhibited at various times during the digestion was the following: 40 ml of fibrinogen of 17.8 mg/ml concentration in 0.3 *M* KCl was mixed with 5 ml of 1 *M* potassium phosphate buffer (pH 7.12). The mixture was placed in a water bath at 24.5° and 3 ml of trypsin solution of 1.56 mg (3820 units)/ml concentration added. In the mixture the trypsin to fibrinogen weight ratio was 1 to 152. At various time intervals 5-ml samples were withdrawn and mixed with 1 ml of soybean trypsin inhibitor solution of 1 mg/ml concentration. For the zero time sample, a similar fibrinogen mixture, scaled down to 5 ml was prepared, but with the inhibitor added first to the fibrinogen before the trypsin. The

samples were kept at 4°, and no changes in their properties were observed over a period of one week. They were analyzed in the ultracentrifuge, viscosimeter, and polarimeter, and their non-protein nitrogen content was estimated. All measurements were performed within three days of sample preparation. For a limited purpose inhibited samples were also prepared by a somewhat different procedure described later.

Viscosity measurements

An Ostwald type viscosimeter was used with an outflow time of 95.1 sec at 24.5° with a solution of 0.208 *M* KCl in 0.086 *M* phosphate buffer (pH 7.1). Viscosities were calculated relative to the above solvent neglecting the slight density and kinematic corrections.

Sedimentation studies

Sedimentation runs were made in the Spinco Model E ultracentrifuge at 59780 rev./min, employing a standard 4° cell with a Kel-F centerpiece, and using the Schlieren optical system. Temperature was maintained constant during each run in the range 21° to 24° with the Rotary Temperature Indicator and Control Unit supplied by the manufacturer. The photographic plates were measured in a Bausch and Lomb Bench Comparator, Cat. No. 33-12-11. The sedimentation coefficients were converted to standard conditions of water at 20° with the use of the viscosities and densities of the solvent at the various temperatures of the runs.

The relative concentrations of the sedimenting components were determined from the areas under the Schlieren curves. These areas were measured by tracing the curves projected by an Omega Type D2 photographic enlarger (found to be free from distortions) on a thin cardboard, Strathmore paper, single ply, cutting them out with scissors and weighing them on the analytical balance. The weight per unit area of the cardboard and its constancy were determined by cutting out and weighing squares of measured surfaces. The weight was constant to better than 1%, and the reproducibility of the areas under the curves was also of this order of precision. Runs using only the solvent under every set of experimental conditions were made to delineate the base line under the areas. The area measurements were made on the patterns obtained after 80 min of centrifugation. The areas were corrected for radial dilution, but no attempt was made to correct for the Johnston–Ogston effect.

Optical rotation measurements

Rotation measurements were performed with the Rudolph Precision ultraviolet polarimeter, Model 80, equipped with the Rudolph photoelectric polarimeter attachment and an oscillating polarizer prism. The Rudolph, Model 610 xenon-arc lamp was used as a light source. Readings were made at 370 mμ with the samples contained in a 1-dm tube.

Non-protein nitrogen determinations

In preliminary experiments it was established that, with native fibrinogen, variation of the trichloroacetic acid concentration, incubation time, and temperature

had little effect upon the absorbancy of the supernatant solution, which was of the order of 0.2% of the total. On the other hand, variation of the above conditions had a pronounced effect on the amount of absorbing materials in the supernatants of tryptic digests of fibrinogen. Increasing the trichloroacetic acid concentration from 5 to 15% decreased the absorbancy of the supernatants by about 40%, and a further, but smaller, decrease was obtained by lowering the temperature to 0° and prolonging the incubation. Thus a clear-cut separation of non-protein nitrogen and protein nitrogen is not possible. Arbitrarily, for the non-protein nitrogen determinations, the following conditions were chosen: 15% final trichloroacetic acid concentration and 45 min incubation time at 0°. After incubation the samples were centrifuged in the Servall Model SS 1 centrifuge for 20 min and the absorbancies of the supernatants measured. Aliquots were then digested with H_2SO_4 and $CuSO_4$–$KHSO_4$ catalyst; the ammonia was liberated in Conway dishes and estimated with Nessler's reagent.

