The Activation of Proaccelerin by Bovine Thrombokinase
(with Notes on the Antithrombic Activity of Phenylmethyl Sulfonfonylfluoride)

By Robert T. Breckenridge and Oscar D. Ratnoff

Recent studies in this laboratory have attempted to clarify the role of proaccelerin (Factor V) in human coagulation. Our experiments seem to demonstrate that proaccelerin is converted to a prothrombin-converting principle by the enzymatic action of activated Stuart factor (Factor X) in the presence of phospholipid and calcium. Studies with bovine clotting factors, however, have provided data which could not be reconciled with this view.

One explanation for the differences obtained with human and bovine materials is that preparations of the different clotting factors are not equally deficient in contaminating substances. The present experiments examine the interaction between activated bovine Stuart factor and bovine proaccelerin under conditions minimizing the influence of contaminating substances.

Milstone has purified a clot-promoting agent from bovine plasma to which he has given the name of "plasma thrombokinase." In the studies reported now, bovine plasma thrombokinase, treated to reduce its contamination by thrombin, appeared to activate bovine proaccelerin in an enzymatic manner. Bovine plasma thrombokinase corrects the abnormality in plasma deficient in Stuart factor, and seems to be similar in behavior to human activated Stuart factor. Under these conditions, then, the activation of proaccelerin by activated Stuart factor appeared to be similar in bovine and human systems.

METHODS

Citrated and oxalated plasmas were obtained from normal persons and from patients with various coagulative abnormalities by previously published methods. They were used immediately or stored in silicone-coated Lusteroid tubes at -20 C until used.

Oxalated beef plasma in 50-liter lots was purchased in the frozen state from Armour Laboratories. Upon receipt of the plasma it was immediately thawed, centrifuged at 1200 g for 10 minutes, and stored in 250 ml aliquots at -20 C until used.

From the Department of Medicine, Western Reserve University School of Medicine and University Hospitals of Cleveland, Cleveland, Ohio.

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Robert T. Breckenridge, M.D.: Senior Instructor in Medicine and George and Anna Bishop Fellow in Medicine, Western Reserve University School of Medicine, Cleveland, Ohio, Oscar D. Ratnoff, M.D.: Professor of Medicine and Career Investigator of the American Heart Association, Western Reserve University School of Medicine, Cleveland, Ohio.

Phenylnethyl sulfonylfluoride* was prepared in a $4 \times 10^{-2}$M solution in isopropyl alcohol in the manner described by Fahrney and Gold.8,9 A solution of bovine thrombokinase, prepared by Dr. J. H. Milstone,5 was kept at $-20$ C. until used. This solution had approximately 30 times the capacity of normal pooled human plasma to correct the defect in Stuart factor-deficient plasma. No increase in its ability to shorten the clotting time of Stuart factor-deficient plasma was noted when bovine thrombokinase was tested in the presence of Russell's viper venom. These properties, similar to those reported by Nemerson and Spaet4 are in agreement with the view that bovine thrombokinase has properties indistinguishable from activated Stuart factor, and does not contain a significant proportion of Stuart factor in its inactive form.

The preparation of bovine thrombokinase was incubated with an equal volume of $4 \times 10^{-2}$M phenylmethyl sulfonylfluoride for 15 minutes at room temperature. Sufficient powdered bovine Fraction V (Armour) was then added so that the mixture contained 1 per cent albumin. The mixture was then dialyzed in cellophane casings† against frequent changes of water for 1 hour and then against $500 \times$ its volume of barbital-saline buffer overnight. The resultant solution, or a dilution thereof, was used as a source of activated Stuart factor in the experiments to be described. As will be noted subsequently, phenylmethyl sulfonylfluoride completely inhibited the clot-promoting and esterolytic activities of a solution of bovine thrombin. This treatment reduced the ability of bovine thrombokinase to correct the defect in Stuart factor-deficient plasma and to hydrolyze $p$-toluenesulfonyl-L-arginine methyl ester hydrochloride (TAMe) but did not completely inhibit these activities.

