Kinetics of Conversion of Prothrombin to Thrombin by Biological Activators

I. DEMONSTRATION OF A PROTHROMBIN DERIVATIVE WHICH REACTS WITH PROTEOLYTIC ENZYME INHIBITORS*

N. RAPHAEL SHULMAN AND JOHN Z. HEARON

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda 14, Maryland

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Purified prothrombin can be converted into thrombin in different ways (2), namely, by the use of biological activators, by the action of high concentrations of sodium citrate, and by treatment with trypsin. Biological activation of prothrombin requires lipoprotein tissue-factor(s) that have prothrombin-converting or thromboplastic activity, protein serum-factor(s) which augment thromboplastic activity, and ionic calcium. Thrombin formed in the presence of these quasi-physiological activators has been referred to as "biothrombin." Most experimental evidence indicates that biothrombin is formed enzymatically from prothrombin; but the evidence has been interpreted variously as indicating that the enzymatic process is autocatalytic (3a), first order with respect to prothrombin (4), or involves a more complex sequence of reactions (2, 5).

Prothrombin conversion in citrate (6) resembles autocatalysis in that the time course of thrombin formation is a sigmoid curve and preformed thrombin must be added to initiate the reaction (5, 7, 8). The process of thrombin formation in citrate may involve extensive degradation or dissociation of prothrombin, for before thrombin formation, much of the carbohydrate and nitrogen content of the original prothrombin preparation becomes nonprecipitable in trichloroacetic acid; and prothrombin initially present becomes refractory to biological activators (9). "Citrate thrombin," which subsequently forms, has a lower molecular weight than prothrombin (4). These observations have led to the conclusion that one or more prothrombin derivatives may form before formation of "citrate thrombin." However, recent studies by Alexander (10) and Goldstein et al. (11) indicate that purified prothrombin that can be converted to thrombin by citrate is contaminated with other clotting factors, and therefore the significance of initial changes in physical properties of prothrombin preparations exposed to citrate is not clear.

Tryptic digestion of prothrombin results in release of large amounts of acid soluble material and formation of thrombin which has a lower molecular weight than prothrombin (4). It appears that "trypsin-thrombin" is an incidental consequence of nonspecific proteolysis.

There is little, if any, change in the physical properties of prothrombin when biothrombin forms; for acid soluble material is not released in significant amounts (2) and biothrombin appears to have the same molecular weight and electrophoretic mobility as prothrombin (2, 4). These observations suggest that transformation of prothrombin to "biothrombin" may not involve formation of other prothrombin derivatives.

STI (12) is a crystalline protein that inhibits a number of proteolytic enzymes and also delays coagulation of blood. Glendenning and Page (13) concluded from their study of the inhibition of "biothrombin" formation by STI that the inhibitor does not react with thrombin or with biological activators of prothrombin, but that it interferes with the generation of thrombin by forming a highly dissociable complex with prothrombin. Since STI also inhibited "citrate thrombin" formation, they suggested that the inhibitor might form a highly dissociable complex with a prothrombin derivative. Alkaerts, Deutsch, and Seegers (14) interpreted results of their study as supporting the latter possibility.

Our earlier investigations on the nature of the anticoagulant activity of a proteolytic enzyme inhibitor obtained from human plasma and urine, and of STI, indicated that these inhibitors combine with a derivative of prothrombin formed during the conversion of prothrombin to thrombin by biological activators (1, 15). The detailed kinetic analysis in the present report demonstrates the manner in which these inhibitors complex with a prothrombin derivative and elucidates some of the properties of the derivative and of the complex. The mechanism of inhibition is unusual, for it involves an intermediate derivative of a proenzyme participating in two essentially irreversible, competitive processes.

EXPERIMENTAL PROCEDURE

Prothrombin was separated from bovine plasma by the method of Seegers, Loomis, and Vandenbelt (16). The preparation used in this work contained 6.24% tyrosine by the Folin-Ciocalteu method (17) based on dry weight of protein, and its ultraviolet absorption curve in 0.1 N sodium hydroxide had essentially the same configuration as those reported by Seegers et al. (16, 18); the $\varepsilon_{280}$ at 280 mp was 10.8. Its specific activity, determined by the method of Ware and Seegers (19), was 23,200 units per mg of tyrosine, based on a prothrombin assay that gave 220 to 260 units of prothrombin per ml of human plasma (see below). Thus, this preparation had the characteristics of high quality

* Part of this work was presented at the Fifth Annual Symposium on Blood, January 21, 1956, Detroit (1).

