The Effect of Certain Proteolytic Enzymes on the Thrombin-Fibrinogen Interaction*

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Trypsin promptly (0–2 seconds) retards the coagulation of fibrinogen by subsequently added thrombin. Prolonging the exposure to trypsin results in greater clotting interference, to complete incoagulability. If added soon (0–5 seconds), soybean trypsin inhibitor blocks this action. A progressive increase in fibrinogen relative to fixed trypsin also obviates the action of trypsin. When thrombin is added *before* trypsin, however, clotting is virtually unimpaired. The same phenomena are obtained with plasmin, which can be blocked by ϵ-aminocaproic acid as well as by soybean inhibitor, and with α-chymotrypsin preparations. The blocking effect of the latter is inhibited by soybean inhibitor and by β-phenyl propionic acid, and partially by indole. The data suggest that interference with thrombin clotting by trypsin, plasmin, and α-chymotrypsin is related to proteolysis at specific peptide site(s), and not to random fragmentation of fibrinogen at several different bonds or to evolution of an antithrombin. If trypsin, plasmin, or chymotrypsin reaches the susceptible loci first, the loci are rendered subsequently nonsusceptible to thrombin, and *vice versa*. In this way opposite biologic effects are obtained. Thus, trypsin, plasmin, α-chymotrypsin, and thrombin competitively seek and promptly reach certain vulnerable bonds in fibrinogen whose proteolytic cleavage by thrombin is crucial in clotting. From the known action of trypsin and plasmin it is inferred that the site of their blocking action is arginyl-glycyl peptide bonds. The observation that α-chymotrypsin splits *O*-toluenesulfonyl-L-arginine methyl ester (TAMe) suggests that this enzyme acts similarly, although other mechanisms by which the accessibility of these bonds to thrombin may be blocked by chymotrypsin cannot be excluded.

Proteolytic phenomena have long been considered closely related to the coagulation sequence (Alexander *et al.*, in press), and it has been established that the conversion of fibrinogen to fibrin by thrombin is due to the highly specific protease activity of thrombin. This enzyme acts by catalyzing the cleavage of two peptides from the fibrinogen molecule (Bailey *et al.*, 1951; Bettelheim and Bailey, 1952; Lorand, 1951), yielding fibrin monomer which subsequently polymerizes to give fibrin. The specific bonds influenced by thrombin are certain arginyl-glycyl linkages (Laki *et al.*, 1960). The high specificity of thrombin is also demonstrable on some synthetic substrates such as arginyl esters or amides (Sherry and Troll, 1954). Other proteases which catalyze hydrolysis at the same bonds are trypsin, papain, and plasmin (Kimmel and Smith, 1954; Neurath, 1957a; Troll *et al.*, 1954).

In connection with extensive studies on the proteolytic activation of certain clotting components (Alexander *et al.*, in press; Alexander and Pechet, 1962; Alexander and Pechet, 1958), a detailed investigation was undertaken on the action of several proteases on fibrinogen. The results with trypsin, plasmin, and thrombin herein described suggest that these enzymes competitively seek and reach at least one bond whose cleavage is crucial in the conversion of fibrinogen to fibrin. Similar observations with chymotrypsin raise other possibilities.

MATERIALS AND METHODS

Trypsin was two-times crystallized salt-free lyophilized material (Lot No. 595–6, Worthington Biochemical Corporation, Freeway, N.J.) with an activity of 9500 units of BAEe[1] esterase activity. Its activity was also measured by TAMe[1] assay (Sherry and Troll, 1954): 100 μg hydrolyzed 6.83×10^{-6} M of TAMe in 10 minutes at 37° in veronal buffer, *p*H 8.0. Trypsinogen (Worthington) was one-time crystallized, contained 50% $MgSO_4$, and when tested before use was found to be devoid of TAMe esterase activity. Plasmin was human material spontaneously activated in glycerol, kindly provided by Dr. Alan Johnson of New York University. It contained 70 caseinolytic units (Remmert and Cohen, 1949) per ml. α-Chymotrypsin (Lots No. 6021 and 6029) was

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[1] The following abbreviations are used in this paper: BAEe, benzoyl-arginyl-ethyl ester (assay quoted by Worthington Biochemical Corp.); TAMe, *O*-toluenesulfonyl-L-arginine methyl ester.

three-times crystallized, obtained from Worthington.

Thrombin was derived from purified prothrombin that had been separated from bovine plasma by barium sulfate adsorption, elution with sodium citrate, and fractionation by ammonium sulfate (Goldstein *et al.*, 1959). The prothrombin thus prepared was converted to thrombin by incubation in a 25% sodium citrate solution. The citrate was subsequently removed by dialysis (Seegers *et al.*, 1950). In most of the experiments Armour Company bovine plasma Fraction I, containing 50% sodium citrate, provided the fibrinogen. Material obtained by the method of Laki (1951) (90% clottable), as well as more highly purified fibrinogen[2] (97+% clottable) gave similar results. Soybean trypsin inhibitor (SBI) was crystalline material obtained from Worthington Biochemical Corporation. Indole was obtained from International Biochemical Corporation, Lot No. 1413, and from California Corporation for Biochemical Research, Lot No. 500230: β-phenyl propionic acid was obtained from K & K Laboratories, Inc., Lot No. 20070. All reactions were carried out in veronal-buffered isotonic saline solution, *p*H 7.4, and at room temperature.

