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THE SITE OF INHIBITION OF BLOOD CLOTTING BY SOY BEAN TRYPSIN INHIBITOR[1]

By MARY BETH GLENDENING and ERNEST W. PAGE

(From the Division of Obstetrics and Gynecology, University of California School of Medicine, San Francisco, Cal.)

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Two of the principal reactions involved in the clotting of blood are:

$$1.\quad \text{Prothrombin} \xrightarrow[\text{Ac-Globulin}]{\text{Thromboplastin} + Ca^{++}} \text{Thrombin}$$

$$2.\quad \text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin.}$$

There are, of course, accessory factors concerned with the first reaction, such as platelet derivatives, soluble plasma factors and various inhibitors, but this study deals only with the main reactions listed above. The studies of Chargaff (1) as well as our own observations indicate that thromboplastin in the presence of calcium ions acts as an enzyme on the substrate prothrombin. Serum accelerator globulin (Factor VI, labile factor) appears to be a cofactor which accelerates the rate of formation of the enzyme-substrate complex (2).

A trypsin inhibitor, isolated from soy beans by Kunitz (3), is also capable of delaying the coagulation of blood. Previous studies by six groups of investigators have shown that it inhibits the first phase of coagulation and has no effect on the activity of thrombin (4–10). None of these groups of workers, however, was able to ascertain whether the soy bean trypsin inhibitor (SBTI) combined with prothrombin, accelerator globulin, or thromboplastin. Preliminary incubation of SBTI with each of these components individually does not increase the degree of inhibition.

Further pursuit of this problem seemed important because its solution might shed light on the nature of prothrombin conversion and because the methodology developed might be applicable to the study of other inhibitors. A series of kinetic studies on the reactions between SBTI, plasma, thromboplastin, calcium, accelerator globulin and prothrombin were therefore undertaken in an attempt to find a definitive explanation for the inhibition.

Effect of SBTI on Clotting Time of Recalcified Plasma

This experiment was carried out to test the hypothesis that thromboplastin was either reversibly or irreversibly inactivated or "bound" by SBTI.

The plasma was obtained from blood collected in a chilled silicone-lined syringe and added to 1 volume of 2.5% sodium citrate for each 9 volumes of blood. All tubes and pipettes subsequently used were coated with silicone, using the method of Jaques (11). The blood was centrifuged for 20 minutes at 1,100 r.p.m. in an angle centrifuge in the refrigerator. The plasma was kept in an ice bath for the duration of the experiment. All determinations were carried out as quickly as possible because there was a tendency for the recalcified clotting time to become shorter when the plasma was allowed to stand. The thromboplastin or inhibitor to be tested was dissolved in buffer consisting of 1 part imidazole buffer and 9 parts saline. For the determination of clotting time, 0.5 ml. of plasma and 0.2 ml. of buffer or of the reagents to be tested were transferred to a Wasserman tube. After incubating at 30° C. for one minute, 0.1 ml. of 0.5% calcium chloride solution was blown in at the same time that a stop watch was started. The tubes were gently tipped back and forth until the first fibrils of fibrin appeared.

The thromboplastin used was a stock solution prepared from human placenta by the method described below for beef lung. For each concentration of SBTI used, varying dilutions of the thromboplastin solution were added, as indicated in the first column of Table I.

Over a wide range, the logarithm of the thromboplastin concentration was consistently found to be a straight line function of the negative logarithm of the clotting time. From the line showing this relationship in the absence of SBTI, the apparent concentration of thromboplastin which remained effective could be determined. The percentage of added thromboplastin which was apparently "bound" is shown (Table I) in the data from a typical experiment. It will be noted that this apparently "bound" fraction remains constant —within the limits of error—at each level of inhibitor.

[1] This investigation was supported by research grants from the National Heart Institute of the National Institutes of Health, Public Health Service; the Christine Breon Fund; and the American Foundation for High Blood Pressure.