\* The abbreviation used is: STI, soybean trypsin inhibitor.
purified prothrombin (18). Although some purified prothrombin preparations may be relatively unstable (18), it is noteworthy that the preparation used in this work showed no detectable loss of activity and no spontaneous activation when kept at 5°C in the lyophilized state over a 6-month period, or when kept at 5°C in water solution for several days.

The method used for converting prothrombin to thrombin by biological activator was essentially the "two-stage" procedure devised by Warner, Brinkhous, and Smith (20). Activation was carried out in siliconized tubes to prevent adsorption of thrombin on glass (18). The thromboplastin used was a NaCl suspension of acetone-extracted human brain (21); it contained no detectable accelerator or antithrombin activity. Highly diluted bovine serum was used as a source of accelerator activity (19) and is hereafter referred to as accelerator. The optimal accelerator concentration, 2.5 × 10⁻³ ml per ml; Ca⁺⁺, 2 × 10⁻³ m; thromboplastin, optimal by previous titration. Imidazole buffer, pH 7.4, in 0.147 M NaCl was used as a diluent. Time measured from addition of Ca⁺⁺.

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optimal without changing the thrombin yield or rate of prothrombin conversion. These various results agree with those obtained by Owren (3c) but differ somewhat from those obtained by Roka (5).

Ware and Seegers (23) have reported that purified prothrombin in NaCl solution at concentrations in the order of 10,000 units per ml may be inactivated by low concentrations of purified thrombin (in the order of 1 to 10 units per ml) or converted to thrombin by high concentrations of thrombin (in the order of 1000 units per ml). In the present work it was found that “biothrombin” from which thromboplastin was removed by sedimentation at 100,000 × g (22) neither inactivated nor activated prothrombin when NaCl solutions containing a mixture of as much as 40 units of thrombin per ml and 40 units of prothrombin per ml were incubated for as long as 6 hours at 28°. Our findings are similar to results obtained by Owren (3c).

The addition of increasing concentrations of ST1 to mixtures containing a fixed concentration of prothrombin and optimal concentrations of conversion factors produced progressive decreases in the final yield of thrombin (Fig. 2). The rates of thrombin formation in the inhibited reactions were proportional to the final yields of thrombin and the same as the rates of uninhibited reactions which produced equivalent final yields of thrombin (Fig. 2). Once the thrombin yield reached a plateau in an inhibited reaction, it remained fixed (Fig. 2); and no further formation of thrombin could be induced at this point by adding a surplus of conversion factors. Increasing the initial concentration of thromboplastin fourfold, of accelerator sixfold, and of ionic calcium threefold above optimal in mixtures that otherwise contained the same concentration of prothrombin and varying concentrations of ST1 as in Fig. 2, did not change the rates of thrombin formation or final yields of thrombin from those obtained with optimal concentrations of conversion factors.

2. Final Yield of Thrombin as Function of Relative Concentrations of ST1 and Prothrombin—The curves of Fig. 3 show the units of thrombin that were prevented from being formed when various concentrations of ST1 were added to each of five different concentrations of prothrombin. Reduction of thrombin yield approached a limiting value as the concentration of ST1 was increased, and a single concentration of inhibitor (7 µg per ml, vertical broken line) produced approximately 50% reduction in the final yield of thrombin with each concentration of prothrombin used. As shown in Fig. 4, the amount of thrombin prevented from being formed by a fixed concentration of ST1 was directly proportional to the concentration of prothrombin used.

3. Dependence of Inhibition on Rate of Prothrombin Conversion—All of the above results were obtained at the maximal rate of prothrombin conversion, i.e. with optimal or above optimal concentrations of conversion factors. The effect of decreasing the rate of prothrombin conversion on the inhibition of thrombin formation by ST1 is shown in Fig. 5. All curves in Fig. 5 were...
The optimal rate of prothrombin conversion, the thrombin yield obtained in the presence of the same concentration of ST1 when optimal accelerator concentration (Curve S) was slowed, the final yield of thrombin was the same as at the optimal rate of prothrombin conversion (Curve 1). The yield of thrombin obtained at a suboptimal rate of prothrombin conversion due to suboptimal accelerator concentration. Although the rate of prothrombin conversion in the uninhibited reaction with suboptimal accelerator concentration (Curve 3) was slowed, the final yield of thrombin was the same as at the optimal rate of prothrombin conversion (Curve 1). The yield of thrombin obtained in the presence of 1.0 \mu g of ST1 per ml at the slower rate of prothrombin conversion (Curve 4) was much lower than the yield obtained in the presence of the same concentration of ST1 when the rate of prothrombin conversion was optimal (Curve 2). At the optimal rate of prothrombin conversion, the thrombin yield was reduced by 3.8 units per ml, and at the slower rate by 18 units per ml.