Thrombin clotting times were determined with or without acacia-calcium in the reaction mixture (Seegers and Smith, 1942). The first appearance of visible granules was taken as the end-point. This was usually, but not always, followed by gelation.

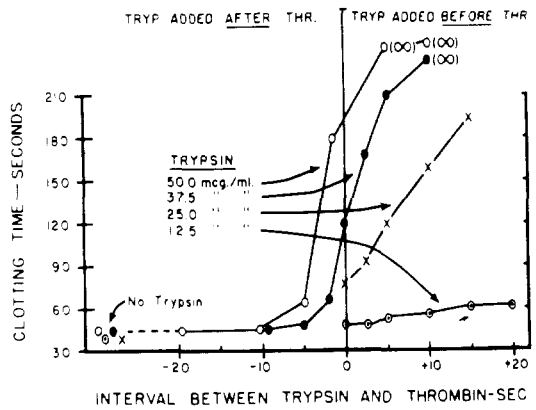

FIG. 1.—Inhibition by trypsin of thrombin-fibrinogen clotting. Abscissa indicates time interval between admixture of trypsin and thrombin with the fibrinogen. The 'O' value indicates simultaneous addition of both enzymes. Negative values refer to intervals at which addition of trypsin *followed* the addition of thrombin to the clotting mixture; positive values, intervals at which trypsin *preceded* the addition of thrombin. Reagents comprised 0.3 ml of acacia-Ca^{++} veronal-buffered isotonic saline (*p*H 7.4); 0.1 ml of fibrinogen solution containing 5.0 mg per ml, uncorrected for citrate content (50%); 0.1 ml trypsin solution; and 0.1 ml of thrombin solution containing 1.7 units per ml.

[2] Generously provided by Dr. M. Mosesson, National Institutes of Health. This material was devoid of plasminogen.

RESULTS

The admixture of trypsin with fibrinogen promptly retards or completely blocks its visible coagulation by thrombin (Fig. 1). The effect is increased by increasing the concentration of trypsin in relation to fibrinogen or by lengthening the interval of exposure of fibrinogen to the protease (Fig. 1, 2). When the thrombin is added after a 10-second exposure of the fibrinogen (0.42 mg[3] per ml) to trypsin (6.25[3] or 8.33[3] μg per ml) clotting is abolished. At higher trypsin concentrations this occurs even earlier. In the absence of acacia-calcium, the effects of trypsin are even more profound.

Experiments in which the thrombin was preincubated with trypsin before addition to fibrinogen indicated that the effects of trypsin were not referable to tryptic destruction of thrombin. Interaction of 25 μg of trypsin with 1.7 units of thrombin for 5 to 120 seconds did not affect the clotting action of thrombin. Much longer exposure and higher concentrations of trypsin were required to inactivate thrombin.[4]

Next explored was the quantitative relationship between the concentrations of trypsin and fibrinogen. After 10 seconds' exposure as little as 2.08[5] μg per ml of trypsin retarded the clotting of 0.42 mg per ml of fibrinogen in the presence of acacia-Ca^{++}; 4.2 μg produced a more profound effect, and at 6.25 μg per ml coagulation was completely abolished (Fig. 1). The last concentration represents a trypsin-fibrinogen molar ratio of about 0.11. Retarded clotting was evident at a ratio of 0.034.

Although remote, the possibility still remained that the action of trypsin was referable to proteolytic degradation of fibrinogen or fibrin monomer taking place at bonds other than those specifically involved in thrombin action. Were this true, the prompt, if not instantaneous, clot-delaying action of trypsin should also be demonstrable with reversal of the sequence with which the reactants are admixed. Thus, clotting should similarly be abolished when thrombin is added to fibrinogen *before* trypsin. As evident from Figure 1, however, trypsin added 10 seconds after thrombin to the fibrinogen in the presence of acacia-Ca^{++} did not retard clotting at all; when it was added 5 seconds after thrombin, clotting was only slightly retarded. It should be noted that the experi-

[3] The concentrations in the text are those in the final reaction mixture. Correction is made for the citrate content of Fraction I.

[4] Unpublished observations.

[5] The concentration figures in this paragraph represent concentrations in the final reaction mixtures. M.W. of trypsin assumed to be 24,000 (Neurath, 1957b) and of fibrinogen, 160,000 (Laki *et al.*, 1960).

ments were so designed as to give a thrombin-fibrinogen clotting time of 25–40 seconds, an interval sufficiently long to permit interference by trypsin, as demonstrated by the experiments (*vide supra*) where the adverse effects of trypsin were evident within several seconds after fibrinogen was exposed to trypsin. Even at a trypsin concentration of 8.3 μg per ml, which abolished coagulation completely when the trypsin was added *prior* to thrombin, reversal of the sequence, namely, addition of trypsin soon after thrombin, gave a clotting time not significantly different from that with thrombin alone.

Next studied were the kinetics. When trypsin was added to the fibrinogen 2–2.5 seconds *after* the thrombin, clotting was somewhat retarded (Fig. 1), but far less than when the trypsin was added *before* the thrombin. As the interval between the addition of thrombin and subsequent addition of trypsin was lengthened, interference by trypsin lessened, and when the hiatus was 5–10 seconds, clotting was virtually unimpaired. It is also noteworthy that the *simultaneous* addition of trypsin and thrombin to fibrinogen (the observation at "0" time), while retarding coagulation significantly, did not abolish it at a concentration of trypsin which, if it had been added *before* the thrombin, would have induced total incoagulability.