TABLE I

The effect of thromboplastin and soy bean trypsin inhibitor on the clotting time of recalcified plasma

Dilution of thrombo-plastin added	Concentration of SBTI in micrograms/ml.										
	0	12.5		31.3		87.5		175		625	
	C. T.*	C. T.	Bound†	C. T.	Bound	C. T.	Bound	C. T.	Bound	C. T.	Bound
0	8.6	9.9	—	9.9	—	16.0	—	37.2	—	98.0	—
1/100	4.6	6.2	69.0	9.7	87.0	16.5	96.2	31.1	99.2	78.0	99.9
1/50	3.2	5.8	72.0	7.7	86.0	14.6	97.3	27.8	99.4	61.0	99.9
1/25	2.7	4.6	76.8	6.3	89.0	9.3	95.8	15.8	98.9	32.0	99.8
1/10	1.9	3.0	70.5	3.9	85.0	3.8	93.9	8.0	97.6	21.9	99.8
1/5	1.4	2.4	73.5	2.1	85.2	4.0	93.2	4.7	95.2	14.4	99.7

* C. T. = clotting time of recalcified plasma in minutes.
† Bound = per cent of added thromboplastin apparently "bound" by the SBTI.

Now if the enzyme and the inhibitor formed a complex, the fraction of total enzyme inactivated by a given amount of inhibitor should decrease with each increment of enzyme added. Since this was not the case, the results suggest that some component of the plasma which is not being varied is the factor which is actually being inhibited.

The Effect of SBTI on the Conversion of Prothrombin to Thrombin

In order to determine the precise site of inhibition, the isolated components of the clotting system were studied individually in a two stage system.

MATERIALS

Soy bean trypsin inhibitor: Two preparations with similar anticoagulant properties were used. One was a crystalline product prepared by Dr. Kunitz and kindly given to us by Dr. Lineweaver. The other was a purified product prepared for us by E. C. Loomis of Parke, Davis and Company, Detroit. Fresh solutions of convenient concentrations were prepared in 0.9% sodium chloride for each experiment.

Prothrombin: Two highly purified lots of prothrombin were given to us, one by E. C. Loomis and the other (lot number 500518) by W. H. Seegers, Wayne University School of Medicine, Detroit. Each of these preparations had been heated in aqueous solution at 52° C. to destroy any contaminating accelerator globulin. Stock solutions which contained 1.0 mg./ml. of distilled water were stored in 1 ml. aliquots in the deep freeze.

Accelerator globulin: Highly purified products were kindly prepared for us by Dr. A. G. Ware and Dr. W. H. Seegers (lot numbers 490112 and 500127). Stock solutions which contained 200 micrograms/ml. of 0.9% sodium chloride solution were stored in 1 ml. aliquots in the deep freeze.

Fibrinogen: Bovine Fraction I, purchased from Armour and Company, was used throughout. Seventy to 80% of the protein was clottable with thrombin. Solutions containing 1.2% Fraction I were treated with freshly precipitated barium sulphate (approximately 8 mg./ml.) to adsorb the small amount of contaminating prothrombin. Solid sodium citrate was added to the fibrinogen solution to a concentration of 1.2% so that thrombin formation would be halted during the measurement of thrombin concentration. It was found necessary to prepare fresh solutions daily.

Thrombin: Parke-Davis Thrombin (Topical) was used for preparing standard curves.

Thromboplastin: A variety of preparations was used. Unless otherwise specified, the thromboplastin was prepared from beef lung as follows: Fresh beef lung, washed with running tap water and stripped free of major bronchi and blood vessels, was ground in the meat grinder and mixed with 1 volume of 0.9% sodium chloride. This was allowed to stand overnight in the refrigerator. The mixture was centrifuged at 2,000 r.p.m. The supernatant solution contained a large amount of thromboplastin but was discarded because it also contained blood proteins. The sediment was mixed in a Waring blendor for three minutes with an equal volume of 0.9% sodium chloride. This homogenate was centrifuged at 2,000 r.p.m. and the supernatant was saved. This extract, containing as much or more activity than the first supernatant described above, was kept frozen pending further purification. When thawed and again homogenized in the Waring blendor, there was always some insoluble material which was removed by centrifuging at 2,000 r.p.m. The supernatant solution was then centrifuged at 18,000 r.p.m. (20,000 g.) for two hours. The pellet obtained was thoroughly mixed with one-half the original volume of 2.5% sodium citrate and centrifuged again at 20,000 g. for one hour. The pellet was now homogenized with one-half the original volume of 0.9% sodium chloride. Following centrifugation at 2000 r.p.m. for 10 minutes, the supernatant solution was again subjected to 20,000 g. for one hour. The resulting pellet was homogenized with one-tenth the original vol-