The amount of inhibition produced by a fixed concentration of inhibitor was always inversely related to the rate of prothrombin conversion. It did not matter which conversion factor was used to vary the rate. Decreases below optimal of accelerator, thromboplastin, or ionic calcium concentration all increased the inhibition of thrombin formation produced by a given concentration of ST1. After the final yield of thrombin had been reached in an inhibited reaction carried out in the presence of a suboptimal concentration of any one of the conversion factors, addition of more of that conversion factor did not increase the thrombin yield.

In experiments done as in Fig. 5, but with accelerator decreased to approximately 2 \times 10^{-4} ml per ml or thromboplastin reduced to 0.1 optimal, the rate of prothrombin conversion was slowed to the extent that the final yield of thrombin in uninhibited reactions was reduced by 20 to 30\%. Decreased thrombin yield at very slow rates of prothrombin conversion is a well known phenomenon (e.g., (3c)). At these very slow rates of prothrombin conversion, STI at a concentration of 0.20 \mu g per ml further reduced the thrombin yield by as much as 20 units per ml when the initial concentration of prothrombin was 60 units per ml. In these experiments, increasing prothrombin concentration above 60 units per ml in the presence of 0.20 \mu g of ST1 per ml produced relatively small increases in the amount of thrombin formation inhibited, i.e., inhibition was not directly proportional to the final yield of thrombin, as it was in Fig. 4.

4. Indications that STI Combines with Prothrombin Derivative—
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Because the only identified substances in conversion mixture with STI were prothrombin, conversion factors, and thrombin, these various findings suggested that STI might combine with prothrombin. However, this possibility could not be reconciled with results of the following experiments in which the order of adding reagents was varied.

In one case, prothrombin and STI were incubated together for 30 minutes before conversion factors were added; and in the other case, STI and conversion factors were added simultaneously to prothrombin. A range of STI concentrations and prothrombin concentrations shown in Fig. 3, and the optimal and suboptimal concentrations of conversion factors shown in Fig. 5, were used in these experiments. Regardless of the order of adding reagents, both the thrombin yield and the rate of thrombin formation was the same at a given ratio of STI to prothrombin and a given concentration of conversion factors.

The finding that the order of adding reagents had no effect on the rate of thrombin formation or final thrombin yield in inhibited reactions indicated that any hypothetical combination between STI and prothrombin would have to occur so rapidly as to be completed before conversion factors, at any concentration, could act on prothrombin. Since it was evident from Figs. 2 and 5 that the substance that combined with STI was no longer available for transformation into thrombin by conversion factors, if prothrombin were the substance that combined with STI, then simply changing the rate of prothrombin conversion by varying the concentration of conversion factors should not have affected the yield of an inhibited reaction. The fact that inhibition of thrombin formation varied inversely with the rate of prothrombin conversion (Fig. 5) therefore was inconsistent with the possibility that the inhibitor combined with prothrombin. Since STI apparently did not act on conversion factors, thrombin or prothrombin, a remaining possibility was that the inhibitor combined with an intermediate derived from prothrombin during the formation of thrombin.

5. Nature of Prothrombin Derivative that Combines with STI—
Kunitz (27) has shown that trypsin and STI react together almost instantaneously to form a highly stable, chemically inert compound, the dissociation constant of which is in the order of 10^{-19} M (31). In view of the rapidity of formation and high stability of the STI-trypsin complex, it was considered that trypsin might displace STI from a possibly less stable STI-derivative complex. Results of adding trypsin to the reaction mixture at different times after the final yield of thrombin had been reached...
Fig. 6. Effects of adding trypsin to an inhibited reaction. Curve 1 was obtained with 25 units of prothrombin per ml without inhibitor; Curve 6 was obtained with the same concentration of prothrombin in the presence of 20 μg of STI per ml; optimal concentrations of conversion factors used for both curves. A large volume of reaction mixture containing reagents of Curve 6 was divided into aliquants and treated as follows. Curve 2 was obtained by adding 24 μg of trypsin per ml after 16 minutes; Curve 3, by adding 24 μg of trypsin per ml after 40 minutes; Curve 4, by adding 30 μg of trypsin per ml after 40 minutes, and Curve 5, by adding 20 μg of trypsin per ml after 40 minutes. Trypsin was added over a period of 10 to 15 seconds with vigorous stirring, the volume of trypsin solution added being 2/3 volume of the aliquant. Aliquants were brought to 0o just before trypsin was added and returned to 22o immediately afterwards. Controls for Curves 2 through 5 were aliquants of Mixture 6 to which the different amounts of trypsin were added along with sodium citrate to give a final 0.013 M citrate concentration. There was no thrombin formation in the controls for Curves 2, 3, and 5, but in the control for Curve 4, approximately 4 units of thrombin per ml formed within 10 minutes. Curve 7 was obtained by adding 40 μg of STI per ml to the mixture used to obtain Curve 2 ten minutes after trypsin was added, or by adding sodium citrate to a similar mixture to produce a 0.013 M citrate concentration.