That clotting interference by trypsin appeared related to its proteolytic function was demonstrated with soybean trypsin inhibitor, which, *if added soon enough*, prevented this action (Fig. 2). As the interval between the addition of trypsin and the subsequent addition of soybean trypsin inhibitor was lengthened, trypsin interference with coagulation became progressively greater. Apparently the inhibitor must interact with the trypsin very early, before sufficient protease can reach the fibrinogen to exert significant blocking action. In this connection, it should be noted that the inhibitor interacts with trypsin instantaneously and irreversibly, blocking proteolytic activity (Kunitz, 1947).

Replacement of tryspin with albumin (Armour Co.) or casein, or their initial addition to the fibrinogen in the presence or absence of trypsin, did not affect either the clotting of fibrinogen or the clot-interfering action of the trypsin when it was added before thrombin. All of these data are in harmony with the concept that trypsin acts via a specific enzymatic property.

Nevertheless, the remote possibility remained that this action was not proteolytic, although as far as we know no other enzymatic activity except esterase and transpeptidase (Waley and Watson, 1951), has been attributed to trypsin. In this connection it was significant that negative results were obtained with trypsinogen, which in most, if not all, properties other than proteolytic resembles trypsin more closely than any other known naturally occurring protein.

Some question arises regarding the possible

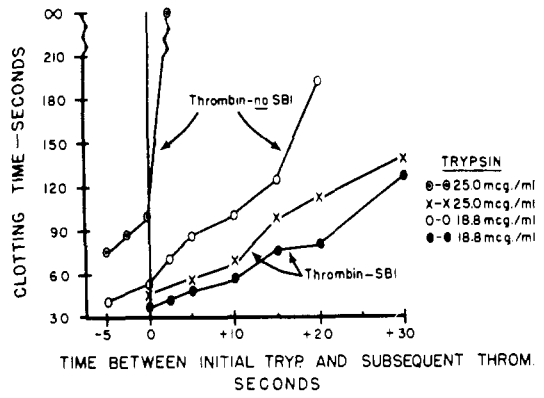

FIG. 2.—Effect of soybean inhibitor (SBI) on reaction to thrombin of fibrinogen exposed to trypsin. The inhibitor was added to the trypsin-fibrinogen combination in order to stop tryptic action at intervals shown on the abscissa, followed immediately by thrombin. For the soybean inhibitor experiments the 'O' point on the abscissa indicates *simultaneous* addition of trypsin and soybean inhibitor to the fibrinogen, immediately followed by thrombin.

role in the observed phenomena of acacia-Ca^{++}, which is ordinarily used in thrombin clotting-time determinations. Acacia and other colloids have been found to accelerate the thrombin-fibrinogen reaction (Seegers and Smith, 1942; Waugh and Livingstone, 1951), for reasons as yet obscure. When acacia-Ca^{++} was omitted from the reaction mixture, however, the interfering effects of prior exposure to trypsin were again evident, and, in fact, the competitive balance between trypsin and thrombin for fibrinogen seemed to be shifted in favor of trypsin at the expense of thrombin. Thus, with 4.2 μg per ml of trypsin, the clotting time observed when trypsin and thrombin were added simultaneously was 80 seconds in the presence of acacia-Ca^{++} (Fig. 1), in contrast to 96 seconds in their absence, despite the fact that the amount of thrombin employed in the latter experiment was 2.4 times greater.[6]

Also in this connection, it is noteworthy that in the absence of acacia-Ca^{++}, and with 4.2 μg trypsin per ml, thrombin had to be added at least 10 seconds *before* trypsin in order to escape trypsin interference entirely. With larger amounts of tryspin (6.25 μg per ml) this interval had to be even greater, and even then trypsin interference was only partially prevented. Of additional interest was the effect of acacia-Ca^{++} on fibrinogen reactivity to thrombin when trypsin was added *after* thrombin. The slope of the curve reflecting tryptic interference with the clotting of fibrinogen by thrombin added before trypsin was different when acacia-Ca^{++} was omitted. One may thus conclude that these reagents do not influence trypsin activity but rather

[6] This higher concentration was selected in order to obtain a control clotting time comparable to that of Fig. 1, when acacia-Ca^{++} was present.

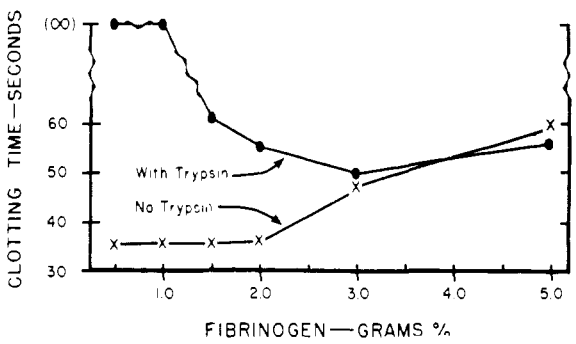

FIG. 3.—Effect of increasing fibrinogen concentration on tryptic interference with the thrombin-fibrinogen interaction. Abscissa indicates fibrinogen concentrations (uncorrected for citrate content) of the solutions of which 0.1 ml were incorporated in the reaction mixture containing other reagents as in Figure 1. Trypsin solution contained 37.5 μg per ml. Thrombin solution added 15 seconds after trypsin.