ume of 0.9% sodium chloride and centrifuged at 2000 r.p.m. for 10 minutes. One or 2 ml. aliquots of the resulting supernatant solution were placed in test tubes and stored in the deep freeze until used.

Imidazole buffer: 1.72 gm. of imidazole (Edcan Laboratories) were dissolved in 90 ml. of 0.1 N. hydrochloric acid and made up to 100 ml. with distilled water. If necessary the pH was adjusted to 7.25.

METHODS

Rate of thrombin formation: Modifications of the two stage method for prothrombin determination (12) were used. The reaction mixture consisted of : 4 parts of buffered calcium solution (which contained 7 parts 0.9% sodium chloride, 3 parts imidazole buffer and calcium chloride to 0.05 M.) ; and 1 part each of the following solutions: thromboplastin, inhibitor (or saline), accelerator globulin (or saline) and prothrombin.

Except for early experiments conducted in glass, the determinations were carried out in silicone-lined vessels because it has been shown by Seegers that appreciable quantities of thrombin are adsorbed by glass (13). The mixture was kept in a water bath at 28° C. At intervals, 0.4 ml. of the reaction mixture was added to 0.1 ml. of fibrinogen solution in a Wasserman tube and the length of time required for the formation of fibrils was measured. The amount of thrombin present could then be determined from previously prepared standard curves which related the clotting time to units of thrombin. Silicone-lined tuberculin syringes bearing blunted 18 gauge needles coated with silicone were used for sampling the reaction mixture.

Standard curves for thrombin: Each lot of fibrinogen required the construction of a separate curve relating clotting time to units of thrombin. At no time was the thrombin solution permitted to contact any surface which was not coated with silicone. A stock solution of thrombin was prepared by dissolving 40 mg. in 10 ml. of 0.9% sodium chloride, and 1 ml. aliquots were stored in the deep freeze. Immediately prior to the determination of a given point on the curve, a tube was removed from the freezer, thawed and the desired dilution quickly made with saline. One-half ml. of the freshly diluted thrombin was added to 1 ml. of buffer solution plus 0.5 ml. of saline and placed in the water bath at 28° C. Of this mixture, 0.4 ml. was added to 0.1 ml. of fibrinogen and the exact clotting time determined.

The clotting times over a wide range of thrombin concentrations were determined in triplicate. The amount of thrombin which would result in a clotting time of 60 seconds was obtained by interpolation and called one unit. (This is the same amount of thrombin which will clot untreated fibrinogen in the presence of acacia in 15 seconds, as in the method of Seegers and Smith [14].) Multiples or fractions of this amount were then assigned their respective unit values. When the units of thrombin were plotted against clotting times on logarithmic paper the resulting curve was usually a straight line over a wide range of concentrations. Occasional lots of fibrinogen were encountered which gave a slightly curved line.

Determination of prothrombin: The amount of active prothrombin being used in a given experiment was checked by using a 1/25 dilution of purified stock beef lung thromboplastin and a 1/10 dilution of stock accelerator globulin solution in the reaction mixture; *i.e.*, the final dilution of thromboplastin was 1/200 and the final concentration of accelerator globulin was 1.25 micrograms/ml. Under these conditions the maximal yield of thrombin was taken as a measure of the prothrombin present.

A. Variation in thromboplastin

In the first stage reaction mixture described above, the concentrations of accelerator globulin, prothrombin and SBTI were kept constant, whereas the concentration of thromboplastin was varied.

The results of a typical experiment are shown in Figure 1. The velocity represents the amount of thrombin formed per minute during the initial phase (five minutes) of the reaction when the yield of thrombin per unit of time was relatively constant. The enzyme concentration is expressed as the final percentage of the purified stock beef lung solution contained in the reaction mixture.