Reactivity toward thrombin

The clotting ability of fibrinogen at various stages of tryptic digestion was tested with thrombin. Because of the rapidity with which trypsin destroys clottability, the digestion was run with a much lower trypsin concentration than that described earlier. 4 ml of fibrinogen of 19.15 mg/ml concentration was mixed with 0.5 ml of 1 *M* potassium phosphate buffer (pH 7.1). To the above mixture, held at 25° and stirred by a magnetic stirrer, was added 0.5 ml of trypsin of 0.091 mg (223 units)/ml concentration, resulting in a trypsin to fibrinogen ratio of 1 : 1680. At a timed interval, as short as 10 sec for some runs, 1.0 ml of inhibitor solution of 1.0 mg/ml concentration was added to the mixture. The procedure was repeated with different digestion times to complete the sample series.

2-ml aliquots from each sample were pipetted into 50-ml Servall centrifuge tubes, each containing 8.0 ml of a solution of 0.3 *M* KCl and 0.025 *M* KH_2PO_4. The resulting mixture had a pH of approximately 6.5 which is well within the range in which fibrinogen forms a compressible clot. 0.1 ml of thrombin solution of 30 units/ml concentration was then added to each tube and the tubes were allowed to incubate overnight at 4°. The tubes were spun down for 20 min in the Servall SS 1 centrifuge in the cold and the supernatant solutions from which the clots separated well were read at 280 mμ in the Beckman DU spectrophotometer. The readings were corrected for the amount of non-clottable material originally present in the fibrinogen solution.

RESULTS

pH-stat studies

The digestion reaction was followed in the pH-stat at 0.5-pH intervals over the range of pH 7.0–9.5. The curves obtained were analyzed in the manner proposed by LÉONIS[35]. It was immediately apparent that the prolonged digestion of fibrinogen very likely involves all the bonds in the molecule theoretically susceptible to trypsin, divided into three, or possibly more distinct reaction rate classes. A kinetic analysis of exhaustive digestion did not seem feasible, for, obviously, complex kinetic curves of this type are susceptible to mathematical description in terms of various combi-

nations of simultaneous first order reactions, none of which may have any reflection in reality. However, the first 20–30 min of the base uptake can be analyzed in terms of only two distinct first order reactions since, as will be shown, the slower reactions have progressed insignificantly in this short time interval. These two reactions can be correlated, moreover, with certain well-defined physical alterations thus lending credence to the analysis as a true description of the digestive process. Because attention was focused upon these initial reactions, the digestion was followed in the pH-stat for only 20 min.

The reaction curves are shown in Fig. 2. Reproducibility of the curves was ex-

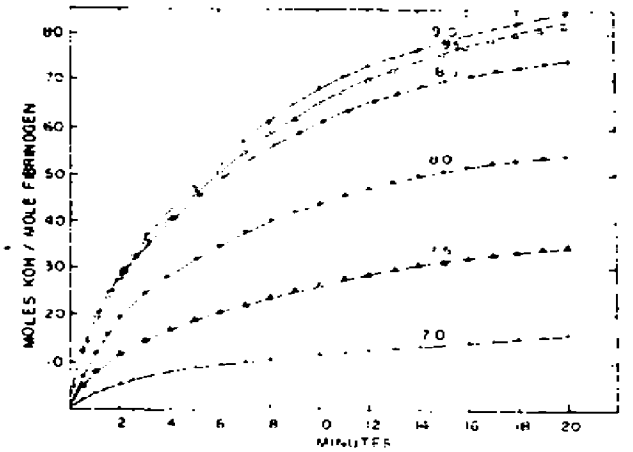

Fig. 2. Base uptake during the digestion of fibrinogen at various pH values. Curves shown were traced by the pH-stat, while the points were calculated on the basis of the assumed reaction mechanism, as explained in the text. The number on each curve indicates the pH of the experiment. In each experiment there were 153 mg of fibrinogen and 2470 units of trypsin in 10.5 ml volume.

