STUDIES ON THE INHIBITION OF PROTEOLYTIC ENZYMES BY SERUM

I. The Mechanism of the Inhibition of Trypsin, Plasmin, and Chymotrypsin by Serum Using Fibrin Tagged with I^{131} as a Substrate

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Inhibition of proteolytic enzymes by serum of normal persons and patients with various diseases has been the subject of numerous investigations since Camus and Gley in 1897 (1) first observed that normal serum markedly inhibits the activity of trypsin. Serum proteolytic inhibition was found to be increased in association with many diseases. Jobling and Petersen presented a comprehensive review of the subject in 1914 (2), and Grob reviewed the interval literature in 1942 (3). The physiological significance of proteolytic inhibition and the reasons for its increase with disease are not yet known. Trypsin has been the enzyme usually employed in studying proteolytic inhibition by serum. More recently there has been interest in the inhibition of other proteolytic enzymes, *e.g.*, plasmin (4, 5) and chymotrypsin (6).

The detailed mechanisms by means of which serum inhibits proteolytic enzymes have received relatively little attention. Using gelatin as a substrate, Hussey and Northrop in 1922 (7) studied the inhibition of trypsin by plasma and obtained results indicating that the inhibition was a stoichiometric, readily reversible reaction. Using casein as substrate, Grob in 1942 (3) studied the inhibition of trypsin by serum and came to a similar conclusion. The mechanism of the inhibition of plasmin and chymotrypsin by serum has not been studied.

The present work is concerned with comparing the mechanisms of the inhibition of trypsin, plasmin, and chymotrypsin by serum using fibrin tagged with radioactive iodine as a substrate. The effect of relative concentrations of enzyme and inhibitor on the enzyme-inhibitor relationship, and also the effect of substrate concentration on inhibition were studied.

In subsequent papers the findings obtained here are applied in the demon-

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stration of specific proteolytic inhibitors in serum and in the study of physiological aspects of variations in proteolytic inhibition.

Materials and Methods

Trypsin.—A crystalline preparation of trypsin of bovine origin was obtained from Armour and Company. It contained approximately 50 per cent magnesium sulfate; 25 mg. of this

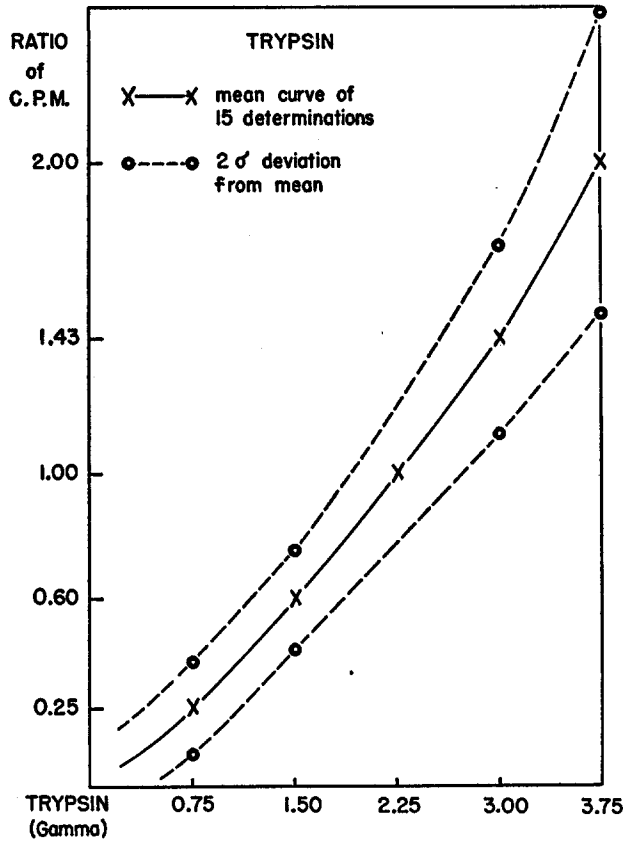

FIG. 1. Proteolysis of iodinated fibrin by trypsin. Reaction carried out at pH 7.4. Unclotted substrate, 0.14 per cent tagged fibrinogen solution. Thrombin, 1 ml. = 100 units. Total volume in each tube, 1.1 ml. Temperature, 28°C. Incubation time, 30 minutes. Control tubes contained same materials as experimental tubes, but no enzyme.

trypsin preparation produced 0.341 mg. of tyrosine in 10 minutes at 25°C. when assayed by the method of Anson and Mirsky (8). One γ of this preparation was equivalent to approximately 4×10^{-6} hemoglobin trypsin units.

Chymotrypsin.—A crystalline preparation of chymotrypsin of bovine origin was obtained from the Plaut Research Laboratory. It contained approximately 50 per cent magnesium sulfate.

Plasmin.—A preparation of plasmin from bovine plasma, prepared by the method of Loomis *et al.* (9), was kindly supplied by Dr. Eugene Loomis.

Fibrinogen.—Fraction I from bovine plasma was obtained from Armour and Company and was further purified by the method of Ware, Guest, and Seegers (10). The final product contained 85 to 90 per cent clottable protein.

Thrombin.—A preparation of bovine thrombin called topical thrombin was obtained from Parke, Davis and Company. The solution used contained 100 units per ml.

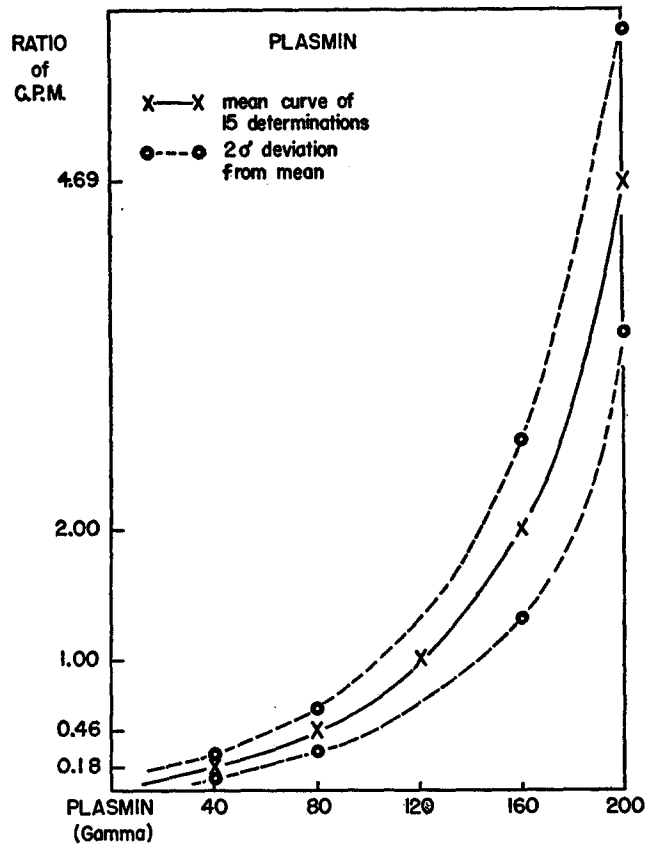

FIG. 2. Proteolysis of iodinated fibrin by plasmin. Conditions as in Fig. 1.