In order to insure that the presence of accelerator globulin did not influence the interpretation, the experiment was repeated in its absence. The results of a typical experiment are shown in Figure 2.

In both instances, increasing the concentration of thromboplastin up to 0.05% caused progressive

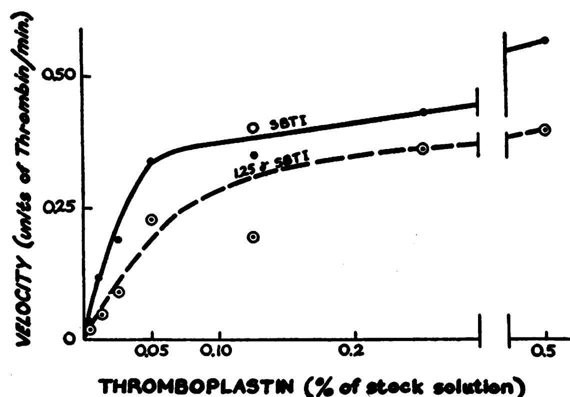


FIG. 1. EFFECT OF THROMBOPLASTIN ON INITIAL VELOCITY OF THROMBIN FORMATION

1.25 micrograms Ac-globulin/ml. and 1.3 units prothrombin/ml. in all reaction mixtures. Solid line—no inhibitor. Broken line—1.25 micrograms SBTI/ml.

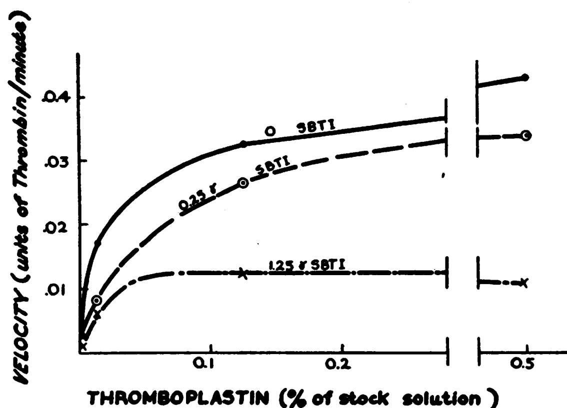


FIG. 2. EFFECT OF THROMBOPLASTIN ON INITIAL VELOCITY OF THROMBIN FORMATION IN ABSENCE OF ACCELERATOR GLOBULIN

6.1 units prothrombin/ml. in all reaction mixtures.

increases in the rate of thrombin formation regardless of the presence or absence of inhibitor. Above concentrations of 0.05%, increases of velocity were relatively slight. The omission of accelerator globulin simply caused a limitation of the maximal velocity which could be achieved at any level of thromboplastin. The presence of SBTI likewise limited the maximal velocity at any given enzyme concentration.

If the inhibitor were acting directly upon thromboplastin, it would be predicted that a ten-fold increase in the enzyme concentration would overcome to some degree the effect of the inhibitor. This is not the case. Above the thromboplastin concentration of 0.15%, there is, in fact, a constant difference between the velocities of the inhibited and uninhibited reactions.

Further evidence that the inhibitor and enzyme did not form a complex was provided by the following experiment: Equal quantities of purified beef lung thromboplastin were diluted with saline, and placed in two tubes. To one was added an excessive amount of SBTI. Both tubes were centrifuged at 20,000 g. for two hours. The activity of the recovered thromboplastin was the same in both cases. It would seem unlikely that a complex of thromboplastin and SBTI could be completely separated by simple centrifugation.

