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KINETIC STUDIES OF THE DIGESTION OF FIBRINOGEN BY α-CHYMOTRYPSIN

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SUMMARY

The kinetics of the digestion of fibrinogen by α-chymotrypsin (EC 3.4.4.5) has been studied in the pH-stat, by non-protein nitrogen determinations, and by changes in the sedimentation patterns, clottability and precipitability with $(NH_4)_2SO_4$ of the digested protein.

In not too prolonged digestions, the pH-stat revealed 2 simultaneous reactions, of which the fast one comprised 7, the slow one 96 bonds per mole of fibrinogen. During the slow reaction the molecule was fragmented into fragments similar to those obtained with trypsin (EC 3.4.4.4). Clottability was lost at a rate intermediate between those of the slow and fast reactions in the pH-stat. The action of thrombin upon the digests could be shown in the pH-stat, even after complete loss of clottability.

INTRODUCTION

In our previous work[1,2] we have shown the splitting of fibrinogen by trypsin (EC 3.4.4.4) into 3 fragments of approximately equal weight. The bonds susceptible to thrombin were split much before fragmentation occurred, a result not surprising in view of the similar specificity of the 2 enzymes. It was desirable, therefore, to investigate the digestion process with a proteolytic enzyme which does not attack the arginyl–glycine bonds and obtain fragments which still retain their bonds susceptible to thrombin. For this purpose α-chymotrypsin (EC 3.4.4.5) was chosen, and the kinetic results obtained with this enzyme will be reported in this paper.

MATERIALS AND METHODS

Bovine fibrinogen was purified from Fraction I from bovine plasma, obtained from Armour Pharmaceutical Co., Kankakee, Ill., Lot No. V-5201, and thrombin

(EC 3.4.4.13) from Thrombin, Topical of bovine origin from Parke, Davis and Co., Detroit, Mich., by the methods described in the previous communication[1] and they had the characteristics described there.

α-Chymotrypsin was a thrice crystallized, salt-free lyophilized preparation obtained from Worthington Biochemical Corp., Freehold, N.J. The concentrations of the enzyme solutions were estimated spectrophotometrically on the basis of $E^{1\%}_{282\ m\mu}$ equal to 20.0 (ref. 3). Its specific activity, determined as described by BALLS AND JANSEN[4], was equal to that reported by these authors. The extent of trypsin contamination of the enzyme was determined from the rate of splitting of tosyl-arginine methyl ester, a typical trypsin substrate, at pH 8 and 25°. The measured rate could have been ascribed to 0.02% trypsin impurity. However, INAGAMI AND STURTEVANT[5] as well as other authors, showed that these 2 enzymes have a certain degree of cross-reaction with each others substrates, so that the low activity observed may be that of chymotrypsin itself. Indeed, when the activity was determined in the presence of various amounts of soybean trypsin inhibitor, the enzyme–inhibitor association constant was found to be $0.5 \cdot 10^5 \cdot M^{-1}$, which is near to the one characteristic for chymotrypsin and soybean inhibitor[6], 4 orders of magnitude below the association constant of trypsin with the same inhibitor[7].

Soybean trypsin inhibitor of KUNITZ, salt-free crystallized from ethanol, was obtained from Worthington Biochemical Corp.

Since the soybean trypsin inhibitor of KUNITZ only partially inhibits chymotrypsin and the organo-phosphorus compounds are too slow in their inhibitory action for kinetic purposes, a second inhibitor in soybean, first described by BOWMAN[8], was used in these studies. It was prepared according to BIRK[9], omitting the column fractionation. Assuming that the preparation contained 50% active material, its association constant for chymotrypsin was found to be $2.4 \cdot 10^7 \cdot M^1$, which is about 130 times higher than that of the KUNITZ inhibitor. Though inhibition was by no means complete with this inhibitor, it slowed down the reaction sufficiently to permit the investigation of samples inhibited at various stages of the digestion.

The physicochemical techniques and the evaluation of the kinetic data were described in detail in the paper on the trypsin digestion of fibrinogen[1]. Since these were used essentially unchanged, their description will be omitted.