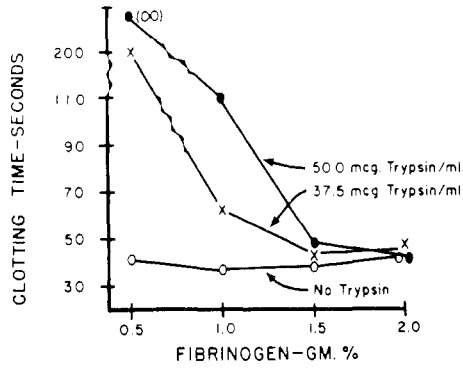

FIG. 4.—Similar to Figure 3 except that trypsin and thrombin were added *simultaneously* to solutions of varying fibrinogen concentrations.

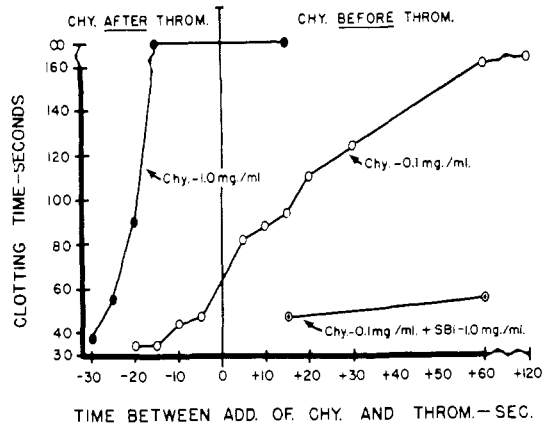

FIG. 5.—Effect of α-chymotrypsin on the thrombin-fibrinogen interaction. Reagents as in Figure 1 except for substitution of acacia-Ca^{++} by buffer, and replacement of trypsin by α-chymotrypsin. Data with soybean inhibitor indicate experiments wherein soybean inhibitor preceded the addition of chymotrypsin.

favorably affect thrombin action on fibrinogen, and at its earliest phase.

It has long been known (Ferry and Morrison, 1947; Waugh and Livingstone, 1951), and is presently confirmed by us (Fig. 3), that with increasing fibrinogen concentrations beyond approximately 2.0 g per 100 ml, visible gelation from a given amount of thrombin becomes progressively retarded. Although the phenomenon is probably referable to interference by fibrinogen or fibrinopeptides with sequential steps (polymerization of monomers, followed by formation of nuclei or fibrin) involved in clotting, it was nevertheless deemed desirable to explore the effect of increasing substrate (fibrinogen) concentration on the trypsin-thrombin phenomena. Experiments were performed in which trypsin was added both *before* and *after* thrombin to mixtures containing 0.42 to 4.2 g of fibrinogen per 100 ml. The addition of trypsin 15 seconds *after* thrombin gave the same clotting times as those obtained with thrombin alone, despite the fact that with each increment of fibrinogen above 1.7 g per 100 ml clotting was progressively retarded. When trypsin was added *before* thrombin, its effect on clotting decreased as the fibrinogen concentration increased (Fig. 3). When trypsin and thrombin were added *simultaneously*, the same effect was observed: as the fibrinogen was increased above 0.42 g per 100 ml, the effect of trypsin decreased progressively, disappearing altogether at 1.7 g per 100 ml (Fig. 4). These data also support the view that trypsin competes with thrombin for fibrinogen.

Similar results were obtained with α-chymotrypsin and plasmin (Fig. 5, 6). Soybean inhibitor abolished the interfering effect of both enzymes. Furthermore, the effects of α-chymotrypsin were partially blocked by indole (1.2×10^{-3} and 2.4×10^{-3} M in 0.021 M phosphate[7] buffer, *p*H 7.6) and completely blocked by β-phenyl propionic acid (3.0×10^{-3} M in phosphate buffer). These two agents also block interference with clotting by trypsin. The effects of plasmin were abolished by ϵ-aminocaproic acid (Fig. 7), which, however, had no effect on trypsin.

In view of possible contamination of α-chymotrypsin preparations with trypsin, which was used to obtain the active enzyme from chymotrypsinogen, additional experiments were performed with another lot of chymotrypsin (No. 6029). This preparation had the same effect on the thrombin-fibrinogen interaction. Moreover, both preparations had considerable TAMe esterase activity. At *p*H 8, 37°, in veronal buffer (0.02 M) and incubated for 30 minutes, batch No. 6021 split 2.80×10^{-6} M TAMe[8] per 100 μg, and sample No. 6029 split 3.0×10^{-6} M per 100 μg.

[7] It is noteworthy that indole does not block chymotrypsin in veronal buffer, in contrast to phosphate buffer.

[8] Calculated for 10-minute incubation period, in accordance with the method of Sherry and Troll (1954).

By the same method, 100 μg trypsin split 6.83 $\times 10^{-6}$ M TAMe. By the method of Ronwin (1956), 100 μg α-chymotrypsin (batch No. 6029) split 2.4 $\times 10^{-6}$ M TAMe. Thus, the chymotrypsin exhibited considerable TAMe esterase activity, about one third that of trypsin, irrespective of the method.

Of further interest is the observation that the plasmin or trypsin added, either after the thrombin or simultaneously, subsequently lyse the clot formed, irrespective of their effect on clotting time.