Serum.—Serum was separated within 1 hour after blood was drawn and was used immediately or was frozen at −10°C. and used within a week.

Buffer.—All solutions and dilutions were made with M/30 phosphate buffer in physiological saline at pH 7.4 unless otherwise indicated.

Measurement of Proteolytic Activity.—The method employed, recently developed by Shulman and Tagnon (11), depends upon the measurement of radioactivity in the soluble products of digestion released from an insoluble fibrin clot tagged with radioactive iodine. The reaction is carried out by mixing tagged fibrinogen with enzyme, adding thrombin immediately to form a clot, and filtering the mixture rapidly at the end of various periods of incubation. The radioactivity released by digestion is measured in an aliquot of the filtrate.

In the present work measurements were made after incubating for 30 minutes at 28°C. and pH 7.4. The reaction mixture consisted of 0.5 ml. of a 0.14 per cent solution of tagged fibrinogen as substrate, varying amounts of enzyme, 0.1 ml. of thrombin, and suitable amounts of phosphate buffer to make the volume up to 1.1 ml. Control tubes contained the same reagents as experimental tubes except for enzyme. Control values were subtracted from experimental values to obtain counts per minute.

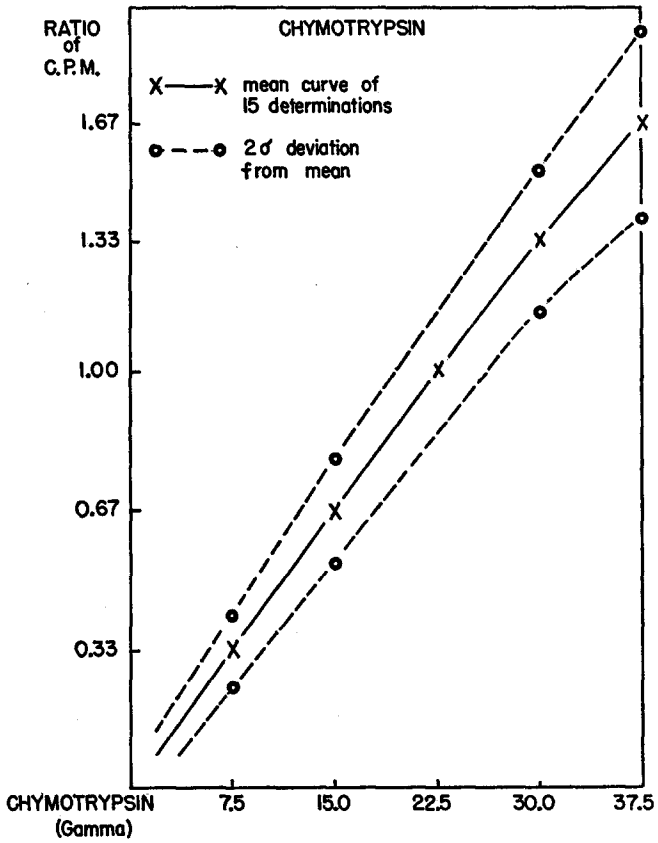

FIG. 3. Proteolysis of iodinated fibrin by chymotrypsin. Conditions as in Fig. 1.

By incubating varying amounts of enzyme with a constant amount of substrate under standard conditions, relationships could be developed between concentration of enzyme and extent of action on the substrate as judged by the radioactivity (C. P. M.) in the filtrate. Such curves are shown for trypsin (Fig. 1), plasmin (Fig. 2), and chymotrypsin (Fig. 3). Each curve represents the average of 15 series of determinations; two times the standard deviation is shown in each case. For purposes of utility, the ordinates are expressed as the ratio of C. P. M. at any concentration of enzyme to the C. P. M. obtained with an amount of enzyme arbitrarily chosen as a reference point. (The reference amount of enzyme for trypsin was 2.25 γ, for plasmin, 120 γ, and for chymotrypsin, 22.5 γ.) These curves permit the comparison of results obtained when the same concentration of substrate contains different amounts of radioactivity.

All subsequent enzyme measurements were made within the range of activity of the standard curves. In measuring an unknown amount of enzyme, the unknown was run in duplicate and the reference amount of enzyme was run in quintuplicate. The ratio was determined from the averaged results and the unknown amount of enzyme was read from the standard curve. In the standard curves, approximately 60 per cent of the radioactivity in the substrate was made soluble by the greatest amount of each enzyme used. Thus 3.75 γ of trypsin, 200 γ of plasmin, and 37.5 γ of chymotrypsin released approximately the same number of C. P. M. from the standard substrate in 30 minutes. (As will be shown later, this does not mean that these amounts of enzyme are precisely comparable in terms of proteolytic activity.)

In some instances it was necessary to measure small degrees of enzyme activity with greater accuracy than was permitted by the above standard curves. It was found that half the amounts of enzyme with half the amount of substrate (other conditions remaining the same) produced the same ratios of C. P. M. as those in the standard curves. It was, therefore, possible to measure smaller increments of enzyme activity by simply decreasing the reference amount of enzyme and the concentration of substrate proportionally and using the original standard curves with a proportional decrease in the values of the abscissa.

Measurement of Inhibition by Serum.—In the experiments concerned with inhibition of enzymes by serum, the serum and enzyme were mixed to give the desired amounts of each substance per 0.5 ml. of enzyme-serum mixture. After incubation at room temperature for 30 minutes, 0.5 ml. of the enzyme-serum mixture was used for determining residual enzyme activity in the manner described above for measuring unknown amounts of enzyme. Control tubes contained the same reagents as experimental tubes but no enzyme. Incubation periods as short as 15 minutes or as long as 45 minutes did not change the amount of enzyme inhibited. Throughout this work amounts of serum are expressed as milliliters of whole serum per 0.5 ml. of enzyme-serum mixture and amounts of enzyme are expressed as micrograms per 0.5 ml. of enzyme-serum mixture.