RESULTS

pH-stat studies

Over a not very extended digestion period, the reaction curve could be described by 2 simultaneous reactions, being similar in this respect to the digestion with trypsin. The difference between the rates of the 2 reactions, however, was large, so that it proved difficult to characterize both reactions at a single enzyme concentration. This difficulty was circumvented by performing the reaction, at each pH, with 2 different enzyme concentrations, one being 6 times larger than the other. Fig. 1A shows a representative example of the runs with the 2 different enzyme concentrations and Fig. 1B their analysis by the log rate *vs.* time plot. The points on Fig. 1A were calculated with the parameters of the 2 reactions obtained from the log rate plots and show that the mathematical analysis gives a very accurate description of the experimental curves.

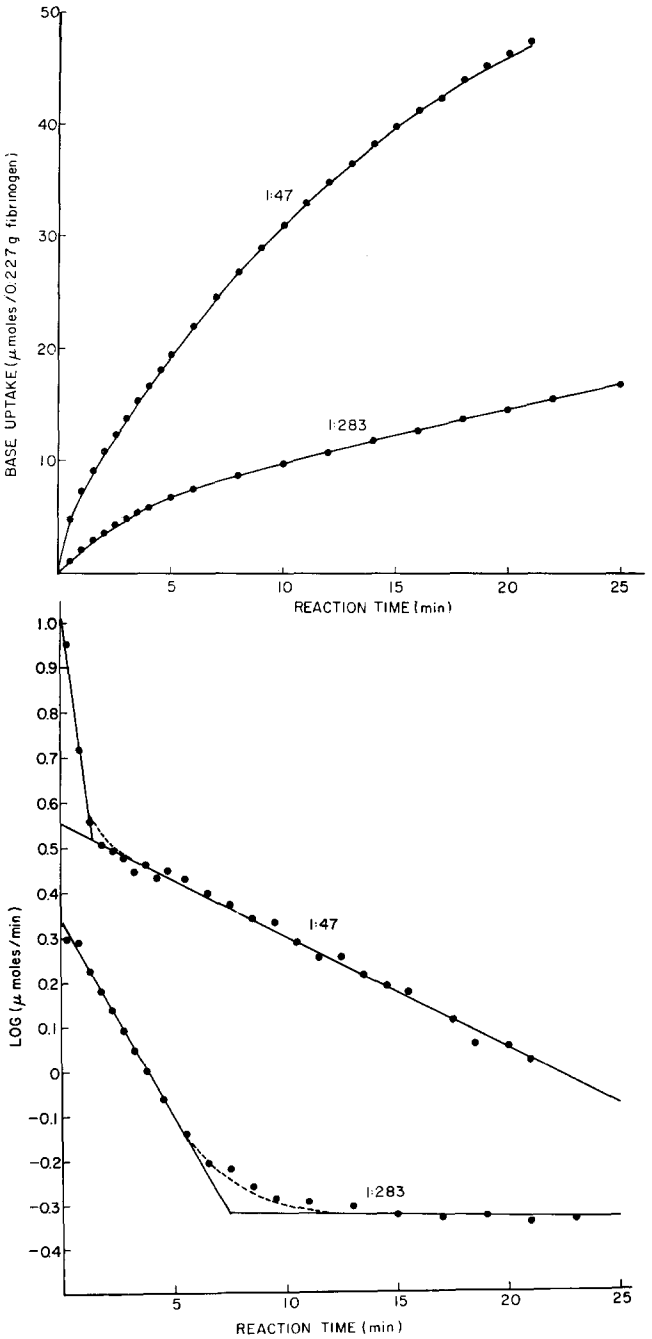

Fig. 1. A, Base uptake during the digestion of fibrinogen at pH 9.0 and 20°. Curves were traced by the pH-stat, while the points were calculated as explained in the text. The enzyme to fibrinogen weight ratio is indicated above each curve. Fibrinogen concentration was 2%. B, Analysis of the 2 pH-stat curves shown in A. Logarithm of the rate of base uptake plotted against time.