B. Variation of accelerator globulin

In this group of experiments, all factors remained constant except for a variation in the concentration of accelerator globulin. Increasing the accelerator globulin always resulted in greater velocities of thrombin formation. The final yield of thrombin was not appreciably affected, except at very low concentrations of accelerator when the reaction then proceeded so slowly that deterioration of the reactants became a limiting factor. Experience has taught us that dilute solutions of accelerator globulin and thromboplastin became less active upon standing at 28° C. Variations of thromboplastin concentration produced the same results. These observations regarding the constancy of the final yield regardless of thromboplastin or accelerator globulin levels differ from the results reported by others (15–17). When using concentrations of prothrombin below 50 units/ml., we confirmed the fact that the final yield is decreased whenever the concentration of accelerator globulin or thromboplastin is reduced provided that the experiments are conducted in glass. This is not true when the same experiments are conducted in silicone-lined vessels. We have not reinvestigated the problem using the high substrate concentrations employed by Mertz, Seegers and Smith (15).

The addition of SBTI definitely limited the final yield of thrombin and slowed the velocity. Figure 3 presents data from a typical experiment in which two levels of accelerator globulin were used with and without SBTI. The marked reduction in the final yield of thrombin could not be explained on

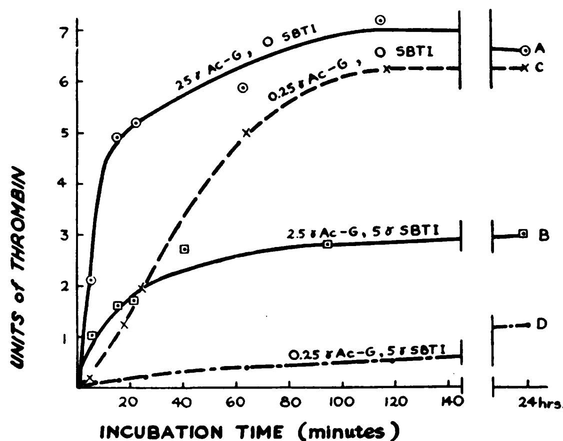


FIG. 3. EFFECT OF ACCELERATOR GLOBULIN ON PRODUCTION OF THROMBIN WITH AND WITHOUT SBTI

10 units prothrombin/ml. and 0.01% thromboplastin in all reaction mixtures.

the basis of accelerator globulin inhibition. Furthermore, if SBTI simply limited the amount of active accelerator globulin, it should produce changes in the shape of the curves comparable to the differences between curves A and C. Such is not the case; rather the curves for the inhibited reactions suggest a limitation of substrate (prothrombin).

From a series of such experiments, the curves illustrated in Figure 4 were constructed. They

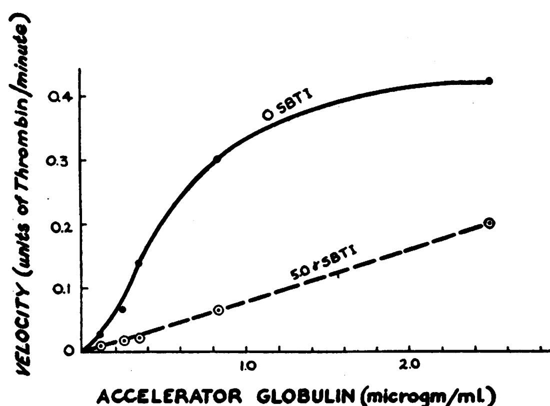


FIG. 4. EFFECT OF ACCELERATOR GLOBULIN ON INITIAL VELOCITY OF THROMBIN FORMATION WITH AND WITHOUT SBTI

10 units prothrombin/ml. and 0.01% thromboplastin in all reaction mixtures.

demonstrate that accelerator globulin will increase the initial velocity of even an inhibited reaction. The reasons for this will be discussed below.

C. Variation of soy bean trypsin inhibitor

In these experiments, the concentrations of accelerator globulin and of thromboplastin were the same as those described under "Methods" for the assay of prothrombin. The amount of prothrombin remained constant at 12.5 units. The effects of adding increments of SBTI to such a system are illustrated in Figure 5. Whereas the initial velocity is reduced, the major effect of SBTI is a limitation in the final yield of thrombin.