RESULTS

The Effect of Relative Concentrations on the Enzyme-Inhibitor Relationship

Three types of experiments gave information concerning the nature of the enzyme-inhibitor relationship; namely, (*a*) the effect of adding varying amounts of serum to a constant amount of enzyme; (*b*) the effect of adding varying amounts of enzyme to a constant amount of serum; and (*c*) the effect of varying the volume of enzyme-serum mixtures (*i.e.*, the effect of dilution).

a. In the first series of experiments, varying amounts of normal serum were added to fixed amounts of each enzyme. In one experiment, the largest amount of enzyme on the standard curves for each enzyme was used. Serum dilutions were chosen which would not completely inhibit this amount of enzyme. In the other experiment, a smaller amount of enzyme was used with the same dilutions of serum. This amount of enzyme was small enough to be completely inhibited by some of the serum dilutions used. The results with trypsin, plasmin, and chymotrypsin are shown in Figs. 4 to 6.

The curves represent the averaged results for 10 different sera with each enzyme. The amount of each enzyme inhibited was *directly proportional* to the amount of serum present throughout the entire range of inhibition.

b. The results of adding varying amounts of enzyme to a fixed amount of serum are shown in Tables I to III.

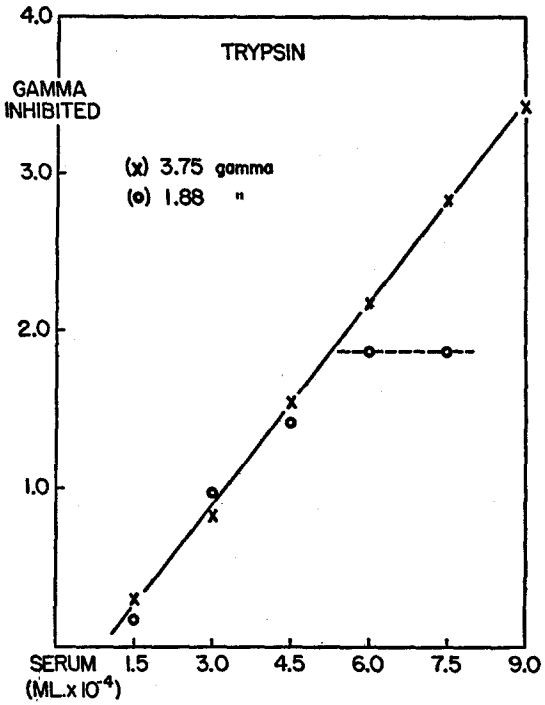

FIG. 4. The inhibition of trypsin by varying amounts of serum. Description in text. Amounts of serum used, 1.5 to 9.0 ml. $\times 10^{-4}$ per 0.5 ml. of enzyme-serum mixture. Conditions otherwise as in Fig. 1.

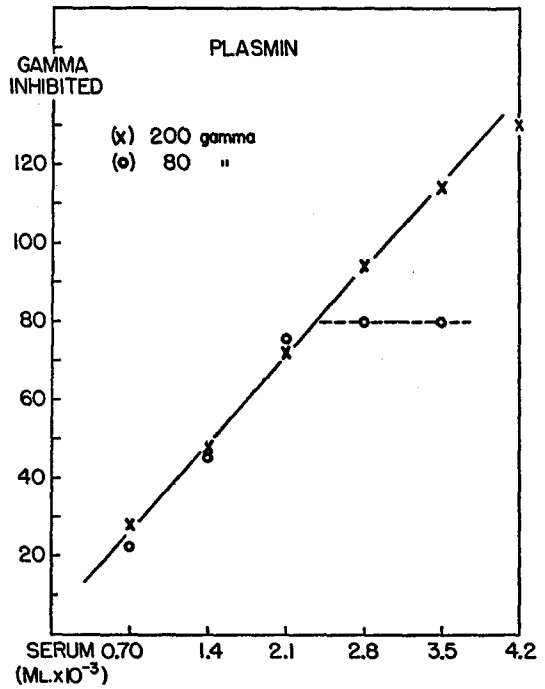

FIG. 5. The inhibition of plasmin by varying amounts of serum. Description in text. Amounts of serum used, 0.7 to 4.2 ml. $\times 10^{-3}$ per 0.5 ml. of enzyme-serum mixture. Conditions otherwise as in Fig. 1.

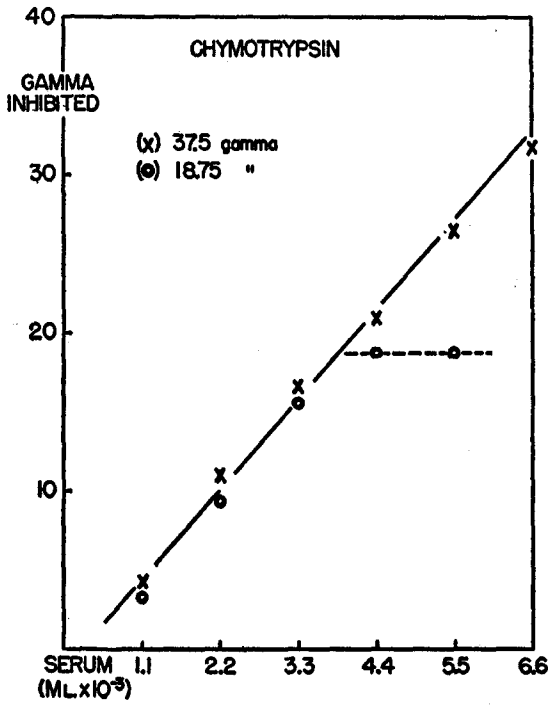

FIG. 6. The inhibition of chymotrypsin by varying amounts of serum. Description in text. Amounts of serum used, 1.1 to 6.6 ml. $\times 10^{-3}$ per 0.5 ml. of enzyme-serum mixture. Conditions otherwise as in Fig. 1.

TABLE I

Effect of Adding Varying Amounts of Trypsin to a Constant Amount of Serum

Serum concentration constant at 1.13 ml. $\times 10^{-3}$ per 0.5 ml. of enzyme-serum mixture. Experimental error in measuring trypsin activity = $\pm$ 0.4 γ.

Trypsin added	Trypsin activity	Trypsin inhibited
γ	γ	γ
2	0	All
3	0	All
4	0–0.4	$\sim$ 3.8
5	1.0	4.0
5.5	1.7	3.8
6.0	2.1	3.9
6.5	2.3	4.2
7.0	3.0	4.0

In each case the amount of enzyme inhibited by a fixed amount of serum showed no significant variation although considerably different amounts of enzyme were used. There was no change in the amount of enzyme inhibited even at low enzyme concentrations.

c. To ascertain the effect of dilution on the enzyme-inhibitor relationship, an initial enzyme-serum mixture containing 0.1 ml. of serum and 4500 γ of plasmin per 0.5 ml. was progressively diluted and 0.5 ml. of the various dilutions was used to determine enzyme activity. The dilutions of the initial mixture were allowed to stand for 30 minutes at room temperature before activity was determined. Results with plasmin are shown in Table IV, parts *A* and *B*.