Fig. 7. Formation of additional thrombin was much slower than the rate of formation of an equal amount of additional thrombin after STI was neutralized in the presence of optimal concentrations of conversion factors. Further formation of thrombin after neutralization of the inhibitor with trypsin could be prevented if thromboplastin was removed from the reaction mixture by sedimentation at 30,000 × g before trypsin was added. Thus it appeared that formation of thrombin from the derivative required the presence of the conversion factors that are necessary to convert prothrombin into thrombin.

In view of the fact that trypsin is capable of enzymatically converting prothrombin directly into thrombin in the absence of ionic calcium or biological converting factors (32), it should be emphasized that trypsin, in the amounts used to reverse an inhibited reaction (Fig. 6, Curves 2, 3, and 5), was not producing thrombin by direct enzymatic activity. If trypsin had acted to produce thrombin enzymatically, thrombin formation would not have been dependent on the concentration of ionic calcium or other conversion factors. Moreover, the amount of thrombin that formed when trypsin was used to reverse an inhibited reaction was much more than the amount of thrombin that could be formed from the same prothrombin preparation by the enzymatic action of trypsin. The maximal yield of thrombin that could be obtained by converting prothrombin to thrombin with trypsin was found to be approximately 40% of the yield obtained when prothrombin was converted to thrombin by biological activators (Fig. 7). In contrast, when trypsin was used to reverse
an inhibited reaction, the subsequent yield of thrombin approached the maximal yield that could be obtained with biological activators (Fig. 6, Curves 2 and 3). Trypsin added to solutions of preformed “biothrombin” in the absence of inhibitor produced only inactivation of thrombin, but the maximal yield of thrombin was progressively lower and there was subsequent inactivation of thrombin which had formed (e.g., Fig. 6, Curve 4). Results of experiments in which more than equimolar amounts of trypsin were added were no doubt due to the combined effects of neutralizing STI and the direct tryptic digestion of thrombin that formed. Relationships between the molar ratios of trypsin to STI, and additional amounts of thrombin formed, are considered further under “Discussion.”

6. Anticoagulant Activity of Various Proteolytic Enzyme Inhibitors—The purified proteolytic inhibitor obtained from human urine was substituted for STI in the series of experiments shown in Figs. 2, 3, 4, and 5; and in each case results with the urine inhibitor were qualitatively similar to those obtained with STI. When the two inhibitors were compared in terms of their trypsin-inhibiting activity (see “Experimental Procedure”), the amounts of urine inhibitor required for inhibition of thrombin formation, although similar, were not quantitatively the same as the amounts of STI required. For example, when the concentration of prothrombin and conversion factors was the same as in Fig. 2, an amount of urine inhibitor (13.2 μg) sufficient to inhibit the activity of 6 μg of trypsin prevented formation of 19 units of thrombin per ml, whereas an amount of STI (5 μg) sufficient to inhibit 6 μg of trypsin prevented formation of 12 units of thrombin per ml. The initial slope of curves plotted as in Fig. 3 (with abscissa changed to trypsin-inhibiting activity) were steeper, and asymptotic values were higher with the urine inhibitor than with STI; but otherwise the curves had the same characteristics as those in Fig. 3. Just as in Fig. 4, the amount of thrombin formation inhibited by a fixed concentration of urine inhibitor was directly proportional to the concentration of prothrombin used. In experiments done as in Fig. 5, decreases in the rate of prothrombin conversion had less influence on the degree of inhibition obtained with the urine inhibitor than with STI. For example, with the decreased rate of prothrombin conversion in Fig. 5, there was approximately a fivefold increase in the inhibition produced by a fixed concentration of STI, but a two- to threefold increase in the inhibition produced by a fixed concentration of urine inhibitor.