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 min. The base uptake is given as moles of base per 340 000 g of fibrinogen. Fibrinogen concentration was 2%; fibrinogen to chymotrypsin ratio, 47:1.

pH	*Fast reaction**		*Slow reaction**	
	k	A_0 *(moles base)*	$k \cdot 10^2$	A_0 *(moles base)*
7.0	1.30	1.8	2.39	15.6
7.5	1.50	3.1	3.26	35.0
8.0	1.34	5.1	2.99	59.2
8.5	1.34	6.5	2.90	79.5
9.0	1.10	6.8	2.47	93.6
9.5	0.93	7.5	2.37	95.7

* The data of the fast reaction are averages of 3 runs at each pH, those of the slow reaction of 2 runs.

The rate constants and the maximal base uptakes of both reactions were determined at every 0.5 pH interval between pH 7.0 and 9.5. The data are assembled in Table I. All the k values, regardless of the actual enzyme concentration, were normalized to correspond to an enzyme to protein ratio of 1:47. The rate constants of both reactions show parallel pH dependence, with a maximal rate at approximately pH 7.5.

The A_0 values represent the titration of the new α-amino groups formed by the splitting of peptide groups. It could be assumed that these groups were in completely uncharged form at the highest pH where they were determined, because this pH was far enough from the usual pK of such groups. The value of A_0 at that pH, therefore, corresponds to the number of peptide bonds split by the enzyme. Thus, from Table I it appears that there are about 7 bonds split in the fast reaction and

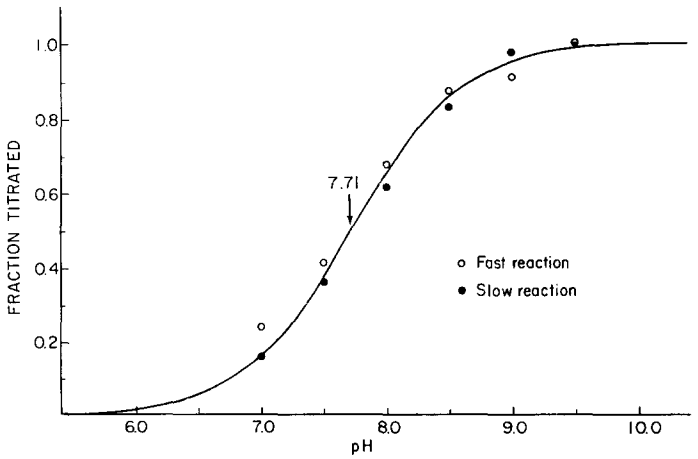

Fig. 2. Maximal base uptake in each reaction, calculated from the pH-stat curves, plotted against pH. Ordinates are expressed as fractions of the uptakes obtained at pH 9.5. The curve is a theoretical titration curve with a pK of 7.71.

about 96 in the slow reaction in 1 molecule of fibrinogen. The degree of titration of these groups, expressed as fraction of the maximal, is shown as a function of pH in Fig. 2. The drawn curve is a theoretical titration curve with no electrostatic correction. It appears that the α-amino groups produced in both of these reaction classes have a pK of 7.7, which is reasonable for an α-amino group in a protein, or peptide.

Besides the 2 reactions mentioned above, chymotrypsin splits a large number of other bonds in still slower reaction classes. These are apparent at prolonged reaction times, or with high enzyme concentrations. Since these led eventually to the complete breaking up of the molecule, their investigation did not seem to be of any particular interest from the viewpoint of our investigations.

Sedimentation studies

Chymotrypsin digestions for sedimentation analysis were performed at pH 8.0, 20°, with an enzyme to protein ratio of 1:103, and fibrinogen concentration of 2%. The course of the slow reaction was calculated under these conditions with the data obtained from the pH-stat studies, and samples were withdrawn from the reaction mixture at various degrees of completion of the slow reaction. These were then mixed with BOWMAN'S soybean inhibitor in a 3.4-fold excess over the chymotrypsin present and refrigerated immediately. Since the inhibitor does not cause complete inhibition, the samples were run as early as possible in the Spinco, Model E, analytical ultracentrifuge. The series of patterns obtained is shown on Fig. 3. From this it appears that the fragmentation of the fibrinogen molecule occurs during the slow reaction seen in the pH-stat. The relative proportion of the components was determined by planimetry of the patterns superimposed on a base line obtained with all the ingredients, except

