Effect of Trypsin on Prothrombin.* (20670)

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It has long been known that trypsin clots plasma(1,2) and that it speeds the clotting of whole blood(3). Furthermore, it has been demonstrated that the rapid intravenous injection of trypsin causes intravascular clotting(4,5). The mechanism of the clotting effect of trypsin has not been completely clarified. A number of reports state that trypsin acts on prothrombin, converting it into thrombin(4,6,7), but there is disagreement as to the need for calcium in this reaction. Eagle and Harris(4) believed that calcium is not essential for conversion of prothrombin by trypsin, while Ferguson and associates(6,7) have stressed the essentiality of calcium. A more complex view of the clotting action of trypsin was presented by Lenggenhager(8). He thought that trypsin activated a hypothetical plasma factor and that this factor, together with calcium, converted prothrombin into thrombin. More recently, Schultze and Schwick(9) demonstrated that trypsin speeds the activation of purified prothrombin in concentrated citrate solutions. This effect was also noted for prothrombin solutions in distilled water, 50% glycerin, and 20% serum albumin. However, the role of calcium in the reaction was not considered.

Experiments have been performed which were designed to clarify the mechanism of the clotting effect of trypsin. Three new approaches to the problem were used. The first was the use of purified clotting factors; the second was the use of soybean trypsin inhibitor(10) to neutralize tryptic activity at any desired time; and the third was the use of a quantitative prothrombin assay.

Methods. Prothrombin assays. These were performed by the 2-stage method of Warner, Brinkhous, and Smith(11), as modified by Flynn and Coon. Their modifications involve the use of the purified thromboplastic "accessory factors" needed for the conversion of prothrombin into thrombin(12). *Thrombin assays.* Thrombin solutions were assayed by the second stage of the 2-stage prothrombin assay. *Activation of prothrombin by trypsin.* Trypsin† was added to purified prothrombin‡ in various reaction mixtures. The exact composition of these mixtures is described under Fig. 1. At measured intervals, aliquots of the mixtures were removed and immediately added to trypsin inhibitor.§ The use of inhibitor prevented the trypsin from acting during the subsequent procedures. Thrombin and prothrombin assays were then performed. Calcium and the thromboplastic "accessory factors" (labile factor (factor V, etc.) and stable factor (factor VII, etc.)(13,14))‡ were added to the prothrombin along with the trypsin to study their effect on the activation reaction. *Plasma prothrombin utilization.* This was determined by assaying plasma prothrombin concentration before and after addition of trypsin to the plasma. *Antithrombic activity of heparin.* Purified thrombin (Parke, Davis & Co.) was added to oxalated plasma containing heparin (sodium salt, Eli Lilly & Co.). The same procedure was performed with control oxalated plasma without heparin. At measured intervals, an aliquot was removed from each mixture and a thrombin assay done. The reduction in thrombin concentration of the heparinized plasma be-

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† A twice-recrystallized, salt-free, and lyophilized preparation was used (Worthington Biochemical, Freehold, N. J.).

‡ Prothrombin, labile factor, and stable factor were kindly supplied by Drs. J. E. Flynn and R. W. Coon. Methods of preparation are described elsewhere(12).

§ A 5-times recrystallized preparation of soybean origin was used (Worthington Bio.).