Crude bovine proaccelerin was prepared from oxalated plasma in the identical manner to that described for the preparation of human proaccelerin.1 In essence, oxalated beef plasma was adsorbed 3 times with barium sulfate. The fraction of adsorbed plasma soluble at 33 per cent saturation, but insoluble at 50 per cent saturation with ammonium sulfate, was dialyzed against diluted sodium acetate buffer at pH 5.2. The insoluble euglobulin fraction was diluted 1:10 in barbital saline buffer and adsorbed with one-tenth its volume of dilute aluminum hydroxide gel before use. This last adsorption proved necessary to remove the remaining traces of prothrombin. This preparation was free of detectable prothrombin, fibrinogen, Stuart factor and Factor VII. No attempt was made to free it of Hageman factor (Factor XII) or PTA (plasma thromboplastin antecedent, Factor XI).

Crude prothrombin was prepared by a previously published technic.1 Essentially, this preparation was a calcium phosphate eluate of twice bentonite-adsorbed fresh citrated human plasma. The prothrombic activity of this eluate was 50 per cent and the Stuart factor activity was less than 1 per cent of the plasma from which it was prepared. It was diluted 1:10 in buffer prior to use. No contaminating proaccelerin could be detected in this preparation.

Two preparations of fibrinogen were used. Human fibrinogen was prepared from Parenogen as previously described.1 Bovine fibrinogen was prepared from Armour Fraction I.§ One gram of the Armour preparation, containing approximately 50 per cent protein and 50 per cent sodium citrate, was homogenized in 25 ml of distilled water. This protein solution was then adsorbed with one-tenth its volume of concentrated aluminum hydroxide gel. After centrifugation at 2100 g for 10 minutes, the supernatant protein solution was filtered through Whatman No. 1 filter paper. The filtrate was used immediately.

Barbital-saline buffer, pH 7.4-7.6, was prepared as previously described.10 Rabbit brain "cephalin," a crude chloroform-soluble fraction of acetone-dried brain, was prepared by the method of Bell and Alton.11 This "cephalin" was suspended by homogenization in 0.15 M sodium chloride solution at a concentration of 6 mg./ml. and stored at $-20$ C. A portion was diluted 100-fold with buffer before use.

Cation exchange resin. Amberlite IRC-50 (Mallinckrodt Chemical Works. St. Louis. Mis-

*Calbiochem, Los Angeles 63, California.
†Visking Company, Chicago, Illinois.
ACTIVATION OF PROACCELERIN

Esterolytic activity was determined upon a substrate of TAMe by a previously described method, using an automatic titrator (Auto-Burette, Radiometer, Copenhagen, Denmark) to determine the acid liberated.

Two preparations of aluminum hydroxide gel were used. The concentrated gel was undiluted Amphogel.* Dilute aluminum hydroxide gel (Cutter Laboratories, Berkeley, Calif.) was prepared as previously described.¹

Soybean trypsin inhibitor (5 times crystallized)† was dissolved in buffer to the desired concentration before use.

Calcium chloride was prepared in 0.05 M or a 0.1 M solution in distilled water.

Clotting studies were performed in uncoated Pyrex tubes with an internal diameter of 8 mm. or in Lusteroid tubes with an internal diameter of 11 mm.

Assays for specific clotting factors were performed by published methods employing plasmas from patients with inherited deficiencies of each factor as substrates for the assays."¹

The activation of proaccelerin was accomplished by incubating a series of Lusteroid tubes each containing 0.3 ml. of treated thrombokinase, 0.3 ml. of 0.05 M CaCl₂, 0.3 ml. of the 1:100 "cephalin" and 0.3 ml. of the crude proaccelerin preparation at 37 C. At intervals 0.1 ml. of this mixture was pipetted into 0.9 ml. buffer containing 0.03 M calcium. 1.2 × 10⁻⁵ mg. "cephalin" and 100 μg of soybean trypsin inhibitor per ml.