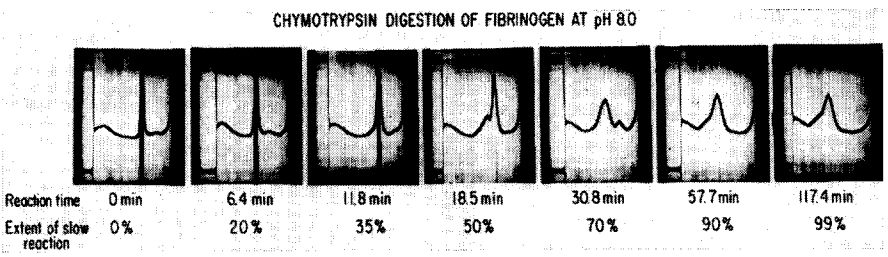

Fig. 3. Sedimentation patterns of fibrinogen digested with chymotrypsin at an enzyme to protein ratio of 1:103, at pH 8.0 and 20°. Fibrinogen concentration in the runs was 1.37%. Patterns obtained after 80-min centrifugation at 59 780 rev./min and 20°, with phase-plate angle at 60°.

fibrinogen, in the same concentration as in the digests. In all the early samples there was a broad peak close to the meniscus, representing 20–22% of the total area, whereas up to 35% of the slow reaction the main peak showed no signs of fragmentation and very little change in sedimentation coefficient. In these mixtures $s_{20,w}$ of the main peak was 6.04 S, as compared with 6.17 S for native fibrinogen at the same concentration (1.37%) and in the same solvent. Thus, the first step in the degradation by chymotrypsin appears to be the removal of approx. 20% of the mass of fibrinogen in the form of fairly large molecular weight peptides. It is possible that modified molecules of this kind account for poor clots seen with some commercial fibrinogen preparations and also for reports of lower molecular weights of this protein.

A slower moving component appears first at the 50% stage and becomes the major constituent of the pattern at 70% completion of the slow reaction. The sedimentation coefficient of this fragment, $s_{20,w}$, was 5.52 S at 50% and 5.18 S at 70% of the slow reaction. With further digestion a shoulder appears on the slow side of the fragment peak, showing degradation to still smaller fragments.

Non-protein nitrogen determinations

Although the trypsin contamination in our enzyme preparation was very low, this and the following experiments were performed with chymotrypsin partially inhibited with the soybean inhibitor of KUNITZ. This should have removed completely whatever trypsin was present. The digestion was performed at pH 8.0, 20°, an enzyme to protein ratio of 1:118, 2% fibrinogen concentration, and with an amount of soybean inhibitor equal to one-third of the weight of chymotrypsin present. In order to determine the extent of inhibition and thus make it possible to correlate this experiment with the ones performed previously without inhibitor, the reaction was followed in the pH-stat, and the rates and quantities involved were determined. The ratio of the rates and the number of bonds split in the slow and the fast reaction were the same within experimental error as those found without inhibitor, demonstrating again that trypsin was not present; however, the rates were 10.2% lower than those corresponding to the amount of chymotrypsin in the mixture. The digestion was then repeated under identical conditions taking out samples at different time intervals and mixing them with an equal volume of 30% trichloroacetic acid. The amounts of nitrogen and the absorbances of the supernatants were then determined. The