The other inhibitors tested were the pancreatic trypsin inhibitor, lima bean trypsin inhibitor, and ovomucoid trypsin inhibitor. These inhibitors did not inhibit thrombin formation when used at concentrations at least 30-fold greater, based on trypsin-inhibiting activity, than the concentrations of STI and urine inhibitor which markedly inhibited thrombin formation (Table I).

**DISCUSSION**

**Analysis of Asymptotic Properties of System**—The following analysis of the characteristics of inhibited and uninhibited prothrombin conversion reactions is based mainly on those proper-

![Fig. 7. Comparison of thrombin yields produced by conversion factors and by trypsin alone. Curve I was obtained with 31 units of prothrombin per ml in a reaction mixture containing optimal concentrations of conversion factors. For Curves 2 through 5, reaction mixtures contained 31 units of prothrombin per ml and varying concentrations of trypsin in imidazole-buffered saline, pH 7.4, at 25°; trypsin concentration, Curve 2, 2.8 μg per ml; Curve 3, 4.25 μg per ml; Curve 4, 1.1 μg per ml, and Curve 5, 0.55 μg per ml. The same yields of thrombin were obtained when 0.013 M sodium citrate was present in the mixtures of prothrombin plus trypsin. The fibrinogen solution used for assaying units of thrombin contained 10 μg of ST1 per 0.5 ml.**

**TABLE I**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Trypsin inhibited per μg of inhibitor</th>
<th>Final concentration of inhibitor in reaction mixture</th>
<th>Thrombin formation inhibited*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy bean</td>
<td>1.2</td>
<td>2.5</td>
<td>1.25 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urine (plasma)</td>
<td>0.46</td>
<td>6.6</td>
<td>1.25 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lima bean</td>
<td>2.2</td>
<td>50.0</td>
<td>4.63 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>0.7</td>
<td>150.0</td>
<td>4.38 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>1.3</td>
<td>80.0</td>
<td>4.34 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Reaction mixture for measuring inhibition of thrombin formation contained 25 units of prothrombin per ml and optimal concentrations of conversion factors.
† Molar concentration based on trypsin-inhibiting activity, assuming a trypsin to inhibitor ratio, 1:1, and molecular weight of trypsin, 24,000.
ties of the system that are related to the final yields of thrombin. For simplicity, STI, or inhibitors that act like STI, prothrombin, and thrombin are denoted by S, P, and T, respectively; the term "factors" refers collectively to Ca^{++}, thromboplastin, and accelerator, any one of which is designated as a factor. The amount of inhibition is defined as the difference between the final yields of T in the absence and presence of S; the degree of inhibition is defined as the amount of inhibition divided by the initial concentration of P. There are several distinctive properties of the system which must be accounted for by any proposed reaction scheme or kinetic model. They may be summarized as follows. (a) For varying initial concentrations of P in the absence of S, the final yield of T is proportional to the initial concentration of P (Fig. 1). (b) At a given concentration of factors, the presence of S decreases not only the rate of formation of T, but also the final yield (Fig. 2). (c) The degree of inhibition produced by a given concentration of S is independent of the initial concentration of P; for example, in Fig. 3, at one concentration of S the degree of inhibition is one-half for each concentration of P. Therefore, the amount of inhibition produced by a given concentration of S is proportional to the initial concentration of P (Fig. 4). (d) For given initial concentrations of P and S, the degree of inhibition is a function of the concentration of the factors; specifically, the degree of inhibition is increased as the rate of conversion of P to T is decreased by lowering the level of one or more of the factors (Fig. 5).

It appears necessary to assume that S enters whatever reactions in which it participates in an essentially irreversible manner. For, if the overall P-to-T conversion is irreversible (and there is no evidence to indicate that T, in the presence of factors, is converted to P or any other species), no reversible combination can account for the decreased final yield in the presence of S. The evidence indicates (Section 4) that S combines with P or some derivative or intermediate formed from P, and not with T or any of the factors. The evidence further indicates that it is not P itself that combines with S. It is demonstrable (Section 5) that in the presence of S there is stored or sequestered, in a form not convertible to T, a species which can be regenerated by the addition of trypsin and converted to T in the presence of the biological conversion factors. On the basis of the above observations it is possible to construct relatively simple reaction schemes which predict properties (a) through (d) above and which are consistent with the qualitative findings of Section 4. When the analysis is confined to the study of final yields there is a fairly broad class of models, any one of which is consistent with the observed properties of the system. In a forthcoming paper, additional models are extensively analyzed. In what follows we present a minimal model which exhibits properties (a) through (d) and which has additional implications that are discussed.