TABLE II

Effect of Adding Varying Amounts of Plasmin to a Constant Amount of Serum

Serum concentration constant at 2.25×10^{-3} ml. per 0.5 ml. of enzyme-serum mixture. Experimental error in measuring plasmin activity = ± 10 γ.

Plasmin added	Plasmin activity	Plasmin inhibited
γ	γ	γ
50	0	All
60	0	All
70	0–5	~ 68
80	10	70
90	17	73
100	30	70
140	63	77
180	112	68
220	145	75

TABLE III

Effect of Adding Varying Amounts of Chymotrypsin to a Constant Amount of Serum

Serum concentration constant at 3.3×10^{-3} ml. per 0.5 ml. of enzyme-serum mixture. Experimental error in measuring chymotrypsin activity = ± 2.5 γ.

Chymotrypsin added	Chymotrypsin activity	Chymotrypsin inhibited
γ	γ	γ
10	0	All
15	0–3	~ 13.5
20	5	15
25	11	14
30	14	16
35	21.5	13.5
40	25	15
50	36	14

In part *A*, with the first 2 dilutions, plasmin activity appeared to increase slightly (column 3). Beginning with the 4th dilution, decreases in activity were proportional, within experimental error, to the degree of dilution. The measured amount of plasmin inhibited (column 4) was significantly greater than the calculated amount (column 5) up to the 4th dilution. In the 4th and subsequent dilutions the values of both were practically equal. The values in column 5 were calculated by extrapolation of the curve in Fig. 5, assuming a

TABLE IV

Part A. Changes in Inhibition upon Dilution of a Mixture of Plasmin and Serum

Substrate, 0.14 per cent tagged fibrinogen solution. Initial enzyme-serum mixture contained 0.1 ml. of whole serum and 4500 γ of plasmin per 0.5 ml. of mixture. pH 7.4 maintained throughout with M/15 phosphate buffer. The data recorded are from one experiment and are typical of the results of three other similar experiments.

Column 1, changes in serum concentration with dilution.

Column 2, changes in plasmin concentration with dilution.

Column 3, plasmin activity as measured at each dilution using 0.5 ml. of enzyme-serum mixture.

Column 4, apparent amount of plasmin inhibited = (column 2–column 3).

Column 5, expected amount of plasmin inhibited calculated from the direct proportionality observed in Fig. 5 between serum concentration and the amount of plasmin inhibited.

Column 6, difference between observed inhibition and calculated inhibition = (column 4–column 5).

The difference in terms of per cent of the amount of plasmin present in the various dilutions is also shown in column 6. The percentage value indicates the relative significance of the differences. Differences of 3.3 per cent or less were attributable to experimental error.

Part B. Effect of Lower Substrate Concentration on Changes in Inhibition with Dilution

Conditions as in part *A* except one-half the amount of substrate used. Substrate, 0.07 per cent tagged fibrinogen solution.

Dilution No.	1 Serum concentration	2 Plasmin in mixture	3 Plasmin activity	4 Plasmin inhibited (observed 2-3)	5 Plasmin inhibited (calculated)	6 Difference (4–5)	
	ml./0.5 ml. × 10^{-3}	*γ/0.5 ml.*	*γ/0.5 ml.*	*γ/0.5 ml.*	*γ/0.5 ml.*	*γ/0.5 ml.*	*per cent*
	100 (initial concentration)	4500	155	4345	3600	745	16.5
1	80	3600	159	3441	2880	581	16.0
2	60	2700	163	2537	2160	377	14.0
3	40	1800	159	1641	1440	201	11.0
4	20	900	150	750	720	30	3.3
5	10	450	75	375	360	15	3.3
6	5	225	40	185	180	5	2.2
7	2.5	113	24	89	90	−1	−1
8	1.67	75	15	60	60	0	0
	Part *B*—Substrate, 0.07 per cent tagged fibrinogen						
	100	4500	48	4452	3600	852	19
1	—	—	—	—	—	—	—
2	60	2700	68	2632	2160	472	17.5
3	—	—	—	—	—	—	—
4	20	900	73	827	720	107	12
5	10	450	53	397	360	37	8
6	5	225	30	195	180	15	6.5
7	2.5	113	20	93	90	3	2.7
8	1.67	75	17	58	60	−2	−2.7

direct proportionality between the amount of serum present and the amount of enzyme inhibited. The observed values (column 4) showed this direct proportionality in dilutions 5 through 9. In part *B* plasmin activity here clearly increased markedly with dilution (column 3). The increase was greatest at the 4th dilution, whereas it was greatest at the 2nd dilution in part *A*. The observed amount of plasmin inhibited was significantly greater than the calculated amount up to the 6th dilution. Decrease in activity proportional to dilution was evident only in the 7th and 8th dilutions.

With progressive dilutions of the plasmin-serum mixture, there was at first an absolute increase (not a relative increase) in enzyme activity. After the first few dilutions the apparent increase in enzyme activity was followed by a decrease in activity which became directly proportional to the degree of dilution.

TABLE V

Effect of Dilution on the Inhibition of Trypsin by Serum

Substrate, 0.14 per cent fibrinogen solution. Initial enzyme-serum mixture contained 8.3×10^{-4} ml. of whole serum and 6.0 γ of trypsin per 0.5 ml.

Serum concentration	Trypsin in mixture	Trypsin activity	Trypsin inhibited
ml./0.5 ml. $\times 10^{-4}$	γ/0.5 ml.	γ/0.5 ml.	γ/0.5 ml.
8.3 (initial concentration)	6.00	3.30	2.7
6.3	4.50	2.50	2.0
5.0	3.60	2.00	1.6
4.2	3.00	1.7	1.3
3.2	2.25	$\sim$ 1.00	$\sim$ 1.2

The apparent increase in enzyme activity with the first dilutions was more marked when a smaller amount of substrate was used with the same initial enzyme-serum mixture (Table IV, part *B*). With the smaller amount of substrate it was also found that proportional decrease in activity did not appear until a higher dilution of enzyme-serum mixture was reached. Serum, in relative abundance compared to the amount of substrate, appeared to effect a decrease in digestion of the substrate independent of its action of inhibiting the enzyme. This effect was less marked when a larger amount of substrate was used and could be completely neutralized by sufficient dilution of the serum. The retarding effect of relatively large amounts of serum on the digestion of the fibrin substrate may be due to competing substrates which are present in serum (see Discussion).