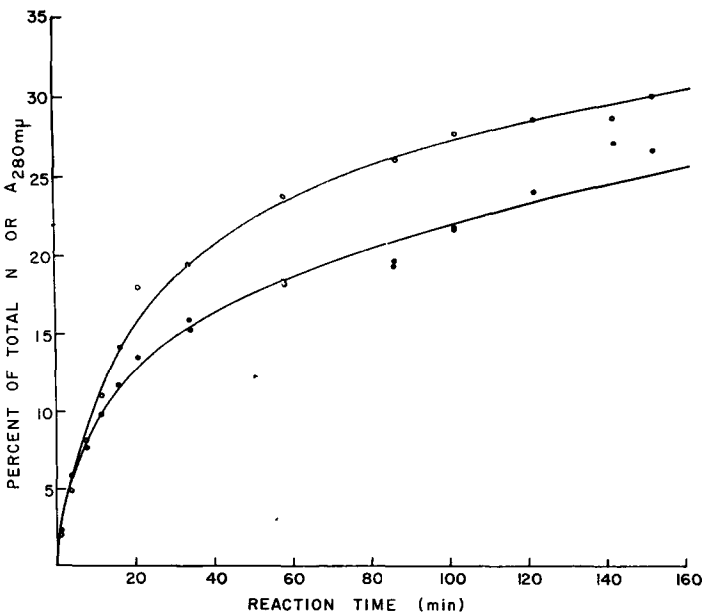

Fig. 4. Amount of nitrogen (●) and of absorbance at 280 mμ (○) in trichloroacetic acid supernatants of digested samples plotted against time of digestion. Reaction conditions during digestion: Fibrinogen concentration 2%, chymotrypsin to fibrinogen ratio 1:118, pH 8.0, 20°.

TABLE II

SUMMARY OF THE KINETIC CONSTANTS OF THE DIGESTION AT pH 8.0

Fibrinogen concentration 2%; fibrinogen to chymotrypsin ratio, 47:1; 20°. Rate constants calculated with decimal logarithms; time expressed in min.

	Fast reaction k	*Slow reaction* $k \cdot 10^2$
pH-stat	1.34	3.0
Sedimentation	Absent	5.4
Non-protein nitrogen formation*	0.10	0.68
Non-protein nitrogen formation**	0.09	1.16
Loss of clottability	0.22	—
Loss of precipitability in 25% saturated $(NH_4)_2SO_4$	0.28	—

* By nitrogen estimations.
** By absorbance determinations.

course of the reaction is shown in Fig. 4. and the rate constants obtained from the log rate plots are given in Table II. The fast reaction liberated 11.6%, the slow reaction 21.1%, of the total nitrogen. On the basis of absorbance the percentages calculated were similar, 13.6% and 20.1%, respectively.

Reaction of the chymotrypsin digests of fibrinogen with thrombin

Clotting ability of the digests. The effect of thrombin upon the arrested digests could be investigated, since neither the KUNITZ, nor the BOWMAN soybean inhibitor has any effect on the clotting or esterase activity of this enzyme. Samples at various stages of digestion were obtained under conditions identical to those described for the investigation of non-protein nitrogen formation. They were mixed immediately with 5 volumes of 0.06 M phosphate buffer of pH 6.3, containing a 5-fold excess over chymotrypsin of BOWMAN'S soybean inhibitor and 8 units of thrombin per ml. The clots were centrifuged after 30 min in the Servall SS-1 centrifuge and the absorbance of the supernatants determined at 280 mμ. Corrections were made for all the non-clottable proteins present. The clottability of the samples declined at a fast rate, and no clottable material could be detected after approx. 10 min of digestion, which corresponds roughly to 25% completion of the slow reaction in the pH-stat.

The change in the precipitability of the digest in 25% satd. $(NH_4)_2SO_4$ solution at pH 6.3 was followed in the same experiment. Under these conditions 98% of the native fibrinogen was precipitated. The amount of precipitate in the digests decreases rapidly, following first-order kinetics, with a rate very close to that of the formation of non-clottable material. The rates of these reactions are given in Table II.

Effect of thrombin upon the digests, followed in the pH-stat. Splitting of peptide bonds by thrombin was apparent in the pH-stat in digests carried well beyond the point where clottability disappeared completely. A large batch of 2% fibrinogen was digested to 75% completion of the slow reaction in the pH-stat at pH 7.0 and then stopped by the addition of BOWMAN'S inhibitor. From the digested mixture 15-ml aliquots were brought to various pH values and the uptake, following the addition of 57 units of thrombin, was recorded in the pH-stat. Titrant was 0.02 M, CO_2-free, KOH.