cellent, of the order of 0.2–0.4 μmoles/150 mg of fibrinogen. The mathematical analysis can be outlined as follows: For a first order reaction:

$$\log dx/dt = \log A_0 k_e - k_{10} t \qquad (1)$$

Thus a plot of log rate *versus* time should give a straight line with the slope equal to $-k_{10}$ (rate constant calculated with common decimal logarithms) and intercept equal to log $A_0 k_e$, *i.e.*, the total number of bonds taking part in the reaction multiplied by the rate constant calculated with natural logarithms. Such a plot is shown in Fig. 3. The projected straight line represents the slow reaction and from it was calculated $A_{02} k_{e2}$. The deviation from the straight line in the initial phase corresponds to the fast reaction. To ascertain its extent the slow reaction was reconstructed from the calculated A_{02} and $k_{10.2}$ and then subtracted from the experimental curve. The resulting difference curve, corresponding to the fast reaction, levels off at a value corresponding to the extent of the fast reaction, A_{01}. The log of the unreacted bonds is plotted against time in the inset of Fig. 3. From the slope of the straight line thus obtained, the rate constant, $k_{10.1}$, of the fast reaction was calculated. With the

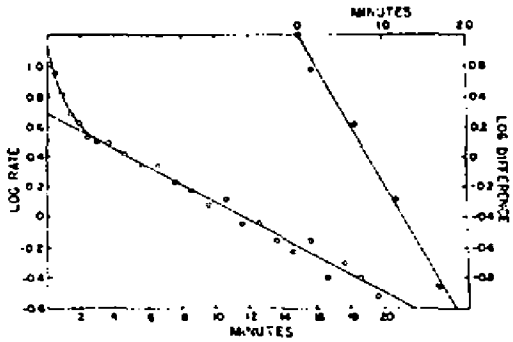

Fig. 3. Analysis of the pH-stat curve obtained at pH 8.5: Logarithm of the rate of base uptake plotted against time. Straight line corresponds to slow reaction; deviation from it is caused by fast reaction. Insert: plot of the fast reaction calculated from the difference of the slow and the overall reaction. For details see text.

constants, A_{01} and $k_{10.1}$, the fast reaction was reconstructed; then the sum of the two reactions was calculated and compared with the experimental curve. As shown in Fig. 2, the agreement is excellent. Save for a short middle section where some of the experimental curves are slightly flatter than the calculated ones, the calculated points fall on the experimental line.

The A_0's and k_{10}'s found for the two reactions at various pH's are given in Table I.

TABLE I

RATES AND BASE CONSUMPTIONS PER MOLE OF FIBRINOGEN IN THE SLOW AND FAST REACTIONS CALCULATED FROM THE pH-STAT CURVES

Rate constants are calculated with decimal logarithms and time is expressed in minutes. The base uptake is given as moles of base per 340 000 g of fibrinogen. Fibrinogen concentration was 14.92 mg/ml; fibrinogen to trypsin ratio, 152 : 1.

pH	Fast reaction		Slow reaction	
	k	Moles base	$k \times 10^2$	Moles base
7.0	0.38	4.5	2.6	37.3
7.5	0.57	6.8	4.3	32.9
8.0	0.51	10.2	5.1	51.3
8.5	0.97	12.2	5.9	69.0
9.0	0.89	12.2	5.5	81.1
9.5	0.86	12.5	4.9	80.5

The rate of the fast reaction is approximately ten times that of the slow, and the rates of both the fast and slow reactions are increased 2–2.5-fold when the pH is increased from 7.0 to 8.5; above 8.5 the rates decrease again. The magnitude of each A_0 depends upon the number of bonds split and their degree of titration. If the same set of peptide bonds were split regardless of pH, the base uptake should follow the titration curve of the amino groups liberated by the splitting. Conversely, it is an indication that the same set of bonds is split at each pH when the base uptake follows a titration curve. The maximum A_0 attained at pH higher than 9.0 for both reactions corres-

ponds to the number of bonds split. It is seen from Table I that there are 12 bonds split in the fast and 80 in the slow reaction per molecule of fibrinogen of 340 000 molecular weight.

In Fig. 4 the base uptakes by each reaction, expressed as fractions of the maximum are plotted against pH. The points fit the theoretical titration curves drawn on this figure reasonably well. As indicated by these curves, the groups liberated by the fast reaction have a pK of 7.37 and those liberated by the slow reaction a pK of 7.67.

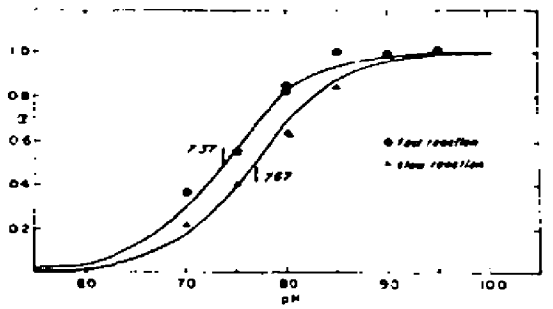

Fig. 4. Maximal base uptake of the slow and of the fast reaction, calculated as indicated in Fig. 3 and plotted against pH. The curves drawn are theoretical titration curves with their pK's indicated.