The final yield of thrombin under these conditions is, by definition, a measure of the quantity of active prothrombin present. So long as the reactions are conducted in silicone, the concentration of prothrombin is the only factor which, in the absence of SBTI, so definitely limits the

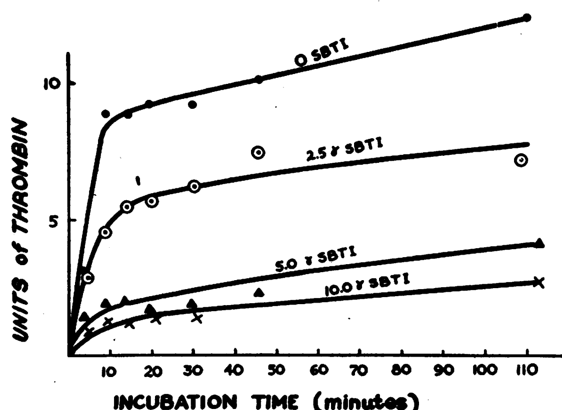


FIG. 5. EFFECT OF SBTI ON THROMBIN FORMATION

12.5 units of prothrombin/ml. and 0.4% thromboplastin in all reaction mixtures.

final yield of thrombin. Identical curves may be obtained in the absence of SBTI when the concentration of prothrombin is progressively reduced. All of the results described thus far can be explained on the assumption that SBTI forms a dissociable complex with prothrombin or an intermediate (see addendum).

D. The effect of SBTI on the spontaneous conversion of prothrombin to thrombin in sodium citrate

The observation by Seegers and Ware (18) that prothrombin in 25% sodium citrate is slowly converted to thrombin provided a means of studying the reaction between SBTI and prothrombin when all other factors were excluded. The prothrombin used was the highly purified product given to us by Dr. Seegers. When 5,000 units of this product dissolved in 1 ml. of saline were allowed to stand at 28° C. for 72 hours, less than 0.1 unit of thrombin was formed. This demonstrated the freedom of the product from any contaminating thromboplastic substances. One mg. of the product, according to our assay, yielded 3,000 units of thrombin.

Approximately 4,300 units of prothrombin were added to each ml. of 25% sodium citrate and incubated in silicone-lined tubes at 28° C. Some of the tubes contained 50 micrograms/ml. of SBTI. At various intervals, aliquots were removed, diluted at least 250 times, and assayed for both thrombin and prothrombin.

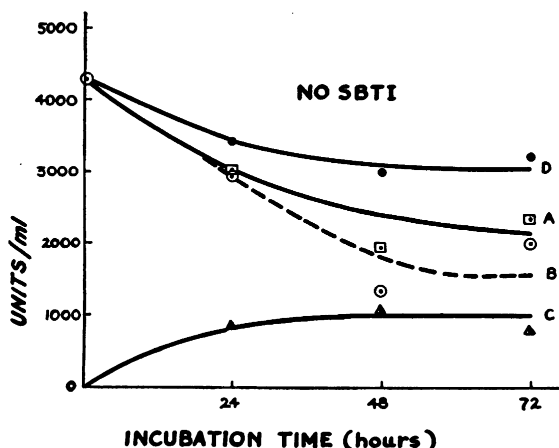


FIG. 6. CONVERSION OF PROTHROMBIN TO THROMBIN IN 25% SODIUM CITRATE WITHOUT INHIBITOR

A (square with center dot). Prothrombin, determined by difference.

B (circle with center dot). Prothrombin, determined in the presence of SBTI (see text).

C (triangle with center dot). Thrombin.

D (solid circle). Control: Prothrombin in saline. No thrombin was formed.

The results of a typical experiment are shown in Figures 6 and 7. In the absence of inhibitor, about 25% of the prothrombin was converted in the first 48 hours. A greater conversion might have occurred if we had added the triaminodiphenyl sulfone described by Seegers, McClaughry and Fahey (19), but we did not wish to confuse the interpretation. While there was an unaccountable loss of several hundred units of prothrombin during 72 hours, most of the total drop was accounted for by thrombin production. The prothrombin assays in the control experiment (Figure 6) were conducted both without (curve A) and with (curve B) the presence of that concentration of SBTI existing in the final dilutions from the inhibited reaction (Figure 7).