One possible mechanism whereby the proteases interfere with the thrombin-fibrinogen interaction is via antithrombin activity of the split products arising from fibrinogen proteolysis. "Antithrombin"[9] activity has been demonstrated *in vivo* after papain injection (Monkhouse, 1955) and *in vitro* after fibrinogen exposure to plasmin (Kowalski, 1960; Niewiarowski *et al.*, 1959). One of the products of the plasmin-fibrinogen interaction, "antithrombin VI," is said to inhibit thrombin protease activity by competitive inhibition (Niewiarowski *et al.*, 1959), and blocks fibrinogen clotting also by inhibiting the polymerization of fibrin monomers (Kowalski, 1960). Antithrombic activity has also been attributed to the fibrinopeptide part of fibrinogen released during coagulation (Laskowski *et al.*, 1952).

Experiments were performed to explore this possibility. Fibrinogen was exposed to the enzymes in the usual manner. In the controls soybean inhibitor was added before the enzyme (Fig. 8: *1A*, *1B*, *5A*, *5B*); in the test system the inhibitor was added at selected intervals after the protease (15 seconds: *2A*, *2B*, *3A*, *3B*, *6A*, *6B*, or 120 seconds: *4A*, *4B*, *7A*, *7B*) in order to stop proteolytic action. Thrombin was then added for clotting time determination. Fresh fibrinogen was added to comparable parallel mixtures, followed by thrombin. If the clot-interfering action of the protease were due to the antithrombic effect of a product of fibrinogen proteolysis, one would expect interference with the coagulation of fresh fibrinogen added to the mixture after an interval sufficient for the clotting of the original fibrinogen to be retarded. The data disprove the concept. The controls initially containing soybean inhibitor showed the usual thrombin clotting times, and the values obtained without (*1A*, *5A*) or with (*1B*, *5B*) additional fibrinogen were identical. As expected, after a 15-second exposure of fibrinogen to plasmin or trypsin, thrombin-induced clotting was retarded (*2A*, *3A*, *6A*), and this retardation was more pronounced consequent to a 120-second exposure (*4A*, *7A*). When, however, the reaction mixtures were provided with additional fresh fibrinogen after the protease was inhibited, clotting was at

[9] This term is used loosely to refer to interference with the clotting action of thrombin, not with any specific step involved in conversion of fibrinogen to fibrin.

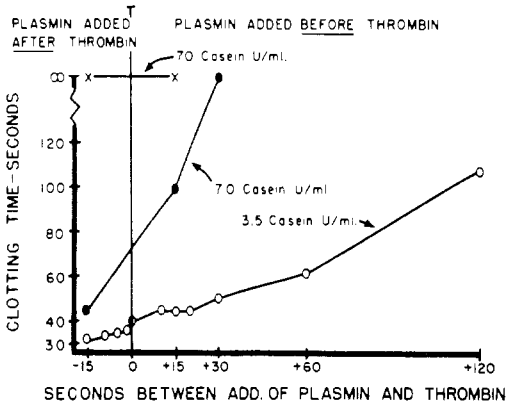

FIG. 6.—Effect of plasmin on the thrombin-fibrinogen interaction. Reagents as in Figure 5 except for replacement of chymotrypsin by plasmin.

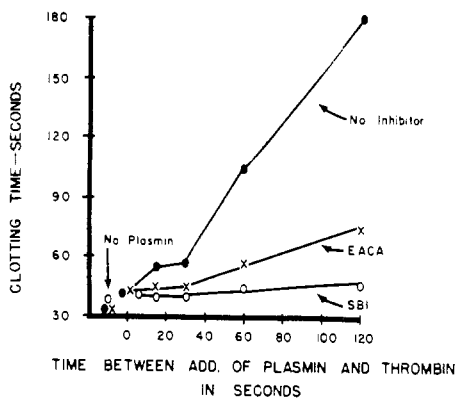

FIG. 7.—Effect of soybean inhibitor or ϵ-aminocaproic acid on clottability of fibrinogen exposed to plasmin. Reagents as in Figure 6; 0.1 ml of ϵ-aminocaproic acid (1.0 mg per ml) or 0.1 ml of soybean inhibitor (1.0 mg per ml) preceded the addition of plasmin.

most only minimally retarded (*2B*, *3B*, *6B*, *7B*), except perhaps in the instance of plasmin, where, after a 120-second exposure to the enzyme, the clotting of the added fibrinogen was retarded to 50 seconds (*4B*) compared with a control value of 36 seconds (*1B*). Without supplemental fibrinogen, plasmin retarded clotting of the initial fibrinogen to 200 seconds. These observations indicate that the prompt effects of the two enzymes cannot be attributed to an antithrombic entity arising from the interaction of fibrinogen with the enzyme. Although some "antithrombic" activity appears in time, it apparently arises long after the very short interval of fibrinogen exposure under the conditions in our experiments. Similar results were obtained with chymotrypsin.