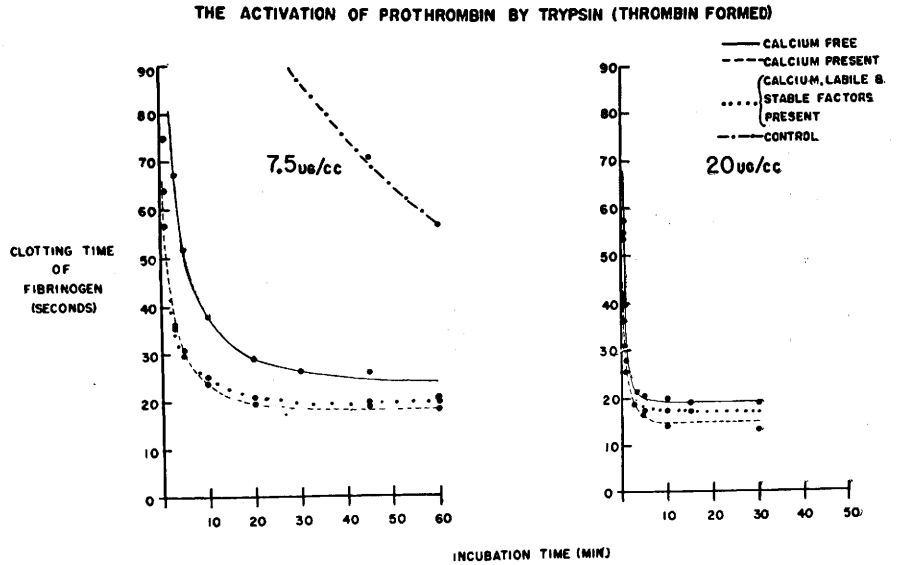

FIG. 1. Activation of prothrombin by trypsin (thrombin formed). *Reaction mixtures:* (a) *calcium-free:* 0.5 ml prothrombin sol. (diluted to 340 units/ml with 7.5% acacia in oxalated 0.85% saline) + 1.4 ml 7.5% acacia in oxal. 0.85% sal. + 0.1 ml trypsin in oxal. 0.85% sal.; (b) *calcium added:* 0.5 ml proth. sol. (dil. to 340 units/ml with 7.5% acacia in 0.85% sal.) + 1.4 ml 7.5% acacia in 0.25% $CaCl_2$ and 0.85% sal. + 0.1 ml trypsin; (c) *calcium, labile and stable factors added:* 0.5 ml proth. (prep. as in b) + 1.2 ml 7.5% acacia in 0.4% $CaCl_2$ and 0.85% sal. + 0.1 ml labile factor + 0.1 ml stable factor + 0.1 ml trypsin; (d) *control-calcium, labile, and stable factors added, but no trypsin:* same as in *c* except that 0.1 ml of 0.85% sal. without trypsin was used. 0.2 ml aliquots were removed and added to 0.2 ml of 0.85% sal. buffered to pH 7.4 and containing trypsin inhibitor in an amt equal to the amt of trypsin added to the react. mixture.

low that of the unheparinized plasma was considered to be an estimate of the increase in antithrombic activity of the heparinized plasma.

Results. The experiment illustrated by Fig. 1 demonstrates that trypsin, by itself, can cause the conversion of prothrombin into thrombin. Within the range of trypsin concentrations used, the rate of this reaction varies with the concentration of trypsin. In addition, calcium is shown to increase the rate of thrombin formation. However, the total amount of thrombin formed is unaffected by the presence of calcium. Furthermore, the thromboplastic "accessory factors," together with calcium, have no effect other than that produced by calcium alone. Although the data presented in Fig. 1 represent trypsin concentrations of 7.5 and 20 μg/ml, essentially similar results were obtained with concentrations of 15 and 50 μg/ml. However, with 50 μg/ml, prothrombin and thrombin activities in the reaction mixtures were rapidly lost. The control mixture, represented by the dash-dot line curve, consisted of purified prothrombin, calcium, labile factor and stable factor, but no trypsin. It will be noted that some thrombin formed in this mixture but the amount was small. This was undoubtedly due to thromboplastic impurities in the reagents. When trypsin was added to such a mixture (dotted line curve), thrombin was formed in considerably greater amounts.

In the above experiment on the activation of prothrombin by trypsin there was a discrepancy between the amount of prothrombin present and the amount of thrombin formed. The available prothrombin could, if fully converted, have given rise to a greater amount of thrombin than that recovered. The question arose as to whether some of the prothrombin had been digested or had remained unconverted. To answer this question, residual prothrombin concentrations were determined at various stages of the reaction. Fig. 2 illustrates the results graphically. As