These are reasonable values for α-amino groups[36]. They do not differ sufficiently, however, to permit conclusions concerning the electrostatic vicinity of the groups split in the two different reaction classes.

We have neglected in our analysis the reaction classes slower than the two fastest ones. These have also contributed to the number of bonds broken in the first 20 min, and our experimental A_0 values must include the contribution from these slower reactions. The A_0 value for the slow reaction is much more affected by this error than the value for the fast reaction. An estimate of its magnitude can be made by examining the curves obtained in prolonged runs. A precise kinetic analysis of these curves is impossible because the digestion is slow and considerable inactivation of the enzyme occurs during the process. However, an approximate evaluation shows that a third reaction class is present in which about 250 bonds are split at a rate some 70 times slower than those in the second class. The contribution of the third class in the first 20 min of the reaction, calculated from the above reaction constants, amounts to 7–8% of the total number of bonds split over this period of time. Our A_0 values for the second reaction class are, therefore, overestimated by about 7–8%. In all, these three reaction classes account for approx. 93% of the total theoretically susceptible bonds to trypsin in the fibrinogen molecule.

Over the 20-min reaction period there was neither significant inactivation of trypsin, nor inhibition by reaction products. By adding TAME or fresh fibrinogen to the reaction mixture after 20 min, it was found that the rates obtained did not differ by more than approx. 10% from the rates obtained with the same concentrations of substrate and enzyme freshly combined.

Sedimentation studies

Samples for the ultracentrifuge runs were obtained from two serially inhibited digestions: one using the usual ratio of fibrinogen to trypsin of 152 to 1 which conveniently allowed for the following of the changes associated with the slow reaction, and a second with a four-fold reduced trypsin concentration for the study of the fast reaction. Fig. 5 shows the patterns obtained with the lower trypsin concentration. It is apparent that the sedimentation coefficient of fibrinogen changes but little

Fig. 5 Sedimentation patterns of fibrinogen digests. Time of digestion in minutes is indicated under each frame. Fibrinogen concentration, 7.50 mg/ml; trypsin to fibrinogen ratio, 1 : 608. Solvent: 0.26 *M* KCl in 0.035 *M* potassium phosphate buffer (pH 7.1). Patterns obtained after 32 min centrifugation at 59 780 rev./min at 23°. $s_{20,w}$ of faster peak: 1 min, 9.85; 2 min, 9.83; 5 min, 9.80; 10 min, 9.69.

during the fast reaction. The principle change is from a hypersharp boundary to one spreading fairly rapidly. Also, a small peak appears on the fast side of the main peak which disappears again toward the end of the reaction.

As shown in Fig. 6 much more pronounced changes are observed with more advanced digestion. After the change of the hypersharp into the spreading boundary (occurring in the first 2–3 min) a new, slower moving boundary appears which increases in size at the expense of the original peak. This transformation seems to be

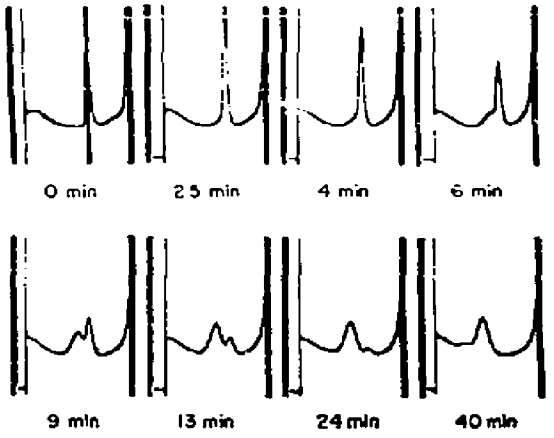

Fig. 6. Sedimentation patterns of fibrinogen digested with trypsin at an enzyme to protein ratio of 1 : 152. Time of digestion in minutes is indicated under each frame. Fibrinogen concentration, 12.36 mg /ml. Patterns obtained after 80 min centrifugation at 59 780 rev./min and 22°. Solvent: 0.21 *M* KCl in 0.09 *M* potassium phosphate buffer (pH 7.1).

nearly complete after 24 min of digestion. On the 24-min digestion pattern one can also see a barely visible, still slower peak, which becomes more apparent after 40 min of digestion.