DISCUSSION

Detailed consideration of the thrombin-induced molecular alterations in fibrinogen leading to visible fibrin formation is particularly pertinent to the blocking action of trypsin, plasmin, and

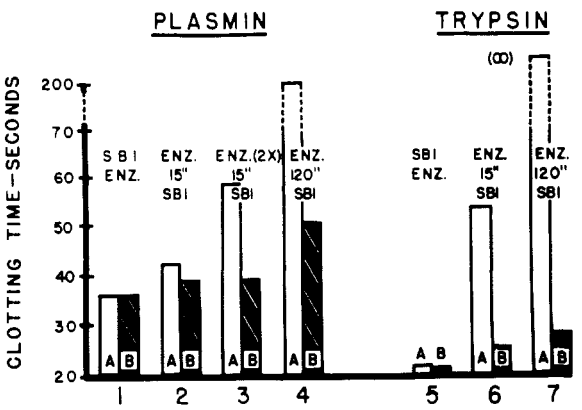

FIG. 8.—Antithrombic activity of products arising from interaction of fibrinogen with trypsin or plasmin. *1A:* 0.3 ml veronal buffered isotonic saline (*p*H 7.4); 0.1 ml of fibrinogen solution (Armour Fraction I), containing 5.0 mg per ml, uncorrected for citrate content (50%); 0.1 ml soybean inhibitor solution, containing 0.10 mg per ml; 0.1 ml plasmin solution, containing 7.0 caseinolytic units per ml. After incubation for 120 seconds, this reaction mixture was admixed with 0.1 ml of thrombin solution containing 9.0 units per ml. *2A:* Same as *1A* except that plasmin was added 15 seconds *before* the soybean inhibitor, and the mixture was incubated for 15 more seconds instead of 120. *3A:* Same as *2A* except strength of plasmin solution was double. *4A:* Same as *2A* except that plasmin was added 120 seconds before soybean inhibitor instead of 15. *5A:* Same as *2A* except that trypsin (50 μg per ml) replaced plasmin. *6A:* Same as *5A* except that trypsin was added 15 seconds *before* soybean inhibitor. *7A:* Same as *6A* except that trypsin was added 120 seconds before soybean inhibitor instead of 15. All "*B*" experiments same as their "*A*" counterparts except that 0.1 ml additional fibrinogen was provided to 0.4 ml of the reaction mixture, which contained the inhibitor. In *2B*, *3B*, *5B*, and *6B* the combination was incubated for an additional 15 seconds before the addition of thrombin. In *1B*, *4B*, and *7B* the incubation prior to addition of thrombin was 120 seconds.

chymotrypsin. Our experimental criterion has been grossly detectable clotting, which obviously makes it difficult to obtain kinetic data relevant to enzymatic action occurring anywhere in the sequence short of visible polymerization.

According to current concept (Scheraga, 1961) the conversion of fibrinogen (*F*) to fibrin by thrombin occurs via three steps: (1) proteolysis whereby $F \rightleftharpoons$ fibrin monomer (f) and two acidic polypeptides (P_A and P_B); (2) polymerization ($nf \rightleftharpoons f_n$); and (3) coagulation ($mf_n \rightleftharpoons$ fibrin). Although there is a recent report (Landaburu and Seegers, 1960) that thrombin also acts in the second step as a polymerase, and preponderance of evidence (Gladner *et al.*, 1959; Folk *et al.*, 1959a,b; Laki *et al.*, 1960; Scheraga, 1961) indicates that the major, if not sole, action of thrombin is as a unique enzyme that exhibits a very "limited proteolysis" operative in step one, where four highly specific arginyl-glycyl peptide bonds are hydrolyzed. As a consequence, donor (tyrosyl) and acceptor (histidyl) groups are thought to be liberated for the hydrogen bonding (Scheraga, 1961) essential for the polymerization in step 2. Arginine appears as carboxyl-terminal residues in both polypeptides, and four glycyl amino-terminal residues appear in the fibrin (Blombäck and Yamashina, 1958). The experiments in which coagulation was found to proceed virtually unimpaired when the thrombin was added shortly before the other proteases suggest that much of this chain of events is not relevant to the blocking action of the proteases.

It is unlikely that the immediate or rapid adverse effect of trypsin, plasmin, and chymotrypsin on fibrinogen clotting is due to random proteolytic fragmentation, since when thrombin was added first and quickly followed by the proteases they should still have had sufficient opportunity to hydrolyze the substrate at random. Yet, under these conditions the conversion of fibrinogen to fibrin proceeded essentially unaffected. From this viewpoint one could also argue that in some way thrombin protects the substrate against proteolysis by these other proteases.

These data indicate the need for critical examination of what transpires under our conditions in this early period when the proteases are added before or after thrombin. Also to be considered is the interval representing the clotting time that would normally follow the addition of thrombin if the proteases were not present. The enzymes presumably might or might not be exerting their effects before it would have become evident one way or the other from the clotting time under observation. In this regard the experiments with soybean inhibitor are especially significant. If we accept the evidence (Kunitz, 1947) that soybean inhibitor blocks trypsin[10] instantaneously by interacting with it stoichiometrically to form an irreversible stable compound, the soybean inhibitor data indicate that trypsin acts (in the absence of acacia-Ca^{++}) in at most 1 or 2 seconds if not instantaneously. Similar temporal relationships hold for plasmin and chymotrypsin, as well as for thrombin. We are thus primarily concerned with what happens during this short interval when the substrate is exposed to the enzymes which alter it in such a way as to give opposite biologic effects.