Determinations of the effect of dilution on the relationship between plasmin and inhibitor were therefore only valid for low concentrations of serum. The same was true when trypsin or chymotrypsin was used. Results of dilution experiments using a low initial concentration of serum with trypsin and chymo-

trypsin are shown in Tables V and VI respectively. In each case the enzyme activity decreased in direct proportion to the degree of dilution and the amount of enzyme inhibited remained directly proportional to the amount of serum present.

TABLE VI

Effect of Dilution on the Inhibition of Chymotrypsin by Serum

Substrate, 0.14 per cent fibrinogen solution. Initial enzyme-serum mixture contained 5×10^{-3} ml. of whole serum and 60.0 γ of chymotrypsin per 0.5 ml.

Serum concentration	Chymotrypsin in mixture	Chymotrypsin activity	Chymotrypsin inhibited
ml./0.5 ml. $\times 10^{-3}$	*γ/0.5 ml.*	*γ/0.5 ml.*	*γ/0.5 ml.*
5.0 (initial concentration)	60	34	26
2.50	30	15	15
1.67	20	12	8
1.25	15	10	6
1.00	12	7	5

The Effect of Substrate Concentration on Inhibition

1. Measurement of Reaction Velocity.—In order to determine the effect of substrate concentration on inhibition it was first necessary to determine what measures could be used to represent the velocity of the enzyme reactions. It was seen above, in the method of measuring proteolytic activity, that the change produced in the substrate in 30 minutes did not vary directly with the amount of enzyme used, and therefore the change produced in the substrate per unit time could not be used directly as a reflection of reaction velocity. In order to determine what measurements could be used to represent reaction velocity correctly, time-action curves were prepared for each enzyme.

The curves were prepared by incubating different amounts of enzyme with a constant amount of substrate for varying periods of time. The results, representing the average values of four experiments with each enzyme, are shown in Figs. 7 to 9.

Bodansky (12), referring to the work of Arrhenius and Osterhout, demonstrated, for the general case, the so called Qt rule: that the product of the time required for an enzyme to effect a given change in a substrate and the concentration of the enzyme is constant. He emphasized that in using the reciprocal of the time necessary to effect a given change in substrate as a measure of reaction velocity, it is necessary that the time-action function remain constant throughout the course of the reaction. If the ratio of the reciprocals of the time required to effect a given change in substrate has the same value for any change in the course of the reaction, it is evident that the time-action function has not changed. Application of the Qt rule to the data obtained with plasmin is shown

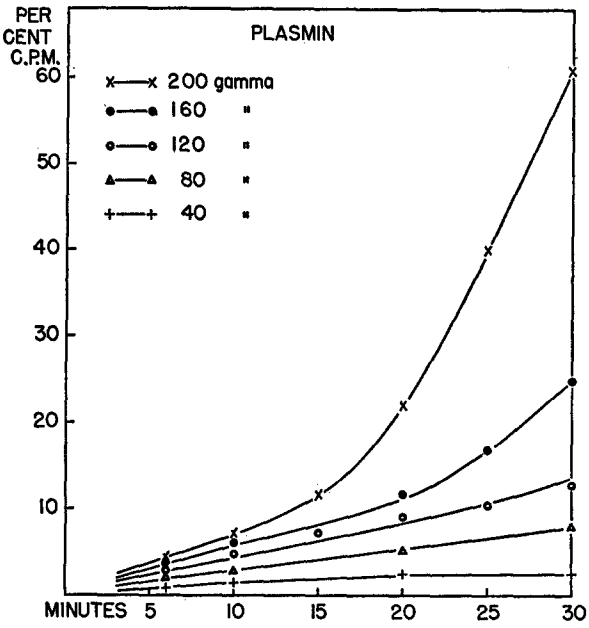

FIG. 7. Proteolysis with time of iodinated fibrin by different amounts of plasmin. Description in text. Conditions as in Fig. 1 except incubation time is varied. C. P. M. made soluble are expressed as per cent of the total C. P. M. in the substrate.

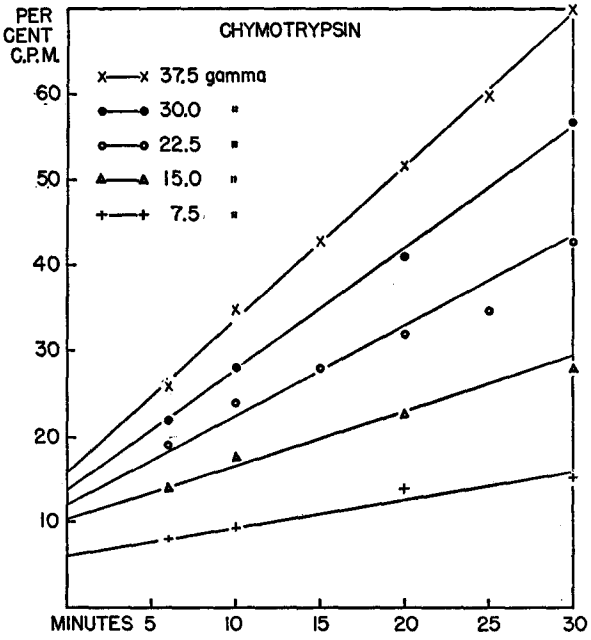

FIG. 8. Proteolysis with time of iodinated fibrin by different amounts of chymotrypsin. Conditions as in Fig. 7.

in Table VII. If the reciprocal of the time required to release up to 20 per cent of the C.P.M. from the substrate is taken as a measure of the reaction velocity,

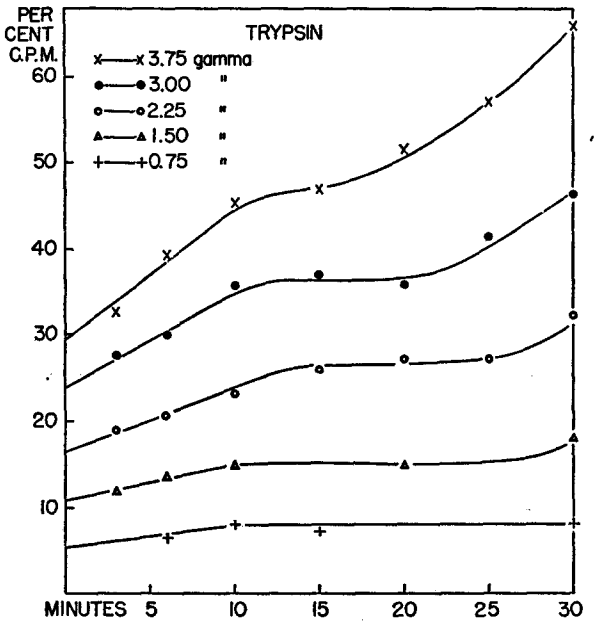

FIG. 9. Proteolysis with time of iodinated fibrin by different amounts of trypsin. Conditions as in Fig. 7.