Consider the model

\[ P \xrightarrow{k} X \xrightarrow{rS} C \xrightarrow{\alpha} T \]

where X is an intermediate, C is a complex between X and S, k is a formal first order rate constant, and \( r \) is the second order rate constant for the combination of S and X. The rate equations for T and C are written as

\[ \frac{dT}{dt} = kX \]

\[ \frac{dC}{dt} = rSX \]

Assuming S has an essentially constant value, \( S_0 \), Equations 1 and 2 can be integrated, under the conditions \( T = C = 0 \) at \( t = 0 \), to give Equations 3 and 4 below. The essential constancy of S can be validated both in terms of the time course of the yield of thrombin and the final yield, but we remark in passing that it is all but evident that no model can lead to property (c) without this condition. Given this condition, we have

\[ T(\infty) = kA \]

\[ C(\infty) = rS_0A \]

where \( A = \int_0^\infty X(t) \, dt \). But clearly if \( P_0 \) is the initial value of P

\[ T(\infty) + C(\infty) = T(\infty) \left[ 1 + \frac{C(\infty)}{T(\infty)} \right] = P_0 \]

which with Equations 3 and 4 gives

\[ T(\infty) = \frac{P_0}{1 + \alpha S_0} \]

where \( \alpha = r/k \). The amount of inhibition, \( I = P_0 - T(\infty) \) is then

\[ I = \frac{P_0S_0}{1 + \alpha + S_0} \]

Equation 6 gives the final yield as a decreasing function of \( S_0 \). It predicts that \( T(\infty) = P_0/2 \) when \( \alpha S_0 = 1 \) and from Equation 7 the corresponding degree of inhibition is one-half when \( S_0 = 1/\alpha \) and this point of one-half inhibition is independent of \( P_0 \) (cf. Fig. 3). The amount of inhibition is proportional to \( P_0 \) and according to Equation 7, the slope of I against \( P_0 \) increases with \( S_0 \), approaching unity as \( S_0 \) becomes large (cf. Fig. 4). Finally, from Equation 7 the degree of inhibition for given \( S_0 \) increases as \( \alpha \) increases. Therefore, since \( \alpha = r/k \), any change in the system which decreases \( k \) should increase the degree of inhibition at a fixed \( S_0 \). This is in accord with experimental fact if \( k \) is an increasing function of the concentration of each factor. That \( k \) is indeed such a function is supported by the facts (Section 5) that in an inhibited system which has attained its final yield, additional \( T \) is formed, after \( S \) is neutralized with trypsin, only in the presence of Ca^{++}, and given the presence of Ca^{++}, the rate of production of \( T \) after neutralization of \( S \) is an increasing function of accelerator and thromboplastin. Therefore, the above reaction scheme correctly predicts all of the asymptotic properties of the system, i.e. the final yield as a function of \( P_0 \) and \( S_0 \), as well as the rate dependence of the degree of inhibition as it is influenced by the concentration of the factors.

In the above scheme, the functional form of Equation 6 and hence of Equation 7 is a consequence of the assumption of a molecular-mole combination of \( S \) with \( X \). The data on the final yields of thrombin are clearly consistent with this assumption. In a
forthcoming paper, it is shown that the data on the time course of thrombin production are inconsistent with any scheme that supposes a different stoichiometry. In view of the evidence that $S$ and $X$ combine mole for mole, the following apparent stoichiometric relationships have significant implications.