The sedimentation coefficients of the various components are listed in Table II. A moderate increase in the $s_{20,w}$ of the original peak is noticeable as the digestion proceeds, probably because of diminishing interaction of the particles. The $s_{20,w}$ of the slow component, on the other hand, after a slight initial drop (which may be a reflection of minor molecular transformations still going on at this stage) remains essentially constant.

TABLE II

RELATIVE CONCENTRATIONS AND SEDIMENTATION COEFFICIENTS OF THE COMPONENTS OF DIGESTED SAMPLES DETERMINED IN ULTRACENTRIFUGAL RUNS

Fibrinogen concentration was 12.30 mg/ml. Trypsin to fibrinogen ratio, 1 : 152 (pH 7.1). Temp. 24.5°

Time of digestion (min)	Fibrinogen		Fragment I		Fragment II		Area deficit Relative concentration
	Relative concentration	$s_{20,w}$	Relative concentration	$s_{20,w}$	Relative concentration	$s_{20,w}$	
0	100	6.29					0
1.0	96	6.00					4
2.5	95	6.11	5				0
4.0	82	6.45	11				7
6.0	68	6.50	25	5.79	7		7
9.0	51	6.47	40	5.48			3
13.0	32	6.60	51	4.98			17
18.0	11	6.82	69	5.12			20
24.0	7	6.76	74	4.86	6		13
40.0	0		69	4.90	8	3.73	23

The relative areas under the curves, corrected for radial dilution, are also given in Table II. By the end of 40 min of digestion the total area has decreased by about 20%, which corresponds approximately to the non-protein nitrogen determined at this stage with trichloroacetic acid precipitation. The decrease in the area of the fast peak follows fairly closely an apparent first order reaction scheme but seems to have an initial lag period approximately equal to the duration of the fast reaction. From a plot of log of remaining fast component *versus* time, a rate constant of $5.0 \cdot 10^{-2}$/min can be calculated.

Viscosity studies

The digestion of fibrinogen is accompanied by a viscosity drop which appears to follow the disappearance of the fibrinogen in the ultracentrifuge. As is frequently observed with elongated particles, the logarithm of the relative viscosity of fibrinogen is a linear function of the concentration. Therefore, in analyzing the viscosity change, the logarithm of the relative viscosity was used instead of the viscosity itself. Because the viscosity continues to decrease slowly even after the completion of the reaction as seen in the ultracentrifuge, it is difficult to choose an end point corresponding to this completion. Guided by the ultracentrifuge patterns one can take the viscosity

of the 40-min digest as that corresponding to the end of the reaction (although the pattern already shows slight secondary changes). The fraction of unreacted fibrinogen will then be given by[37]:

$$C_{x'} = \frac{\log \eta_{x'} - \log \eta_{\infty'}}{\log \eta_{t_0'} - \log \eta_{\infty'}} \qquad (2)$$

Fig. 7 shows the usual first order plot of this quantity, and from it a rate constant of $5.5 \cdot 10^{-2}$/min was obtained. Alternatively, one can use the $d\log\eta_{rel}/dt$ *versus* time plot to obtain the rate constant and the end point. A plot of this kind shows a fairly good adherence to first order kinetics and a rate constant of $5.0 \cdot 10^{-2}$/min, which is in good agreement with the value obtained by the simpler calculation. The

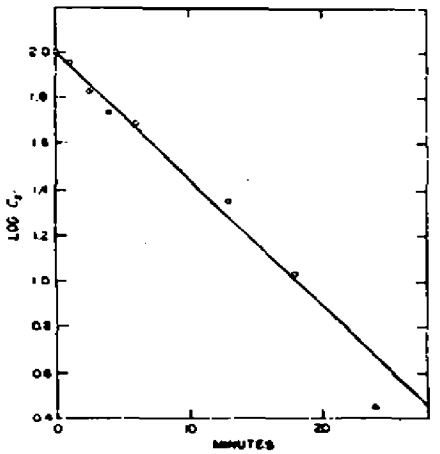

Fig. 7. First order plot of the viscosity chang., as defined in text, against time. Trypsin to fibrinogen rat[illegible], 1 : 152 (pH 7.1).

viscosity change followed first order kinetics very closely when the observations were limited to the first 40 min of the reaction. After this period of time there was a very slow change in viscosity corresponding to the complete digestion of the fibrinogen*.