TABLE VII

Ratios of Reciprocal Reaction Times at Different Stages of Reaction Using 4 Amounts of Plasmin

Description in text.

C.P.M. made soluble (as per cent of the total C.P.M. in the substrate)	Time required to effect the change in substrate with different amounts of plasmin				Ratios of reciprocal reaction times to 200 γ value		
	200 γ	160 γ	120 γ	80 γ	160 γ	120 γ	80 γ
per cent	*min.*	*min.*	*min.*	*min.*			
4	5.4	6.6	8.5	14.4	0.82	0.63	0.38
8	10.8	13.5	17.8	29.0	0.80	0.61	0.37
12	15.5	20.0	26.0	—	0.78	0.60	—
16	17.8	24.0	—	—	0.74	—	—
20	19.4	25.9	—	—	0.75	—	—
24	20.8	29.0	—	—	0.72	—	—
Expected ratios					0.80	0.60	0.40

there is no marked change in relative activity for the various amounts of enzyme. Accordingly, it was possible to use the time-action function to measure the reaction velocity of the enzyme plasmin with sufficient accuracy for

this work if less than 20 per cent of the substrate was affected in the course of the reaction.

Since the time-action curves for trypsin and chymotrypsin did not go through the origin, the Qt rule could not be applied. However, for chymotrypsin (Fig. 8), the slopes of the curves were proportional to the amount of enzyme used; and this proportionality remained constant even to the point at which 60 to 70 per cent of the C.P.M. were released from the substrate. The slopes of such curves provided an accurate measure of the reaction velocity of chymotrypsin.

In the case of trypsin (Fig. 9), the elevation of the curves extrapolated to zero time was more marked and more clear cut than with chymotrypsin. It was found that the degree of elevation of the curves with trypsin was proportional to the concentration of enzyme used, and that the slopes of the curves in the first 10 minutes of digestion were likewise proportional to the concentration of enzyme. The slope of the curve in the first 10 minutes of digestion was most suitable for measuring the reaction velocity of trypsin.

Some of the circumstances which affected the elevation of the extrapolated zero time intercept in the case of trypsin and chymotrypsin were determined. The degree of elevation was related to the amount of enzyme used, as pointed out above, but it was also related to the amount of thrombin used in clotting the fibrinogen. If the amount of thrombin was increased, the C.P.M. value at 3 minutes was lowered but the slope of the digestion curve with either enzyme was not changed. The elevation was present, although to less degree, when the amount of thrombin used was increased tenfold. If a constant amount of enzyme and thrombin were used together, a change in the sequence of mixing the reagents produced changes in the amount of elevation. If enzyme was added first and thrombin was then added, the elevation was higher than if thrombin was added first and enzyme was added in the short interval before clotting took place. It was also found that the time required to produce maximum clot formation was prolonged when trypsin or chymotrypsin was present in the reaction mixture. Iodinated fibrinogen, in the presence of the various other reagents used, was changed maximally to fibrin by the standard amount of thrombin within approximately 30 seconds as judged by the C.P.M. in the filtrate of the clot. In the presence of trypsin or chymotrypsin, the maximum amount of clotting that was possible required approximately 3 minutes to take place (although visible clot formation took place within 15 seconds). It seemed that trypsin and chymotrypsin produced rapid changes in either thrombin or fibrinogen or both, which prevented complete and rapid clot formation. It was possible to obtain reproducible results, however, by using a fixed amount of thrombin and a standard time sequence for adding reagents.

2. Measurement of Reaction Velocity with Varying Substrate Concentration in the Absence and Presence of Inhibitor.—

The effect of the concentration of substrate on the velocity of the reaction in the presence or absence of inhibitor may be evaluated by the graphical method of Lineweaver and Burk

(13), based on a transposition of the Michaelis-Menten expression (14). This mathematical expression was derived from the basic assumption that the enzyme and substrate form an intermediate complex which dissociates into free enzyme and products of the reaction: Enzyme + substrate $\rightleftarrows$ Enzyme-substrate complex $\rightarrow$ enzyme + products. On the basis of assumed theory the rate of the observed reaction is directly proportional to the concentration of the enzyme-substrate complex. The dissociation constant, K_s, of the enzyme-substrate complex is:

$$K_s = \frac{\text{Concentration of free enzyme} \times \text{concentration of substrate}}{\text{Concentration of intermediate}}$$

This dissociation constant does not necessarily represent a thermodynamic dissociation constant. The dissociation constant and the maximum reaction velocity at theoretically infinite concentration of substrate may be evaluated in terms of the rate of the observed reaction at different concentrations of substrate by employing the Lineweaver-Burk transposition of the Michaelis-Menten expression:

$$1/Vo = K_s/V_{max} \times 1/S + 1/V_{max} \quad (1)$$

in which Vo is the reaction velocity in the absence of inhibitor at concentration, S, of substrate; V_{max} is the reaction velocity in the absence of inhibitor at maximal (theoretically infinite) concentration of substrate; and K_s is the dissociation constant of the assumed intermediate enzyme-substrate complex. The reciprocals of the reaction velocities are plotted against the reciprocals of the substrate concentrations and estimates of V_{max} and K_s are made from the ordinate intercept and the slope of the straight line drawn through the experimental points.

A similar plot is made of the reaction velocities in the presence of inhibitor in accordance with the Lineweaver-Burk expression:

$$1/V = K'_s/V_{max} \times 1/S + 1/V_{max} \quad (2)$$

in which V is the reaction velocity in the presence of inhibitor at substrate concentration, S; V_{max} is the reaction velocity at maximal substrate concentration in the presence of inhibitor; and K'_s is the dissociation constant of the intermediate enzyme-substrate complex in the presence of inhibitor. A straight line drawn through the experimental points gives V_{max} in the presence of inhibitor and K'_s.

A comparison of the values obtained with and without inhibitor permits an evaluation of the type of inhibition. If V_{max} in the presence of inhibitor is less than V_{max} without inhibitor and K'_s has the same values as K_s, the findings are consistent with a non-competitive type of inhibition. In a competitive type of inhibition, V_{max} in the presence of inhibitor approaches the value of V_{max} without inhibitor, and K'_s is greater than K_s.