The clot-promoting activity generated was tested by adding 0.5 ml. of the second mixture to 0.1 ml. of crude prothrombin (diluted 1:10 with buffer) and 0.2 ml. of fibrinogen in Pyrex tubes. The clotting time recorded was the time elapsing between the addition of the prothrombin and fibrinogen and the appearance of the first fibrin strands. The final concentration of soybean trypsin inhibitor was 56 μg./ml. in all experiments.

The coagulation system recorded herein relies on the observation recorded later in this manuscript that soybean trypsin inhibitor blocks the interaction between thrombokinase and proaccelerin in a manner previously described for the human coagulation system.¹ To test the effect of this inhibitor, it was necessary to use the human fibrinogen preparation since, unlike beef fibrinogen,¹ this was completely free of detectable proaccelerin. Once the inhibitory action of soybean trypsin inhibitor had been demonstrated, it was possible to use bovine fibrinogen. Any further interaction between thrombokinase and proaccelerin during the actual assay of the clot-promoting agent generated was blocked by the presence of the inhibitor.

The requirement for calcium during the interaction of thrombokinase was tested by adsorption of all reagents with Amberlite IRC-50 before and after their interaction, as previously reported for the human system.¹

Milstone⁵ has reported that his preparation of bovine thrombokinase contains trace amounts of thrombin. To free this preparation from thrombin, it was incubated with phenylmethyl sulfonylfluoride, a known inhibitor of esterases. The effectiveness of the inhibitor was assayed by testing its effect upon bovine thrombin.

The effect of phenylmethyl sulfonylfluoride upon thrombin and bovine thrombokinase was tested in the following manner. Bovine thrombin (Topical Thrombin, Parke, Davis and Company, Detroit, Michigan) was dissolved at a concentration of 50 N.I.H. units per ml. of buffer and mixed with an equal volume of 4 × 10⁻⁵M phenylmethyl sulfonylfluoride or isopropyl alcohol, and incubated at room temperature for 15 minutes. The mixtures were dialyzed against distilled water overnight at 4 C. The esterolytic and coagulant activities of the solution were then tested upon substrates of TAMe and fibrinogen, respectively. Similarly, a bovine thrombokinase solution was incubated with an equal volume of 4 × 10⁻⁵M phenylmethyl sulfonylfluoride or isopropyl alcohol for 15 minutes. Before dialysis, sufficient powdered bovine Fraction V (Armour Pharmaceutical Company, Kankakee, Illinois) was added to all mixtures to make a 1 per cent albumin solution. The presence of albumin seems

*Without flavor furnished through the courtesy of Wyeth Laboratories, Philadelphia, Pennsylvania.
†Nutritional Biochemicals Corporation, Cleveland, Ohio.
Table 1.—Effect of Soybean Trypsin Inhibitor on the Interaction of Thrombokinase and Bovine Proaccelerin

<table>
<thead>
<tr>
<th></th>
<th>Clotting Time (Seconds)</th>
</tr>
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<tbody>
<tr>
<td>Buffer control</td>
<td>120</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor† present during the interaction of thrombokinase and proaccelerin</td>
<td>&gt;600</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor† added after thrombokinase and proaccelerin had interacted</td>
<td>117</td>
</tr>
</tbody>
</table>

*Thrombokinase and proaccelerin were allowed to interact for 10 minutes at 37 C. in the presence or absence of soybean trypsin inhibitor. At the end of this time period the clot-promoting activity generated was assayed by the ability of the mixture to clot a mixture of crude prothrombin and fibrinogen.

†The final concentration of soybean trypsin inhibitor was 10 µg./ml. in all experiments.

RESULTS

The inhibition of the interaction of thrombokinase and proaccelerin by soybean trypsin inhibitor is shown in Table 1. When the inhibitor is added before the interaction of these two factors, the clotting time, as measured by the addition of crude prothrombin and fibrinogen, is greater than 10 minutes. When the inhibitor is added at the end of the interaction of thrombokinase and proaccelerin, the clotting time obtained is identical to that of the buffer control.