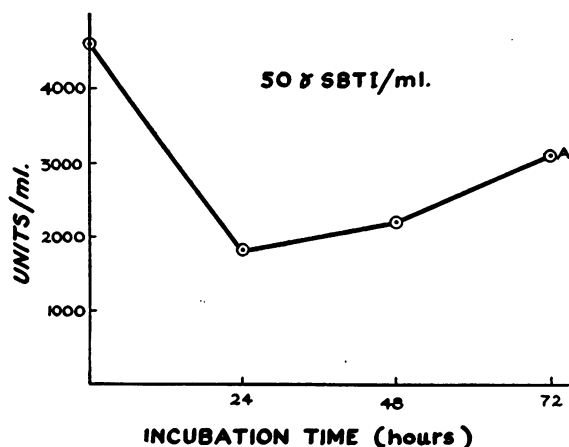


FIG. 7. PROTHROMBIN IN 25% SODIUM CITRATE IN THE PRESENCE OF 50 MICROGRAMS/ML. OF SBTI

Performed simultaneously with experiment shown in Figure 6. Only 12 units of thrombin formed in 72 hours.

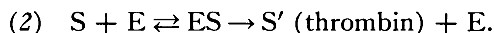
In the presence of SBTI, only 12 units of thrombin were formed in 72 hours. The apparent decrease of assayable prothrombin (Figure 7) might be due to the transient refractory state described by Seegers, McClaughry and Fahey (19). The experiments were repeated four times with comparable results, thus establishing the fact that SBTI does inhibit prothrombin.

DISCUSSION

It has been shown that the site of inhibition of prothrombin conversion by soy bean trypsin inhibitor is the substrate. The existence of a dissociable complex between prothrombin, or an intermediate derivative, and SBTI will explain all of the phenomena observed. It will explain why it is difficult to obtain 100% inhibition of coagulation, because a small amount of substrate would always be free. In the experiments performed with 25% sodium citrate, only a minute quantity of thrombin was formed in the presence of SBTI. Nevertheless, the inhibited substrate could be readily converted by dilution and addition of thromboplastin and accelerator globulin, showing the reversible nature of the inhibition.

In the presence of SBTI, two substances compete for the substrate (S); the enzyme (E) and the inhibitor (I). Inasmuch as the ES complex is also dissociable (20), such a mixture may be represented by the following two equations:

$$(1)\quad S + I \rightleftarrows SI$$



$$(2)\quad S + E \rightleftarrows ES \rightarrow S' \text{ (thrombin)} + E.$$

Any substance which normally speeds the second reaction toward the right in the absence of SBTI would also be expected to do so in its presence. This is because the more enzyme there is to combine with prothrombin, the less prothrombin is available for combination with SBTI.

Evidence to be presented elsewhere (2) indicates that accelerator globulin increases the affinity

between the enzyme and its substrate (*i.e.,* thromboplastin and prothrombin), thus decreasing still further the amount of free prothrombin which might combine with the inhibitor. This explains why accelerator globulin increases the velocity even in the presence of SBTI.

This is the first time, to our knowledge, that inhibition of a biologic reaction is accomplished by the formation of a complex between inhibitor and substrate. The details of the kinetics involved and the mathematical treatment of this type of inhibition will be published separately.

Although SBTI does not now appear to be of clinical importance, it is hoped that the methods utilized in this study for elucidating the site of its action may be applied to the study of other inhibitors of the coagulation of blood.

SUMMARY

Soy bean trypsin inhibitor appears to delay the coagulation of blood by a mechanism unique among biological inhibitions. It apparently forms a dissociable complex with the substrate, prothrombin, or a derivative of the substrate. The inhibitor does not exert its effect upon the enzyme of the first phase (thromboplastin), nor upon the cofactor (accelerator globulin) nor upon the product (thrombin). It will interfere with the conversion of prothrombin to thrombin in 25% sodium citrate when all other conversion factors are absent.

ADDENDUM

In the light of subsequent investigations, still in progress, it appears more likely that SBTI reacts with a compound intermediate between prothrombin and thrombin. This necessitates the concept, already intimated by Seegers, McClaughry and Fahey (19), that the degradation of prothrombin to thrombin proceeds in two or more steps.

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