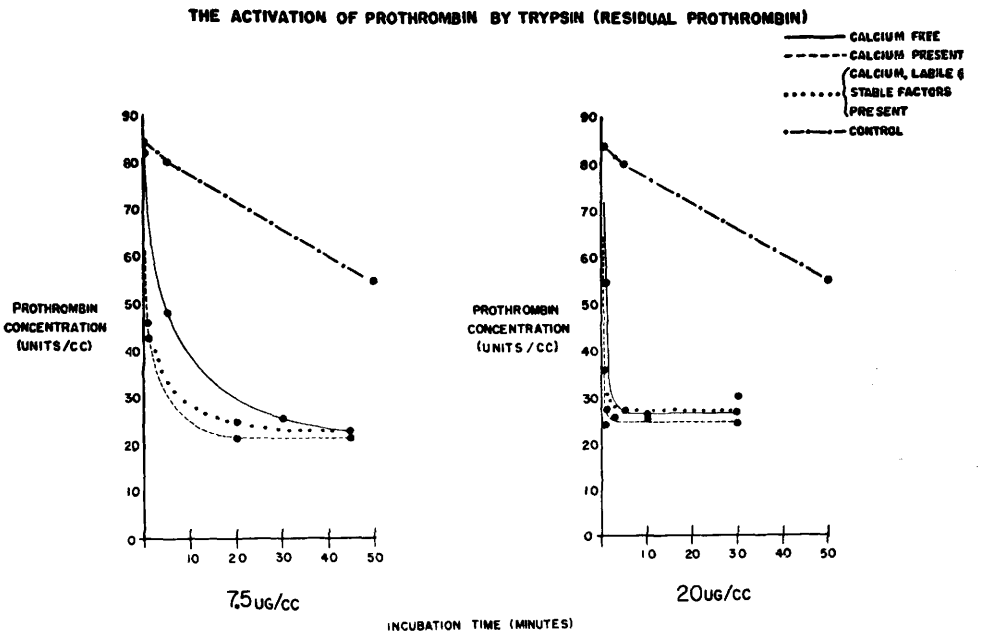

FIG. 2. Activation of prothrombin by trypsin (residual prothrombin). Reaction mixtures same as for Fig. 1.

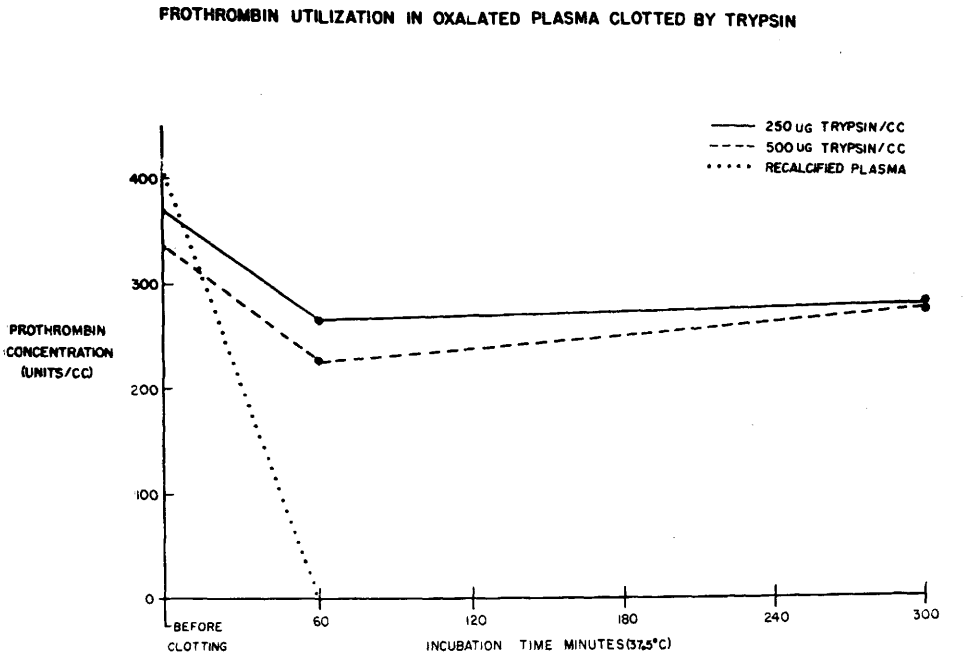

FIG. 3. Prothrombin utilization in trypsin-clotted oxalated plasma. 1 ml samples of oxal. plasma were clotted by adding 0.1 ml of 0.85% sal. containing 250 and 500 μg trypsin. For control, 1 ml plasma was clotted by adding 0.2 ml 2% $CaCl_2$ and 0.3 ml of thromboplastin (Difco). The samples were inc. at 37.5°C and prior to assaying for proth. the clots were removed and 0.1 ml trypsin inhibitor (400 μg) in 0.85% sal. was added to the supernatant plasma.

TABLE I. Inhibitory Effect of Heparin on Trypsin's Clotting Action. Plasma was prepared by diluting 7 pt normal human blood with 1 pt 1.85% potass. oxal. in distilled H_2O. The oxal. blood was then centrifuged at 2500 rpm for 30 min. and the plasma pipetted off. Reagents were kept at 37.5°C and mixed in the order listed in the table. Trypsin was added in sal. sol. in 0.2 ml amounts. Hep. was added as 0.01 ml of liquid hep. 1 mg of protamine was added as 0.1 ml 1% protamine sulf. sol. Controls, consisting of 1 ml oxal. plasma, + 0.2 ml oxal. sal., 0.5 mg hep. and 1 mg prot., did not clot. The clotting effect of trypsin was not inhibited by 1 mg prot.