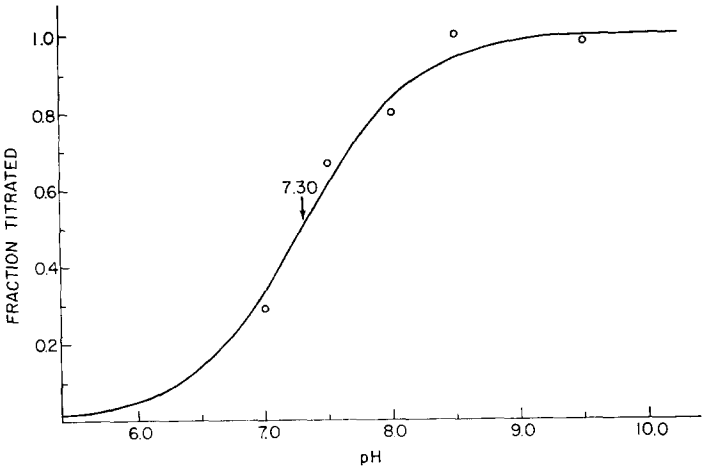

Fig. 5. Final base uptakes caused by thrombin on a chymotryptic digest of fibrinogen arrested at 75% completion of the slow reaction, plotted against pH. Ordinates expressed as fractions of the uptake determined at pH 9.5. Curve is a theoretical titration curve with a pK of 7.3.

Maximal uptake was reached at pH 8.5 and corresponded to 1.6 bond split per mole of fibrinogen. Fig. 5 shows the uptake as a function of pH, expressed as the fraction of the maximum. The drawn curve is a theoretical titration curve, and it appears that the group liberated by thrombin has an apparent pK of 7.3.

End-group determinations[10,11] point to the release by thrombin of 4 peptides per mole of fibrinogen. The experiment described above showed 1.6 bond split when the slow reaction was allowed to proceed to 75% completion. It is obvious that some

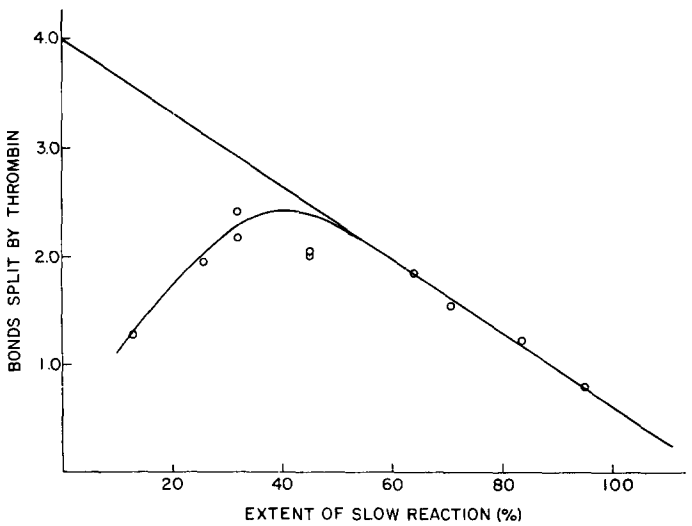

Fig. 6. Bonds split by thrombin in digested fibrinogen, determined by base uptake in the pH-stat at pH 8.5, plotted against the extent of the slow reaction during the preceding chymotryptic digestion of fibrinogen.

of the thrombin susceptible bonds were either split, or became resistant to thrombin during the chymotryptic digestion. This effect was investigated in more detail by digesting fibrinogen to various extents and then allowing it to react with thrombin. Fifteen ml of 2% fibrinogen was brought to pH 8.5 at 25° and digested to predetermined uptakes of base with 3.33 mg of chymotrypsin mixed with 1.11 mg of the soybean trypsin inhibitor of KUNITZ. The reaction was stopped by adding 25 mg of BOWMAN'S soybean inhibitor, and after stabilization of the base line 80 units of thrombin were added. 0.2 M KOH was used as titrant during the digestion phase and 0.02 M KOH during the reaction with thrombin. The results are summarized in Fig. 6. Since at pH 8.5 the titration of the α-amino groups produced should be very nearly complete (to 93% with a pK of 7.3), the final uptakes were taken as a direct measure of the number of bonds split by thrombin. These are plotted against the extent of the slow reaction of the chymotryptic digestion. The curve shows a maximum at approx. 40% completion of the slow reaction. Above this, the number of bonds split by thrombin declines linearly with the extent of digestion. The line extrapolates back at