The protease function of thrombin, trypsin, and chymotrypsin is attributable to an identical small structural common unit, comprising a few amino acids in particular sequence: Gly·Asp··Ser·Gly (Gladner and Laki, 1958; Scheraga, 1961). Also, common to trypsin, plasmin, and thrombin is their ability to attack certain arginyl peptide or ester linkages. In the instance of thrombin the highly limited proteolysis results in release of P_A and P_B[11] and other less well understood alterations in the remaining major core of

[10] In contrast, soybean inhibitor does not inhibit thrombin (Guest and Ware, 1950).

the substrate protein (Scheraga, 1961). These latter associated changes implement end-to-end and side-to-side molecular aggregation, leading later to monomer and thence to polymer formation. Under the conditions of our experiments, this early phase must happen within several seconds at most, and concurrently the affected substrate becomes unassailable by trypsin despite the fact that the functional site of trypsin is identical to that of thrombin, and also that it operates almost instantaneously on at least one and the same locus in the substrate. But with trypsin, the effect is to alter the fibrinogen molecule so as to make it promptly invulnerable to thrombin. Conceivably, this may occur by enzyme-substrate complexing and blocking at the arginyl-glycyl peptide bonds whose cleavage is apparently essential for step one of the fibrinogen-fibrin conversion. It is also possible that the trypsin may simultaneously affect additional peptide structures (*e.g.*, lysyl, tyrosyl, histidyl) whose integrity may be necessary for the hydrogen bonding involved in step one. In this sense this enzyme would be exerting less limited proteolysis than thrombin.

Similar considerations are applicable to plasmin. Although less is known regarding the structure of its active center, it is known that plasmin catalyzes cleavage of the same synthetic esters as trypsin and thrombin (Sherry and Troll, 1954; Troll *et al.*, 1954; Ronwin, 1956), but whether it catalyzes hydrolysis of the arginyl-glycyl bond in particular is not known. From the great similarity in action between plasmin and trypsin, studied in detail by Ronwin (1956), it is reasonable to assume that it does. Such a fact would be in harmony with the concept that plasmin like trypsin acts on fibrinogen by preempting the specific site at which thrombin acts. Conversely, the action of thrombin at this site quickly precludes interference with clotting by plasmin, although the plasmin will lyse the subsequently formed clot in its usual fashion. Of additional interest is the observation that ϵ-aminocaproic acid substantially blocks the clot-interfering action of plasmin at 1.2×10^{-3} M, a level which is reported to have no inhibitory effect on plasmin (Alkjaersig *et al.*, 1959) but which partially inhibits the activation of plasminogen.

The action of α-chymotrypsin deserves special consideration. Preparations of this enzyme, derived from chymotrypsinogen by the action of trypsin, are frequently contaminated with trypsin. It is thus possible that the interference with clotting exhibited by the several chymotrypsin preparations[12] might be referable to trypsin contaminants. According to the manufacturer, both Worthington preparations contained less than 0.1% trypsin. Although soybean trypsin inhibitor has been used to exclude tryptic action in contaminated preparations (Inagami and Sturtevant, 1960; Neurath, 1957b; Neurath *et al.*, 1954), there is evidence that this inhibitor also blocks α-chymotrypsin, although much more weakly than trypsin (Wu and Laskowski, 1955). Thus, our findings that soybean inhibitor obviates clot interference by chymotrypsin is not particularly revealing, especially in view of the relatively high inhibitor concentration. The observations with the chymotrypsin inhibitors indole and β-phenyl propionic acid (Neurath, 1957b; Inagami and Sturtevant, 1960), are also not very helpful. Although clot interference by the α-chymotrypsin preparations was blocked by both, they also acted similarly on trypsin. Thus, no conclusions relevant to trypsin contamination could be drawn. Moreover, both chymotrypsin preparations exhibited considerable TAMe esterase activity. This finding is not in accord with an earlier report that α-chymotrypsin does not catalyze hydrolysis of this synthetic ester (Schwert *et al.*, 1948), although it does cleave α-benzoyl-L-arginine ethyl ester (Kaufman *et al.*, 1949), and simulates trypsin in the hydrolysis of α-benzoyl-arginine amide (Bergman *et al.*, 1949). Nevertheless, whether α-chymotrypsin can affect the hydrolysis of arginyl-*glycyl* bonds is unresolved.

It is generally accepted that chymotrypsin affects primarily tyrosyl-, phenylalanyl-, and tryptophanyl bonds, but not arginyl (Neurath, 1957a). Accordingly, the clot-interfering action of this protease may also be due to complexing with, or hydrolysis of, such linkages in fibrinogen. In accordance with this hypothesis we must at the same time postulate that thrombin added *before* chymotrypsin blocks or otherwise protects those specific sites from chymotryptic action.

The thrombin may do so in any of several ways: *e.g.*, it may induce steric hindrance consequent to its own complexing at arginyl-glycyl bonds that are geographically close to specific bonds vulnerable to chymotrypsin, or it may cause the rapid formation of fibrin polymer (albeit invisible), thereby making the chymotrypsin-vulnerable bonds relatively less accessible. As already mentioned, electron-donor tyrosyl groups functioning in hydrogen bonding (and polymerization) are thought to be liberated as a consequence of thrombic cleavage of polypeptide A. Conceivably the residues in fibrinogen involved in the clot-interfering activities of the several proteases could have the configuration Try·Gly·Arg. With respect to chymotrypsin, the prior addition of thrombin may thus protect certain tyrosyl bonds of special importance in fibrin formation.

Important in clarifying the subtle changes underlying the phenomena would be further studies of fibrinogen, or of the products resulting from fibrinogen, under the influence of the proteases within the extremely short interval under consideration. Little is known in this regard con-

[11] Lorand (1959) suggests that for coagulation only P_A need be formed, and P_B arises only as an extraneous action of thrombin not significant to clotting. If this is true, the number of possible explanations for the interfering effects of trypsin is reduced.