Optical rotation changes

The polarimetric measurements of the digest samples at 370 mμ demonstrated that, as the reaction proceeded, the optical rotation decreased from an initial laevorotatory value. A log rate *versus* time plot showed a good fit to a first order kinetic curve with no evidence of a faster reaction. The maximum rotational change calculated from this plot is 6.3° and the rate constant is $7.8 \cdot 10^{-2}$/min.

* With some trypsin preparations that contained a higher than usual chymotrypsin contamination, the straight line of the logarithmic plot gave an extrapolated zero time viscosity value below the one obtained with a trypsin-free blank, suggesting the presence of a fast initial reaction. This apparent fast reaction was probably caused, however, by the incomplete inhibition of the chymotrypsin impurity by the soybean inhibitor, because it was absent when chymotrypsin-free trypsin preparations were used.

Non-protein nitrogen determinations

Fig. 8 shows the amount of non-protein nitrogen produced during digestion as determined by absorbancy measurements at 280 mμ and by nitrogen determinations of the trichloroacetic acid supernatants. It is seen that the fraction of nitrogen liberated is approximately twice as high as the fraction of the total ultraviolet absorption associated with the non-protein nitrogen. Apparently the low molecular weight products have a lower content of aromatic amino acids than the parent fibrinogen. The log rate *versus* time plot indicated the presence of a fast and a slow reaction with

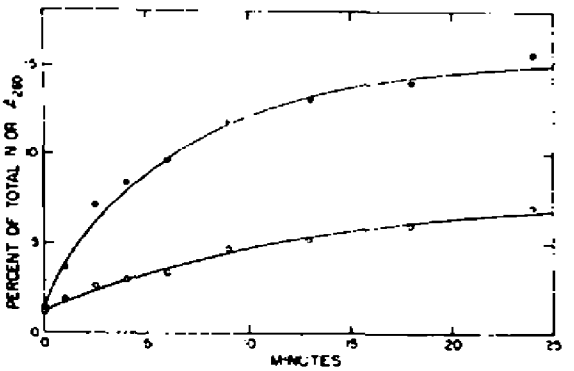

Fig. 8. Amount of nitrogen (●—●) and of the absorbancy at 280 mμ (○- -○), both expressed as percentages of the total, in supernatants of trichloroacetic acid precipitates of digested samples plotted against time of digestion. Trypsin to fibrinogen ratio, 1 : 152. Fibrinogen concentration, 12.36 mg/ml (pH 7.1).

the rate constants: 0.6/min and $5.9 \cdot 10^{-2}$/min, respectively, with 0.92% of the total nitrogen being liberated in the fast and 13.3% in the slow reaction.

Clottability with thrombin

Clotting was completely absent in the sample digested for 1 min with the usual 1 : 152 trypsin to fibrinogen ratio. Therefore, as indicated in METHODS, the trypsin concentration was reduced approximately 10-fold to a trypsin to fibrinogen ratio of 1 : 1665. The series of inhibited mixtures was then tested with thrombin. Soybean trypsin inhibitor does not interfere with the action of thrombin[38].

When the amount of fibrinogen clottable by thrombin was plotted against time of digestion with trypsin a straight line was obtained suggesting zero order kinetics for the inactivation process. However, the data also fit reasonably well a first order plot over more than 50% of the inactivation. In making a choice between the two kinetics the points toward the end of the reaction would be of decisive importance, but obviously these are also the least accurate ones. Incomplete clotting because of the low fibrinogen concentration, inhibition by the inactivated molecules[37], or overcorrection for the originally present unclottable material, would all tend to increase the amount of apparently unclottable material found, thus shifting the reaction towards an apparent zero order kinetics. For the sake of more ready comparison