Reagents (mixed in listed order)	Clotting time in sec. (37.5°C)
1 ml oxalated plasma + 250 μg trypsin	35.7
" " " + 500 μg "	23.2
" " " + 0.5 mg heparin + 250 μg trypsin	No clot
" " " " " + 500 μg "	"
" " " " " + 250 μg " + 1 mg protamine	"
" " " " " + 500 μg trypsin + 1 mg protamine	"
" " " " " + 1 mg protamine + 500 μg trypsin	20.4

might be expected, the rate of prothrombin conversion paralleled the rate of thrombin formation (Fig. 1). However, it should be noted that not all the prothrombin was converted and a constant amount remained despite continued exposure to trypsin. Furthermore, the amount of unconverted prothrombin was the same for the various trypsin concentrations studied (7.5, 15, 20, and 50 μg/ml). Even the presence of calcium and labile and stable factors failed to alter the amount of residual prothrombin. Since about 25 of the original 85 units of prothrombin/ml remained, presumably about 60 units/ml of thrombin were formed. However, antithrombic impurities in the reagents, and the antithrombic effect of trypsin itself(15), prevented complete recovery of the thrombin.

To determine if trypsin required a plasma co-factor for the activation of prothrombin, trypsin was added directly to oxalated human plasma. However, it was again found that only a portion of the available prothrombin was converted (Fig. 3). From the graphs it appears as if a rise in prothrombin concentration occurred at 5 hours of incubation. However, the difference in concentration between the one- and 5-hour samples is within the limits of experimental error.

Other workers(16) have demonstrated that heparin abolishes the clotting effect of trypsin on oxalated plasma. This effect was investigated. The results (Table I) show that concentrations of trypsin which clot oxalated plasma do not clot the same plasma if a small amount of heparin is added prior to addition of trypsin. The addition of protamine to the plasma after addition of trypsin does not reverse the anti-coagulant effect of heparin. However, if protamine is added to the heparinized oxalated plasma prior to addition of trypsin, clotting proceeds as usual. In order to clarify the mechanism of this effect, prothrombin utilization was determined after addition of trypsin. The results (Fig. 4) show that, although no clotting occurs, the reduction in prothrombin levels parallels that observed in ordinary oxalated plasma clotted by trypsin. The trypsin had converted a portion of the prothrombin but no clot formed. Presumably, the heparin raises the antithrombic activity of the plasma to levels sufficient to prevent the newly formed thrombin from being effective. That the heparin was sufficiently antithrombic to have inactivated the thrombin formed through trypsin's action on prothrombin is shown in Fig. 5.

Discussion. These experiments demonstrate that trypsin alone can convert prothrombin into thrombin. In this respect, the work of Eagle and Harris(4) is substantiated. However, it is shown that prothrombin is only partially converted into thrombin by trypsin. Furthermore, calcium and the "accessory

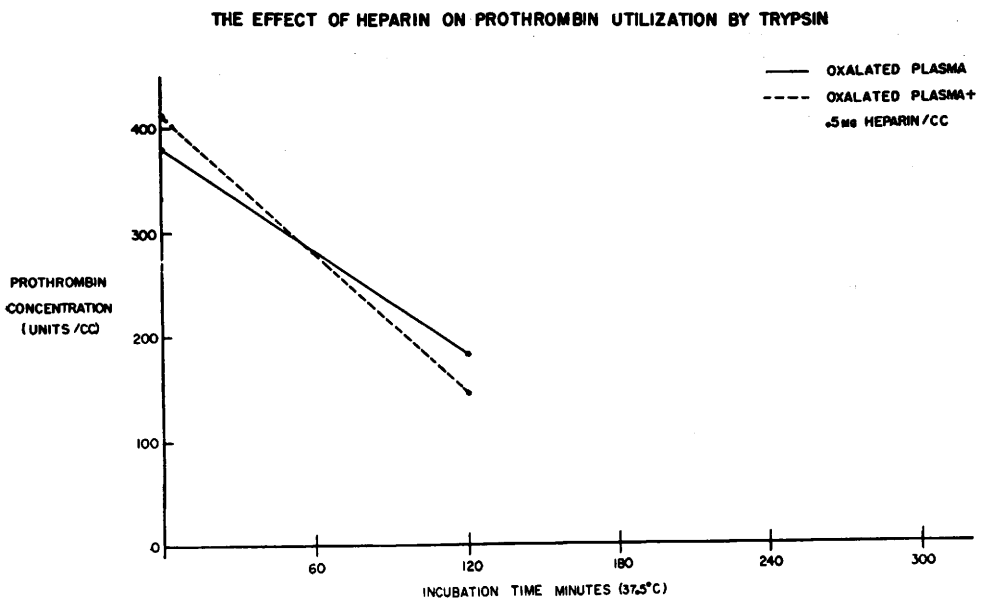

FIG. 4. Prothrombin utilization in heparinized, oxalated plasma.