The inhibitor, phenylmethyl sulfonylfluoride, blocks both the esterolytic and clot-promoting activity of thrombin, as is shown in Table 2. After treatment with phenylmethyl sulfonylfluoride, the preparation of bovine thrombokinase retained one-fifth its capacity to correct the defect in Stuart factor-deficient plasma, and approximately two-fifths its capacity to hydrolyze TAME. Unlike the original preparation, the treated thrombokinase, at a dilution of 1:20, had no effect on a plasma deficient in proaccelerin (Table 2). In the presence of 50y per ml. soybean trypsin inhibitor, a 1:10 dilution cf the untreated thrombokinase clotted bovine fibrinogen in 1 hour. After treatment with phenylmethyl sulfonylfluoride a similar dilution of the thrombokinase caused the formation of only faint fibrin strands after 24 hours at 37 C. These data are consistent with the view that some of the clot-promoting properties of bovine thrombokinase could be attributed to the presence of traces of thrombin, and that treatment with phenylmethyl sulfonylfluoride preserved significant amounts of an activity resembling activated Stuart factor.

The effect of varying the concentration of thrombokinase upon the development of prothrombin-converting activity can be seen in Figure 1. Thrombokinase was incubated with the crude bovine proaccelerin in the presence of
Table 2.—Effect of the Inhibitor Phenylmethyl Sulfonylfluoride (PMSF) on the Esterolytic (TAMEase) and Clot-Promoting Abilities of Thrombin and Thrombokinase

<table>
<thead>
<tr>
<th></th>
<th>TAMe Activity</th>
<th>Clotting Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mM TAMe Hydrolyzed Per Minute)</td>
<td>(Seconds)</td>
</tr>
<tr>
<td><strong>I. TAMease Activities</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin (25u/ml.) + Isopropyl Alcohol</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Thrombin (25u/ml.) + PMSF*</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Thrombokinase + Isopropyl Alcohol</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Thrombokinase + PMSF*</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td><strong>II. Clot-Promoting Ability</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Thrombin (25u/ml.) + Isopropyl Alcohol) + Fibrinogen</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>(Thrombin (25u/ml.) + PMSF*) + Fibrinogen</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>1/20 (Thrombokinase + Isopropyl Alcohol) + Stuart Factor-Deficient Plasma†</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>1/20 (Thrombokinase + PMSF)* + Stuart Factor-Deficient Plasma†</td>
<td>13.0</td>
<td></td>
</tr>
<tr>
<td>1/20 (Thrombokinase + Isopropyl Alcohol) + Proaccelerin-Deficient Plasma†</td>
<td>172.0</td>
<td></td>
</tr>
<tr>
<td>1/20 (Thrombokinase + PMSF)* + Proaccelerin-Deficient Plasma†</td>
<td>&gt;1000</td>
<td></td>
</tr>
</tbody>
</table>

*The PMSF was not present during the actual esterolytic or clotting assays since it had been dialyzed out of the mixture (see text).

†The assay for proaccelerin was performed in an identical manner to that for Stuart factor. The substrate plasma in this case was obtained from a patient congenitally deficient in proaccelerin and stored as oxalated plasma for 9 years at −20 C.

crude “cephalin” and calcium. The clot-promoting activity generated was then assayed by the addition of the incubated mixture to prothrombin and fibrinogen in the presence of calcium and soybean trypsin inhibitor. The presence of this inhibitor prevented further interaction between the thrombokinase and proaccelerin during the actual assay period. When the concentration of the bovine proaccelerin was kept constant and the concentration of thrombokinase was varied over a 20-fold range, the yield of clot-promoting activity generated was essentially the same. The rate at which this activity generated, however, was dependent upon the concentration of thrombokinase in the incubating mixture.