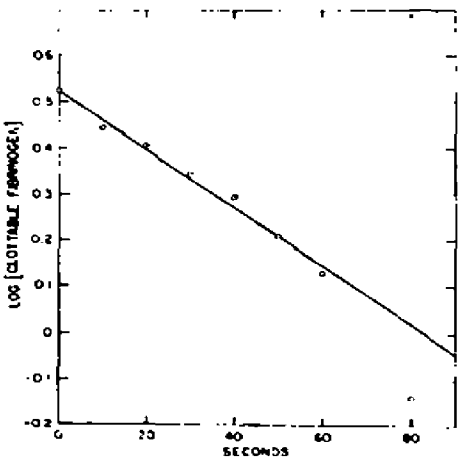

Fig. 9. Logarithm of amount of clottable fibrinogen plotted against digestion time. Trypsin to fibrinogen ratio, 1 : 1665. Fibrinogen concentration 15.5, mg/ml (pH 7.1).

with the rest of the kinetic data, we have described the process by a first order plot of the data (Fig. 9). The rate constant found was 0.39/min at a trypsin concentration of 22.3 units/ml.

DISCUSSION

The sequence of events during the early phase of the digestion of fibrinogen by trypsin can be described as follows: There is a fast reaction involving the splitting of 12 peptide bonds per molecule of fibrinogen of 340 000 molecular weight, accompanied by very little apparent change in the gross structure of the molecule; viscosity and sedimentation coefficient decrease slightly, there is greater spreading of the main boundary during sedimentation, and a faster but smaller boundary appears, which disappears before the end of the reaction. However, these subtle changes are coupled

TABLE III

SUMMARY OF THE KINETIC CONSTANTS AT pH 7*

Fibrinogen concentration was 12.36 mg/ml; fibrinogen to trypsin ratio, 152 : 1.

Method of observation	*Fast reaction* k	*Slow reaction* $k \times 10^3$
pH-stat	0.38	2.6
Sedimentation	Absent	5.6
Viscosity	Absent	5.5
Optical rotation	Absent	7.8
Non-protein nitrogen formation	0.6	5.9
Clottability with thrombin	4.2	—

* Although, the kinetic constants listed in this Table were obtained under slightly varying conditions (pH-stat runs in 0.3 *M* KCl, all the other in 0.3 *M* KCl plus 0.1 *M* phosphate buffer), their comparability was justifyed by the small effect of phosphate ions observed in pH-stat runs performed in the presence and in absence of 0.1 *M* phosphate. These experiments were run at pH 8.4 where the buffering effect of phosphate ions is negligible.

with a dramatic loss of clottability. Parallel to this reaction a slower reaction proceeds that involves the splitting of 80 peptide bonds per molecule of fibrinogen and considerable molecular alteration. This reaction is associated with the appearance in the ultracentrifuge pattern of a peak sedimenting more slowly than the native fibrinogen, production of non-protein nitrogen, a pronounced decrease in the viscosity, and a slight decrease in the optical rotation. To facilitate comparison of the kinetic data, all the constants of the various measurements of the reaction are assembled in Table III.

Focusing attention upon the molecular changes effected by the slow reaction, it is seen that the rate of accumulation of the slower sedimenting material, as well as the rates of the viscosity and optical rotation changes are about twice as fast as the slow reaction in the pH-stat. One can argue that there is a small number of specific bonds, buried in the group of 80, the breaking of which causes these changes and whose rate of splitting is twice that of the whole group. Alternatively, one can discuss the proposition that the splitting of only half of the bonds, taken randomly, is sufficient to cause the above mentioned effects, thus leading again to a two-fold increase of reaction rate over that observed in the pH-stat.

The results cannot be unambiguously interpreted. However, regardless of the specific breakdown process, the decreases in sedimentation rate and viscosity appear to be too large to be attributable to a change in frictional resistance alone, and suggest rather a fragmentation of the molecule. The amount of non-protein nitrogen formed during this process is small, indicating that the enzyme removes a small number of peptide segments from the molecule, thereby setting free large fragments. Significantly, there is no increase in laevorotation during this phase of the digestion; on the contrary, a slight decrease occurs. This demonstrates that unfolding of the secondary structure of the protein does not take place*.

Concerning the significance of the fast reaction, it seems quite probable that loss of clottability, which we shall call inactivation, and this early molecular breakdown are connected. However, this relationship is not readily apparent in the data and a more thorough discussion is necessary to establish it. In the fast reaction, as mentioned earlier, 12 peptide bonds are split. These should include the 4 bonds susceptible to thrombin, because at the end of this reaction no further splitting by thrombin is demonstrable in the pH-stat. Since their splitting causes clotting, these 4 bonds obviously are not connected with the inactivation. Therefore, inactivation must be associated somehow with the splitting of the remaining 8 bonds in this group. Also the rate of inactivation is some 10 times faster than the overall rate of splitting of the bonds in the fast reaction.