[12] Besides the two preparations referred to above, another preparation from Sigma Co. gave the same results.

cerning thrombin, and practically nothing concerning the other enzymes. Thrombin induces early release of nonprotein nitrogen (Lorand, 1952) and P_A and P_B, the latter containing tryosine - *O* - sulfate (Bettelheim, 1954). Under more physiologic conditions where tissue extracts are used to produce thrombin in plasma, P_A becomes demonstrable within 8 seconds (Shainoff and Page, 1960). Whether a detectable change appears sufficiently early, however, to fit our observations remains to be determined. The same holds for many physical properties of fibrinogen. With means at hand for stopping the enzyme-substrate interaction with specific inhibitors, exploration in this direction, already under way, appears promising.

In the meantime at least two possible mechanisms must be considered to explain the interference by the proteases with the clotting of fibrinogen by thrombin. First, thrombin may compete with trypsin and plasmin for fibrinogen. If the trypsin or plasmin precede thrombin they could quickly affect, among others, the few unique arginyl-glycyl linkages that are specifically involved in the proteolysis by thrombin of fibrinogen prior to its conversion to fibrin. Not entirely in accord with this concept are the findings with chymotrypsin, as well as recent evidence that the addition of thrombin to a plasmin digest of fibrinogen[13] still yields four additional moles of amino-terminal glycine residues per mole of original fibrinogen, as well as entities indistinguishable from polypeptides A and B (Wallén and Bergstrom, 1958). These data suggest the alternative possibility: namely, that besides arginyl-glycyl cleavage by thrombin, the integrity of the remaining core, the fibrin monomer, is essential for fibrin formation; that this core is readily vulnerable to the other proteases; and that fibrin polymer, less vulnerable or accessible, is virtually completely formed within a few seconds, long before visible clotting.

Of additional interest are the quantitative relationships. Apparently one mole of trypsin rapidly blocks 15 moles of fibrinogen from reacting with thrombin (2.6×10^{-6} M fibrinogen to 1.74×10^{-7} M trypsin). With lower trypsin concentrations, longer exposure is required before the phenomenon is evident. On the other hand, when thrombin is added before trypsin, the conditions permitting prompt and unimpeded thrombin action are evidently one mole of thrombin[14] for 310 moles of fibrinogen.

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[13] At maximal proteolysis plasmin splits 150 bonds of fibrinogen (Wallén and Bergstrom, 1958).

[14] Specific activity of bovine thrombin considered to be 1000 units per mg, derived from prothrombin calculated to have 2000 units per mg (Alexander, 1958). Molecular weight of thrombin considered to be 33,700 (Seegers, 1962).

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The Effect of Substituents on the Deacylation of Benzoyl-Chymotrypsins

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The effect of substituents on a single step of a chymotrypsin-catalyzed reaction was studied with isolated *para* and *meta* substituted benzoyl-chymotrypsins. Deacylation is facilitated by electron-withdrawing substituents, and a *rho* value of 2.1 was observed for p-CH_3O-, p-CH_3-, H-, p-Cl-, m-F-, and p-CF_3-benzoyl-chymotrypsins. The negative deviations of *para*- and *meta*-nitrobenzoyl-chymotrypsins from the linear relationship are attributed to steric effects of the bulky nitro group. The rates of deacylation of p-nitrobenzoyl-, anisoyl-, and benzoyl-chymotrypsins were found to depend upon the ionization of a group with a pK of 7.25–7.40. The hydrolysis of benzoyl-chymotrypsin at pH 8.24 is 3.6-fold faster in water than in deuterium oxide. This suggests, but does not prove, that the mechanism involves general base catalysis. The nonenzymatic reactions of a series of substituted p-nitrophenyl benzoates, studied for comparison with the enzymatic reaction, display *rho* values of 2.04 for alkaline hydrolysis, 1.19 for nucleophilic catalysis of hydrolysis by imidazole, and *ca.* 1.57 for a general base-catalyzed reaction with imidazole.

Certain acyl transfer and hydrolysis reactions catalyzed by chymotrypsin proceed by transfer of the acyl group of the substrate to the enzyme to form an acyl-enzyme, which is subsequently catalytically hydrolyzed (Neurath and Hartley, 1959). This acyl-enzyme is isolable when the substrate is an effective acylating agent and the rate of enzyme acylation greatly exceeds that of deacylation. The isolation of this covalently bound enzyme-substrate complex makes it possible to study the deacylation independent of the other reactions in the catalytic process, and to investigate the mechanism of this single step in the enzymatic reaction.

The acyl group of acyl-chymotrypsin is believed to be bound to the protein in an ester linkage (Bender, 1960). Since deacylation involves ester hydrolysis, a comparison of acyl-chymotrypsin hydrolysis with nonenzymatic esterolytic creations may be instructive in determining the nature of the enzymatic process. For example, if the important step in the deacylation involves a nucleophilic attack on the ester bond, as in alkaline saponification or nucleophilic catalysis, a large rate enhancement will be produced by electron-withdrawing substituents. If, however, the important catalytic process resembles acid catalysis of ester hydrolysis, in which there is a cancellation of the opposing polar effects on protonation and on hydrolysis of the protonated substrate, the rate would be expected to be insensitive to polar substituents.

The effects of changes in the electronic properties of substrates hydrolyzed by chymotrypsin and a number of other hydrolytic enzymes have been studied previously. In several of these studies the effects of substituents on substrate binding and on the hydrolytic step have not been separated and it is, therefore, not possible to attribute substituent effects solely to the hydro-

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