Implications Concerning Prothrombin and Its Derivatives—The best preparations of purified prothrombin are capable of yielding approximately 2000 units of thrombin per mg under optimal conditions of conversion (18); the molecular weight of prothrombin is approximately 69,000 (4); and the molecular weight of STI is 20,000 (31). In experiments done as in Fig. 5, but with higher prothrombin concentrations and slower rates of prothrombin conversion, the thrombin yield could be reduced by at least 20 units per ml with STI at a concentration of 0.20 $\mu$g per ml (Section 3). Assuming that in these experiments STI was not in surplus, that prothrombin was 100% pure, and that all prothrombin molecules were changed to a derivative in the pathway of thrombin formation, then the apparent molar ratio of derivative to STI in the complex would be a minimum of 16:1. At even slower rates of prothrombin conversion in 25% sodium citrate, the apparent molar ratio of prothrombin to STI required for inhibition was found to be a minimum of 40:1 by Alkjaersig, Deutsch, and Seegers (14). Since all of the characteristics of inhibitory reactions can be explained only on the basis of a 1:1 irreversible combination between STI and derivative, one could conclude that the molecular species that can be converted to thrombin by biological activators (or by 25% sodium citrate) accounts for no more than 6% (and possibly as little as 2%) of a purified prothrombin preparation. Considering the physical homogeneity of purified prothrombin (4), it is unlikely that this percentage represents the degree of purity. It may reflect, rather, the fraction of a single molecular species that is converted by biological activators into thrombin. Trypsin is an example of a prothrombin activator that appears to convert even a smaller fraction of prothrombin molecules into thrombin; for the yield of "trypsin-thrombin" is approximately 25% the yield of thrombin obtained with biological activators (Fig. 7). Thus, trypsin appears to act on prothrombin in more than one way; and for every molecule that is changed to a form that has thrombin activity, apparently many more are altered in such a way that they have no thrombin activity. Biological activators (and 25% sodium citrate), which convert more prothrombin to thrombin than does trypsin, nevertheless may change a large proportion of prothrombin into derivatives that do not yield thrombin. This would suggest that if thrombin could be physically separated from other prothrombin derivatives, or if there were a means of converting every prothrombin molecule to thrombin, then thrombin would have manyfold the specific activity ascribed to it so far.

Approximation of Association Constant of Derivative-STI Complex—In experiments in which trypsin was added to inhibited reactions to produce further formation of thrombin (Section 5, Fig. 6), amounts of trypsin equimolar with respect to STI produced approximately 50% the maximal thrombin yield, amounts less than 0.8 equimolar produced no appreciable additional thrombin, and effects of amounts greater than equimolar were complicated by the presence of free trypsin. Since trypsin reversed inhibition apparently by displacing STI from the derivative-STI complex, the amount of bound derivative freed at a particular molar ratio of trypsin to STI would be a function of the relative affinities of trypsin and the derivative for STI. Given the association constant of the trypsin-STI complex, an order of magnitude estimate of the association constant of the derivative-STI complex was obtained in the following manner. After the addition of trypsin, the competing equilibria are

$$X + S \rightleftharpoons C_1$$

$$E + S \rightleftharpoons C_2$$

where $X$, as before, is the prothrombin derivative, $C_1$ is the complex previously denoted by $C$, $E$ is trypsin, and $C_2$ is the trypsin-S complex. In the absence of trypsin, Reaction 8 is exceedingly far to the right with a vanishingly small concentration of $X$, for it has been seen that stable yields of thrombin obtained in a matter of minutes remain constant for many hours. If the association constants for Reactions 8 and 9 are $K_1$ and $K_2$, respectively, then the mass action expressions are

$$C_1/X = K_1S$$

$$C_2/E = K_2S$$

The totality conditions are

$$X_0 = X + C_1 = X(1 + K_1S)$$

$$S_0 = S + C_1 + C_2$$

$$E_0 = E + C_2$$

Since the data on final yields are consistent with the assumption that $S$ is essentially constant (see above), the term $C_1$ in Equation 13 can be neglected with respect to $S_0$. If $C_1$ is negligible to good approximation in the absence of $E$, it is certainly negligible when $E$ is competing with $X$ for $S$. Neglecting $C_1$ in Equation 13, Equations 10 through 14 give

$$S_0 = S + \frac{E_0S}{K_2 + S}$$

where $K_2$ is the dissociation constant, i.e. $K_2 = 1$. Solution of Equation 15 for $S$ in terms of the molar ratio $f = E_0/S_0$ gives

$$2S = (1 - f)S_0 - K_2 + (((f - 1)S_0 + K_2) + 4K_2S_0)$$

For a fixed value of $S_0$ and given $K_2$, $S$ can be computed from Equation 16 for all values of $f$. Then