With the above factors in mind, two simple alternative hypotheses may be offered: (a) Inactivation follows the splitting of any bond from this group of 8 bonds and there is no distinction as to rate among the members of the group. This will result in an 8 times faster rate of inactivation than the overall rate of splitting of

* A similar conclusion was reached from a spectroscopic investigation of the process. A large fraction of the tyrosine residues in native fibrinogen has an abnormal ultraviolet spectroscopic titration[39,48]. We found by differential spectroscopy, that there is no change in the titration characteristics of these groups when the fibrinogen molecule is fragmented by trypsin. This finding indicates that the secondary and tertiary structure of the protein must be largely conserved; it also demonstrates that no base uptake in the pH-stat can be attributed to normalization of tyrosine residues.

the peptide bonds. (b) Inactivation is caused by the splitting of a single specific group which is split at a rate 10 times faster than the overall splitting rate. The specific group is buried in the fast reaction because our methods of analysis are not sensitive enough to discover its presence. Unfortunately the two mechanisms have identical mathematical forms, thereby preventing any attempt to establish one or the other as the more likely mechanism by testing them against the kinetic data.

With either of the two mechanisms there will be some molecules in which only the bonds susceptible to thrombin are split, and these will be able to polymerize. Indeed, inspecting Fig. 4 one can see that there is a substantial build-up of faster sedimenting material on the ultracentrifuge patterns, which then disappears completely in the later stages. Its maximum area, reached after two to three minutes digestion, is approx. 33% of the total.

The proportion of polymerizable molecules at any time could be calculated if the reaction rates and the number of splits necessary for inactivation and for polymerization were known. But only the number of splits involved in each can be estimated with confidence. Thus, the number of bonds broken in the inactivation reaction probably cannot be larger than one, because the pH-stat shows the splitting of only 4 bonds in the period necessary for complete inactivation. Two of these bonds are from the slow reaction group and at least one is of the non-inactivating kind (attacked by thrombin) of the fast reaction. Thus, it is fairly certain that the splitting of only one bond renders the molecule incapable of polymerizing. By the same reasoning, one bond must be responsible for the polymerizing reaction also, since this reaction takes place also within the period when the initial 4 bonds are broken. Therefore, at least for the restricted kind of polymerization leading only to the appearance of the faster sedimenting material and very likely distinct from the polymerization brought about by thrombin, the splitting of only one peptide bond seems to be sufficient.

Putting aside the question of whether the rates of the inactivation and polymerization reactions are higher because of the presence of one specific group of higher susceptibility to trypsin, or because of the identical effect of any single split taken at random in a larger group, the accumulation of heavy material during this reaction can be used to estimate the ratio of the apparent rates of the two processes. Assuming that both reactions obey first order kinetics and run independently side-by-side, the fraction which was non-inactivated would be given by $e^{-k_1 t}$ and the fraction of this rendered polymerizable by $(1 - e^{-k_2 t})$. Therefore, the polymerizable fraction at any time would be:

$$a = e^{-k_1 t}(1 - e^{-k_2 t}) \tag{3}$$

and

$$a_{max.} = \left(\frac{k_1}{k_2 + k_2}\right)^{\frac{k_1}{k_2}} \left(1 - \frac{k_2}{k_1 + k_2}\right) \tag{4}$$

The experimental value of 0.33 for $a_{max.}$ gives, according to Eqn. 4, a ratio of 0.65 for k_1/k_2; thus, the reaction producing polymerizable molecules appears to be slightly faster than the inactivation one.

It is instructive to compare the data on the digestion of fibrinogen presented in this paper with those previously reported for myosin[8] under similar conditions. Both proteins show a fast and a slow reaction in the pH-stat, but with the same trypsin

to protein ratio the rate of the slow reaction of the fibrinogen digestion is the same as that of the fast reaction of the myosin digestion. The number of peptide bonds split is also comparable: 16 in myosin and 23 in fibrinogen for an arbitrary molecular weight of 100 000. Fibrinogen, as mentioned earlier, has a large number of bonds split at an even slower rate than those in the slow reaction studied herein, and these are the counterpart of the slow reaction of myosin.

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