The effect of varying the concentration of proaccelerin on the development of prothrombin-converting activity is demonstrated in Figure 2. In this experiment the concentration of thrombokinase was left constant at a 1:2 dilution of the original preparation and the concentration of bovine proaccelerin was varied over a 5-fold range. Again, soybean trypsin inhibitor was added at the
Fig. 1.—The effect of variations in the concentration of thrombokinase on the generation of the prothrombin-converting principle. Thrombokinase, treated to minimize contamination with thrombin, was incubated with crude bovine proaccelerin in the presence of “cephalin” and calcium. The clot-promoting activity generated was tested by adding a dilution of this mixture to prothrombin and fibrinogen in the presence of soybean trypsin inhibitor. In this experiment the concentration of the crude bovine proaccelerin was kept constant. The concentration of thrombokinase was varied 20-fold. The results were quantified in terms of per cent of the maximal prothrombin-converting activity generated by serial dilution of the product formed by the interaction of undiluted thrombokinase and crude bovine proaccelerin for 10 minutes.

End of the interaction of thrombokinase and proaccelerin to halt any further interaction of these clotting factors during the assay. Under these conditions, the clot-promoting activity generated was proportional to the concentration of proaccelerin present in the incubating mixture. These results could not be reproduced when concentrated crude proaccelerin solutions were used, for under these conditions this reagent appeared to inhibit the reaction. The nature of the inhibitor in the concentrated solutions of proaccelerin was not investigated.

Calcium ions were necessary for the interaction between bovine thrombokinase and bovine proaccelerin (Table 3). In their absence, virtually no clot-promoting activity generated during the time interval noted.
Fig. 2.—The effect of variations in the concentration of crude bovine proaccelerin on the generation of the prothrombin-converting principle. Thrombokinase, treated to minimize contamination with thrombin, was incubated with crude bovine proaccelerin in the presence of "cephalin" and calcium. The clot-promoting activity generated was tested by adding a dilution of this mixture to prothrombin and fibrinogen in the presence of soybean trypsin inhibitor. In this experiment the concentration of thrombokinase was kept constant at one-half the concentration of the original preparation. The concentration of proaccelerin was varied over a 5-fold range. The results were quantified in terms of per cent of the final prothrombin-converting activity generated by serial dilution of the product formed by the interaction of thrombokinase (one-half the concentration) and crude bovine proaccelerin (1:20 dilution) for 10 minutes.

**DISCUSSION**

The data presented here are consistent with the hypothesis that the steps leading from activated Stuart factor to the conversion of prothrombin to thrombin are similar in both the bovine system and the human system.

Untreated bovine thrombokinase clotted fibrinogen in the presence of soybean trypsin inhibitor. Presumably, then, the thrombokinase preparation contained trace amounts of contaminating thrombin as reported by Milstone. Treatment of this bovine thrombokinase with phenylmethyl sulfonylfluoride inactivated this contaminating thrombin while preserving the activity resembling activated Stuart factor, a result similar to that reported recently by Seegers.
Table 3.—Requirement for Calcium during the Interaction of Thrombokinase and Bovine Proaccelerin*

<table>
<thead>
<tr>
<th></th>
<th>Clotting Time (Seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombokinase + Proaccelerin + Phospholipid + Calcium</td>
<td>5-Minute Incubation + Prothrombin Calcium 124</td>
</tr>
<tr>
<td>Thrombokinase + Proaccelerin + Phospholipid + Buffer</td>
<td>5-Minute Incubation + Prothrombin Calcium 700</td>
</tr>
</tbody>
</table>

*The preparation of bovine proaccelerin was adsorbed with one-third volume of Amberlite IRC-50 to remove divalent cations and centrifuged. The supernatant solution was then divided in half; one-half was incubated with thrombokinase in the absence of calcium and the other half with calcium added. At the end of this 5-minute time period both products were diluted 9-fold in buffer containing calcium, phospholipid and soybean trypsin inhibitor at a concentration of 100 μg./ml. The clot-promoting activity generated was assayed by adding prothrombin and fibrinogen and the clotting time determined. The calcium concentrations were equal (0.028 M) in both tubes during the actual measurement of the clotting times.

and his associates. Once treated in this manner the thrombokinase no longer had a significant capacity to clot proaccelerin-deficient plasma. Thus, in confirmation of the findings of Spaet and Nemerson, bovine thrombokinase behaved like activated Stuart factor, after the trace amounts of thrombin which contaminated the preparation had been inactivated.