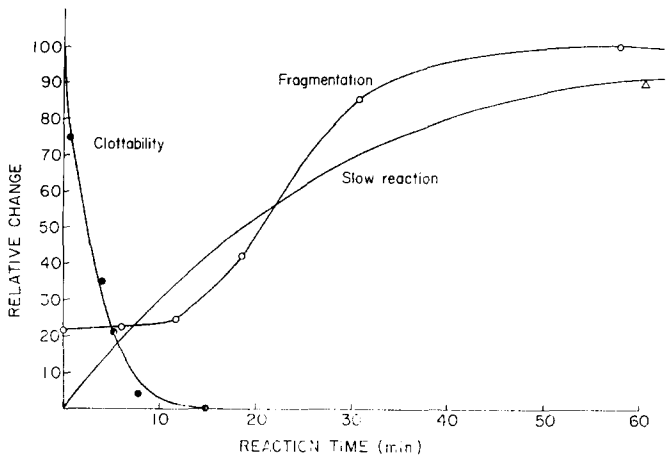

Fig. 7. Clottability, fragmentation of the molecule, determined from the ultracentrifuge patterns, and slow reaction in the pH-stat plotted against digestion time. Reaction conditions: 2% fibrinogen concentration, 1:103 chymotrypsin to fibrinogen ratio, pH 8.0, 20°.

zero digestion to the expected 4 bonds split per mole of fibrinogen. Below 40% digestion the apparent number of bonds split by thrombin decreases again. This effect is caused by the polymerization of the incompletely digested fibrinogen. In our previous studies[12] it was shown that the polymerization reaction absorbs, at this pH, 50–75% of the hydrogen ions liberated during the proteolytic step. The decline of the line below 40% digestion, therefore, represents the extent of polymerization in the digested samples. It should be mentioned here that in the samples there was gel formation up to about 25% and an increase in viscosity and turbidity from here up to about 40% completion of the slow reaction. Samples digested beyond this point showed no changes in their physical properties upon addition of thrombin.

DISCUSSION

The mathematical representation of the pH-stat curves describes the reaction correctly to within experimental error, as proved by the close correspondence of the calculated curves to the experimental ones (see Fig. 1A). However, one should keep in mind that the close fit does not prove that other, more complex kinetic formulations for example, inhibition by reaction products, consecutive reactions, *etc.*, could not give an equally good representation of the process. Moreover, the analysis in terms of 2 reactions is obviously an oversimplification, each reaction in actuality representing the average of a class of closely related kinetic entities.

A comparison of all the kinetic data may give more physical meaning to the reaction classes derived from mathematical analysis, by correlating them with some specific changes in the molecular characteristics observed during digestion. In order to facilitate this comparison, the kinetic constants are assembled in Table II and some of the changes followed are presented in Fig. 7. The fragmentation into the large fragments follows approximately the course of the slow reaction in the pH-stat. The shape of the curve shows that the fragmentation requires a number of bonds split in each molecule, since more than 35% of the total bonds are split before fragmentation occurs. Also, the 2 curves approach each other in the later stages of the reaction, suggesting that nearly all the bonds in this reaction class must be split in order to make the fragmentation complete.

There is no clear-cut correlation between any of the other processes followed and this is probably a reflection of the heterogeneity within the reaction classes seen in the pH-stat. Whereas the latter may give an average of a broad range of rates, some more specific method, for example the change in clottability, may focus on a single discrete group.

As a whole, the digestion of fibrinogen by chymotrypsin appears to be a more complex process than that with trypsin. This is so, probably, because of the more varied specificity of the enzyme, comprising peptide bonds split at widely different rates.

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