The Lineweaver-Burk method of analysis was applied to serum inhibition in the following way. The velocity of the reaction for a constant amount of enzyme was determined with various substrate concentrations. Simultaneously the velocity of the reaction for the same amount of enzyme in the presence of serum (sufficient to inhibit approximately 1/2 the activity at 0.14 per cent concentration of substrate) was determined at each substrate concentration. Velocities were determined by preparing time-action curves as described above (Figs. 7 to 9). With trypsin, the slope of the digestion curve in the first 10 minutes of digestion was used as a measure of reaction velocity, substantiated by the value of zero-time intercept. The intercept plus the slope accounted for approximately 50 per cent of the total C. P. M. in the least amount of substrate used. With chymotrypsin, velocity was measured by the slope of the digestion curve, the reaction being continued until approximately 50 per cent of the C. P. M. in the least amount of substrate used was made soluble. With plasmin, the reciprocal of the time required to effect a given change in substrate was used as a measure of

reaction velocity. The C. P. M. made soluble did not exceed 15 per cent of the total C. P. M. in the smallest amount of substrate used. Since C. P. M. made soluble are a direct reflection of the amount of substrate affected in the reaction, C. P. M. made soluble per unit time were converted to micrograms of fibrin made soluble in order to express reaction velocity in terms suitable for calculating dissociation constants.

Table VIII shows the velocities of action of trypsin in the absence and presence of inhibitor as the concentration of substrate was varied. Straight lines were drawn through the experimental points and the following values were obtained from the plot. For trypsin without inhibitor, $K_s = 1.20 \times 10^{-5}$

TABLE VIII

Effect of Substrate Concentration on Reaction Velocity of Trypsin in the Absence and Presence of Serum Inhibitor

Description in text. Molar concentration of fibrin based on the molecular weight of fibrinogen equal to 500,000. Theoretical reaction velocities in the absence and presence of inhibitor calculated in accordance with equations (1) and (2) in text.

Concentration of iodinated fibrin	Amount of fibrin made soluble per 15 min. using 3.75 γ of trypsin			
	No inhibition		+ serum 5 × 10^{-4} ml. per 0.5 ml. enzyme-serum mixture	
	Observed	Calculated	Observed	Calculated
molar × 10^{-6}	γ	γ	γ	γ
0.9	125	100	80	85
1.8	180	182	114	120
3.6	334	330	227	210
7.2	480	535	350	355
10.8	750	676	386	420
∞	1430*	1430	830*	830

* Extrapolated value.

mole per liter and $V_{max} = 1430\ \gamma$ of fibrin made soluble per 15 minutes. For trypsin with inhibitor, $K'_s = 1.08 \times 10^{-5}$ mole per liter and $V_{max} = 830\ \gamma$ of fibrin made soluble per 15 minutes.

The findings that V_{max} in the presence of inhibitor was less than V_{max} without inhibitor, and that K'_s had, within experimental error, the same value as K_s were consistent with the results expected for a non-competitive type of inhibition. Theoretical reaction velocities in the absence and presence of inhibitor, calculated in accordance with equations (1) and (2) above, were in good agreement with the experimentally determined reaction velocities.

Table IX shows the reaction velocities of chymotrypsin in the absence and presence of inhibitor. Straight lines drawn through the experimental points gave the following values. For chymotrypsin without inhibitor, $K_s = 3.68 \times 10^{-6}$ mole per liter and $V_{max} = 236\ \gamma$ of fibrin made soluble per 15 minutes.

For chymotrypsin with inhibitor, $K'_s = 3.40 \times 10^{-6}$ mole per liter and $V_{max} = 119\ \gamma$ of fibrin made soluble per 15 minutes.

K_s and K'_s were equal within experimental error, and V_{max} in the presence of inhibitor was less than V_{max} without inhibitor. The findings with chymotrypsin, just as with trypsin, were consistent with a non-competitive type of inhibition. The calculated reaction velocities with chymotrypsin and the experimentally determined reaction velocities were in accord.

TABLE IX

Effect of Substrate Concentration on Reaction Velocity of Chymotrypsin in the Absence and Presence of Serum Inhibitor

Conditions as in Table VIII.

Concentration of iodinated fibrinogen	Amount of fibrin made soluble per 15 min. using 37.5 γ of chymotrypsin			
	No inhibition		+ serum 4 × 10^{-3} ml. per 0.5 ml. enzyme-serum mixture	
	Observed	Calculated	Observed	Calculated
molar × 10^{-6}	γ	γ	γ	γ
1.15	53	56	32	30
2.3	86	91	55	48
4.6	140	131	66	68
9.2	156	168	86	87
13.8	189	186	89	96
∞	236*	236	119*	119

* Extrapolated value.

TABLE X

Effect of Substrate Concentration on Reaction Velocity of Plasmin in the Absence and Presence of Serum Inhibitor

Description in text.

Concentration of iodinated fibrinogen	Amount of fibrin made soluble per 10 min. using 120 γ of plasmin	
	No inhibition	+ serum 1.30 × 10^{-3} ml. per 0.5 ml. enzyme-serum mixture
molar × 10^{-6}	γ	γ
0.65	32	21
1.30	40	30
2.60	30	21
5.20	25	16

The effect of substrate concentration on the reaction velocity of plasmin was different from the effect on the other two enzymes. The reaction velocity of plasmin, in both the absence and presence of serum inhibitor, at first increased with additional substrate and then decreased on further addition of

substrate as shown in Table X. It appeared that excessive substrate concentration retarded the action of plasmin. From the data obtained it was not possible to evaluate the competitive or non-competitive nature of the inhibition of plasmin by serum.

DISCUSSION

The Method of Measuring Enzyme Activity.—

Various methods of measuring enzymes have been used in determining the antiproteolytic activity of serum. Casein has most frequently been used as a substrate, enzyme activity being correlated with the refractive index of the filtrate or the amount of acid-soluble tyrosine in the filtrate following precipitation of undigested substrate (3). Hussey and Northrop (7) used viscosity changes in a gelatin substrate to measure proteolytic activity; and the method of Anson and Mirsky (8) employing denatured hemoglobin as a substrate has also been used.

All these methods entail complications in measuring small amounts of enzyme in the presence of biological media. The matter of suitable blanks presents a problem, particularly when impure enzymes and substances such as serum are used with a casein or hemoglobin substrate, since the added reagents contain materials which are similar to the measured products of digestion. It is not possible to determine the effect of substrate concentration on enzyme inhibition using casein or hemoglobin substrates because the value of the blanks becomes so high that experimental variations due to enzyme activity are relatively insignificant. All of the methods require long periods of incubation of enzyme with substrate, during which time (2 to 12 hours), the enzymes employed may undergo considerable spontaneous inactivation (15). The difficulties of accurate measurement with the various methods became especially apparent when the enzyme plasmin was used, for this enzyme is contaminated by various other proteins and protein derivatives and its products of digestion are chiefly large molecular fragments that still have many of the characteristics of proteins (16). More recently, a method based on the time of complete digestion of a standard fibrin clot has been employed to measure small amounts of proteolytic activity (17). This method, while sensitive, has several limitations: the end-point is somewhat uncertain when the time of dissolution is prolonged; amounts of enzyme which are too small to digest the clot completely cannot be measured, and controls have very little meaning since complete clot dissolution is the only criterion of digestion.