Once this small amount of thrombin was inactivated, thrombokinase and bovine proaccelerin underwent a time-consuming reaction in the presence of phospholipid and calcium. The observation that soybean trypsin inhibitor inhibited the interaction between bovine thrombokinase and bovine proaccelerin implies that the activation of proaccelerin follows the activation of Stuart factor in the sequence of clotting and enabled us to determine the nature of this reaction in much the same way in which the human system was described. Mixtures of thrombokinase and bovine proaccelerin could be incubated for varying lengths of time and their further interaction then blocked by the addition of this inhibitor. The prothrombin-converting ability of these mixtures could then be assayed on a substrate of prothrombin and fibrinogen without interference from further interaction during the assay.

Utilizing this technic it was possible to demonstrate that the rate of generation of the prothrombin-converting principle was dependent upon the concentration of thrombokinase present in the mixture. The yield of this principle, however, was dependent upon the concentration of bovine proaccelerin. These rate studies and the demonstration that calcium was needed during the interaction of thrombokinase and bovine proaccelerin provide data similar to those obtained with human activated Stuart factor and human proaccelerin under the same experimental conditions.

One interpretation of the data, then, is that bovine thrombokinase, like human activated Stuart factor, converts proaccelerin to a prothrombin-convert-
ACTIVATION OF PROACCELERIN

The activation principle which we have called “activated proaccelerin” to distinguish it from accelerin, which was defined in a different manner. These observations do not support the view that proaccelerin catalyzes an action of activated Stuart factor upon prothrombin. One explanation for the difference in view is that under the conditions used in the present experiments, it was possible to observe a time-consuming reaction between activated Stuart factor and proaccelerin which under other circumstances has not been apparent.

The esterolytic activity of the treated thrombokinase and the inhibition of the clot-promoting activity of this purified clot-promoting agent by soybean trypsin inhibitor are in agreement with Milstone's observations. Milstone has postulated that bovine thrombokinase is probably an agent which can directly convert prothrombin to thrombin. He left open the possibility that thrombokinase might require proaccelerin for its action or that the final pro-thrombin-converting principle might actually be derived from proaccelerin. Our experiments seem to support the last of these possibilities—namely, that the final prothrombin-converting principle is actually derived from proaccelerin. In addition, our experiments confirm Milstone's view that small amounts of calcium are necessary for the maximal clot-promoting activity of thrombokinase to develop.

SUMMARY

Through the kindness of Dr. J. H. Milstone we were able to perform clotting studies with purified bovine thrombokinase. Phenylmethyl sulfonyl fluoride was found to inactivate bovine thrombin. Bovine thrombokinase, freed of traces of contaminating thrombin by treatment with phenylmethyl sulfonyl fluoride, behaved in a manner similar to activated Stuart factor. Kinetic studies with this purified clotting factor and partially purified bovine proaccelerin supported the hypothesis that bovine proaccelerin is changed by the enzymatic action of thrombokinase to an agent which converts prothrombin to thrombin. The reaction between thrombokinase and bovine proaccelerin requires phospholipid and calcium and is blocked by soybean trypsin inhibitor. The similarities between human activated Stuart factor and bovine thrombokinase are apparent.
calcium e es bloque per inhibitor de trypsin de soja. Le similitudes inter activate factor Stuart human e thrombokinase bovin es apparente.

ACKNOWLEDGMENT

These studies could not have been performed without the expert technical assistance of Mrs. Marjorie M. Smink and Mrs. Edna R. Stone. Plasma from a patient deficient in Stuart factor was obtained through the kindness of the late Dr. Paul Hagen. Dr. J. H. Milstone was kind enough to critically review the manuscript.

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The Activation of Proaccelerin by Bovine Thrombokinase (with Notes on the Antithrombic Activity of Phenylmethyl Sulfonylfluoride)

ROBERT T. BRECKENRIDGE and OSCAR D. RATNOFF