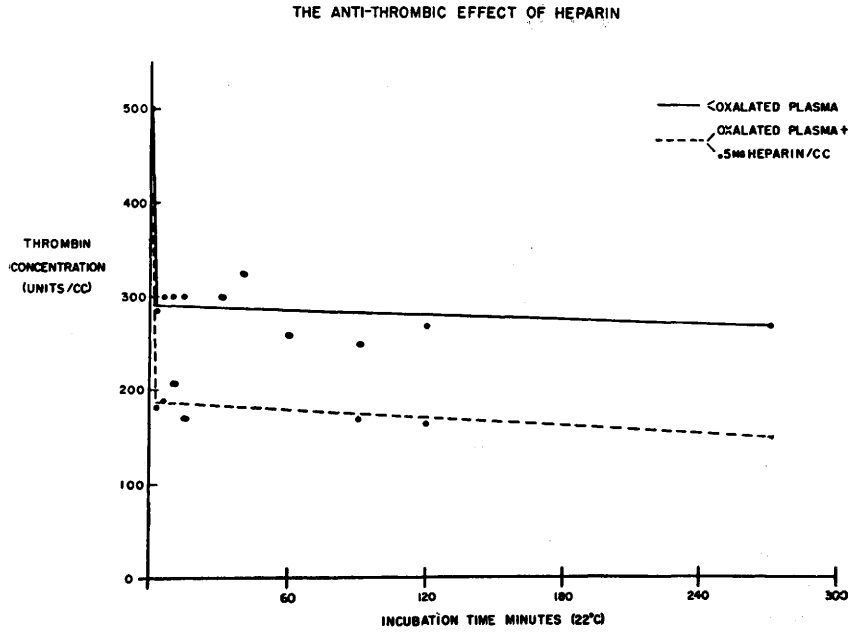

FIG. 5. Antithrombic effect of heparin. 5000 units of thrombin (Parke, Davis & Co.), in 10 ml 0.85% sal., was added to 10 ml oxal. plasma containing 0.5 mg heparin/ml. For control, the same amt of thrombin was added to an equal vol of oxal. plasma without heparin.

factors" are unnecessary for trypsin's action, although calcium does increase the rate of thrombin formation. This acceleratory effect of calcium on the reaction may have led earlier workers to believe that optimal amounts of thrombin were obtained only in the presence of calcium.

It must be emphasized that in the early experiments by other investigators, no attempt was made to inactivate the trypsin before determining the thrombin titer. Thus, the trypsin was free to act on prothrombin,

thrombin and fibrinogen. It was impossible, therefore, to determine which factor the trypsin was affecting. It contrast to these earlier experiments, the overlapping effect of trypsin was avoided by adding a trypsin inhibitor to the sample used for the thrombin assay.

It can also be added that we found no evidence for Lenggenhager's concept(8) that a plasma factor other than prothrombin itself is necessary for the clotting action of trypsin. Nevertheless, there still exists the possibility that our prothrombin was not freed of this hypothetical factor, and we are therefore reluctant to exclude the idea completely.

Because of the recent clinical use of trypsin intravenously, its clotting action has received considerable attention. Tagnon(5) investigated the use of heparin to counteract the intravascular clotting action of trypsin. Working with dogs and rabbits, Tagnon found that the previous injection of one mg/kg of heparin increased the minimal amount of injected trypsin needed to produce intravascular clotting. Other workers(16,17) have shown that heparin inactivates the proteolytic activity of trypsin, the degree of inactivation varying with the concentration of heparin and the incubation time. In this respect, the present experiments demonstrate that concentrations of heparin too low to inactivate the proteolytic activity of trypsin, may still inhibit the clotting action of trypsin. Heparin does this by raising plasma antithrombic activity, thus preventing the thrombin formed from trypsin's action from being effective. The effect observed by Tagnon was due, in all probability to increase antithrombic activity in the blood stream, and not due to direct inactivation of the trypsin.

Summary. 1. Trypsin converts prothrombin into thrombin. Calcium and accessory clotting factors are not essential. Calcium does, however, accelerate the rate of thrombin formation but without affecting the thrombin yield. 2. Conversion of prothrombin by trypsin is incomplete. 3. Heparin, in low concentrations, can inhibit the clotting effect of trypsin by increasing plasma antithrombic activity.

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