In the present work, the method used was one that was developed to increase the sensitivity of the fibrinolytic method and to establish it on a quantitative basis (11). This method was found to be well suited for studying serum inhibition. It permitted accurate measurement of smaller amounts of enzyme than previous methods, and the incubation period could be made short enough to prevent spontaneous inactivation of the enzymes. The ability to measure smaller amounts of enzyme made it possible to use more highly diluted serum as an inhibitory solution. The desirability of using highly diluted serum in this work was evident from the dilution experiments reported above (Table IV), in which low dilutions of serum produced a non-specific retarding effect on the digestion of the substrate. With the method used, the blank depended

solely upon the substrate and was not contributed to by the other reagents used. The value of the blank presented no complication in measuring the effects of substrate concentration on enzyme activity.

The Methods of Measuring Reaction Velocity.—It was found that the time-change course of the reaction of each enzyme (Figs. 7 to 9) differed from that expected for a monomolecular reaction. It was not possible to evaluate the causes of deviation from the monomolecular pattern with the different enzymes. In the case of trypsin and chymotrypsin, some evidence was presented to suggest interaction with thrombin as at least one of the factors involved. The retarding effect of the *substrate* on plasmin activity (Table X) was one factor which would account for a change in the order of the reaction with that enzyme. The rate of change in substrate produced by the different enzymes is no doubt the resultant effect of many factors. Since the order of the reaction was different for each enzyme it was not possible to accurately compare their relative activities, although 3.75 γ of trypsin, 200 γ of plasmin, and 37.5 γ of chymotrypsin released the same number of C.P.M. from the standard substrate in 30 minutes.

With various methods of measurement it has frequently been found that proteolytic enzymes do not follow the law that the velocity of the reaction is directly proportional to the enzyme concentration (18). Bodansky (12), in considering the question of expressing the velocity of biochemical reactions, pointed out the numerous factors that may affect the monomolecularity of a reaction, particularly in an impure system. In the same paper he defined the proper use of various measures of reaction velocity when the course of the reaction cannot be expressed mathematically. It was possible in the present work to obtain values for reaction velocity that were in accord with these principles of measurement.

The Mechanism of Inhibition.—The inhibition of trypsin, plasmin, and chymotrypsin by serum exhibited the following properties: the amount of enzyme inhibited was directly proportional to the amount of inhibitor (serum) used; it was independent of the amount of enzyme used; the reaction between enzyme and inhibitor could not be reversed by dilution (as determined for low concentrations of serum). The inhibition of trypsin, plasmin, and chymotrypsin, therefore, appears to be a *stoichiometric and irreversible reaction.*

The dilution experiments (using high initial concentrations of serum, Table IV) in which enzyme activity increased with the first dilutions might suggest that the inhibitory reaction is reversible. This finding can be explained otherwise. The increase in activity was an absolute increase and not a relative one. If the inhibitory reaction were simply reversed by dilution, one would expect a relative increase in activity, but not an absolute increase. Furthermore, this effect was less marked when a larger amount of substrate was used, and could be completely neutralized by sufficient dilution of serum. These results are consistent with the hypothesis that substances present in serum

compete with the fibrin substrate for the enzyme. It is known that many substances in serum are digested by plasmin (19) as well as by trypsin and chymotrypsin. The apparent increase in enzyme activity with the first dilutions would then be due to the diminution of competing substrates present in serum. Proportional decrease in activity with dilution would appear only when serum was sufficiently dilute to prevent competition with the substrate. Throughout this work serum was used in sufficiently dilute form to prevent competition with the substrate.

The effect of substrate concentration on inhibition was studied in order to give further information concerning the mechanism of inhibition by serum. It was found that the inhibition of trypsin and chymotrypsin by serum was non-competitive with respect to the fibrin substrate. It was not possible to evaluate the competitive or non-competitive nature of the inhibition of plasmin. The finding of non-competitive inhibition is in accord with the finding of irreversible inhibition, for it would be expected that an irreversible type of inhibition would also be non-competitive.

In attempting to evaluate the competitive or non-competitive nature of the inhibition of plasmin it was found that the reaction velocity of plasmin at first increased with additional substrate and then decreased on further addition of substrate. This finding suggests that the substrate contains a specific plasmin inhibitor, or that plasmin is inhibited by excessive concentrations of substrate *per se* (18). It has been shown that iodine or iodide far in excess of the amount present in the substrate has no effect on the activity of plasmin (11). In the second paper of this series it will be shown that the retarding effect of the substrate on plasmin is probably due to a specific plasmin inhibitor present in fibrinogen as an impurity.

Previous workers (3, 7) concluded that the inhibition of trypsin by serum was a stoichiometric, but *readily reversible* reaction (they did not study the inhibition of plasmin or chymotrypsin). The data obtained in the present work indicate that the inhibition of trypsin by serum is a stoichiometric but *irreversible* type of reaction in contrast to the readily reversible type of reaction reported by previous workers. It is not practicable to resolve their results with those of the present work because of the marked differences in materials and methods used in studying an impure enzyme-inhibitor system. For instance, it is difficult to compare the relative concentrations of enzyme, inhibitor, and substrate used in the different studies; and it is not known in what way the different substrates affect the inhibition of trypsin by serum. Moreover, previous workers did not evaluate the effects of substrate concentration on inhibition or the non-specific effects of serum on the digestion of the substrate. The suitability of the various procedures used in studying serum proteolytic inhibition was discussed above; and it appears that the methods of measurement used in the present work are probably better suited for such a study than methods used previously.

SUMMARY

The mechanism of the inhibition of trypsin, plasmin, and chymotrypsin by serum was studied using fibrin tagged with radioactive iodine as a substrate.

Enzyme-inhibitor relationships were studied by: (*a*) varying the concentration of serum; (*b*) varying the concentration of enzyme; and (*c*) by diluting the enzyme-serum mixture. The results indicate that the inhibition of trypsin, plasmin, and chymotrypsin is a stoichiometric and irreversible reaction.

By using the Lineweaver-Burk graphical method of analysis it was demonstrated that the inhibition of trypsin and chymotrypsin is a non-competitive reaction. This finding supports the conclusion that inhibition by serum is an irreversible type of reaction.

The substrate was found to exert a retarding effect on the activity of plasmin. The possibility of a plasmin inhibitor in fibrinogen was suggested.

The suitability of the various procedures used in evaluating serum proteolytic inhibition was discussed.

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