$$X/X_0 - 1/(1 + K_2S)$$

gives the proportion of the derivative freed under these conditions for any assigned value of $K_2$. In the experiments being discussed (Section 5, Fig. 6), the value of $S_0$ was 20 $\mu$g per ml = $10^{-10}$ M. With this value of $S_0$ and $K_2 \approx 10^{-10}$ M (31), values of $S$ were computed for a range of values of $f$ and the corresponding values of $X/X_0$ were calculated for various assignments of $K_1$. These calculated values (expressed as per cent, i.e. $100 X/X_0$) are shown for $K_1 = 10^{16}$ liters per mole in Fig. 8. Approximately 50% of the derivative is freed when $f = 1$. The release of bound derivative is an exceedingly sensitive function of $f$ in the neighborhood of $f = 1$. When $f = 1.05$, approximately 84% of the derivative is freed; when $f = 0.8$, somewhat less than 5% is freed. These results are in fair agreement with the experimental results, and such is not the case when values either higher or lower than $10^8$ are assigned to $K_1$. For example, for $K_1 = 10^{16}$, $f = 0.8$, and $f = 1$ lead, respectively, to 88% and 99% freed; for $K_1 = 10^8$, the corresponding values are 0.5% and 10%. We therefore conclude that $K_1$ is of the order of $10^8$. This relatively firm association between derivative and STI suggests that it may be
inhibitors, it is not known with certainty whether the plasma inhibitors (15), and since it is difficult to separate physically plasma inhibitor which acts as an anticoagulant is present normally in to several different inhibitors (33) which do not act as anticoagulants. Thrombin conversion which occur extravascularly. However, vascularly, but would lose its effectiveness at rapid rates of prothrombin conversion which may occur intravascularly, but would lose its effectiveness at rapid rates of prothrombin conversion which occur extravascularly. However, since almost all of the trypsin inhibiting activity of plasma is due to several different inhibitors (33) which do not act as anticoagulants (15), and since it is difficult to separate physically plasma inhibitors, it is not known with certainty whether the plasma inhibitor which acts as an anticoagulant is present normally in amounts that would influence physiological blood coagulation (15).

We have confirmed the observation of Biggs, Douglas, and Macfarlane (34) that STI interferes with the generation of thromboplastin activity from blood elements; but we have not been able to demonstrate that STI inactivates preformed blood thromboplastin. In view of the results reported herein, and of the body of evidence that indicates that minute amounts of thrombin accelerate thromboplastin formation from blood elements (e.g. 35-37), it appears that the effects of STI on blood thromboplastin activity are due primarily to inhibition of thrombin formation and consequent suppression of thromboplastin formation, rather than to inactivation of preformed thromboplastin.

Proteolytic inhibitor anticoagulants not only inhibit thrombin formation, but also appear to interfere with other coagulation reactions, such as thromboplastin generation, which may be catalyzed by thrombin or some other prothrombin derivative.

At very slow rates of prothrombin conversion, the final yield of thrombin is decreased for reasons which are not yet clear (e.g. 9c). The stoichiometry of the STI-derivative complex (discussed above) suggests that prothrombin may give rise to derivatives which do not yield thrombin. If derivatives outside the pathway of thrombin formation are formed preferentially at slow rates of prothrombin conversion, this may represent still another regulatory process in blood coagulation.

Although the usual increases in trypsin inhibition which occur in various disease states (33) are not due to an inhibitor with anticoagulant activity (15), there is a possibility that physiologically significant increases in a proteolytic inhibitor anticoagulant may occur. The mechanism of inhibition presented herein appears to be relevant to the problem of anticoagulants which arise occasionally in association with lupus erythematosus. These anticoagulants are puzzling because they inhibit thrombin formation to a degree that is dependent on thromboplastin concentration (e.g. (38-40)), yet they do not appear to combine directly with prothrombin or thromboplastin. Since the activity of anticoagulants associated with lupus is influenced by the rate of prothrombin conversion, the possibility exists that these anticoagulants combine with the prothrombin derivative which reacts with proteolytic inhibitors.

**SUMMARY**

Kinetic analysis of the effects of soybean trypsin inhibitor and other proteolytic enzyme inhibitors on the conversion of prothrombin to thrombin by biological activators indicated that a prothrombin derivative formed during the conversion process combines with proteolytic inhibitors. The rate of thrombin production from the derivative was found to depend on the concentration of biological activators and to compete with the rate of formation of an essentially irreversible derivative-inhibitor complex. Degree of association between derivative and inhibitor in the complex and the nature of the conversion of free derivative to thrombin was determined by displacing inhibitor from the complex with trypsin. Stoichiometry of complex formation indicated either that processes of prothrombin activation convert only a small fraction of prothrombin molecules
into the derivative which gives rise to thrombin, or that current methods of purifying prothrombin yield a very impure product. A minimal reaction model deduced from experimental results is described by linear kinetic equations. Physiological implications of the various findings, in particular the possible regulatory effects of plasma proteolytic inhibitors in blood coagulation, are discussed.

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