Effects of Thrombin Treatment on Preparations of Factor VIII and the Ca^{2+}-Dissociated Small Active Fragment

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ABSTRACT When human, canine, or bovine factor VIII preparations are chromatographed on 4% agarose at ionic strength 0.2, the factor VIII activity elutes as a single peak in the void volume with slight tailing. Incubation of such preparations with dilute (0.01 U/ml) highly purified thrombin results in some activation of factor VIII. Chromatography of such incubation mixtures, under the same conditions as before, results in elution of two peaks of factor VIII activity, one in the void volume and one much later with marked tailing. The void volume peak has most of the protein and some factor VIII activity. These void volume fractions also contain all the von Willebrand factor activity of thrombin-treated human factor VIII preparations and all of the platelet aggregating factor activity of thrombin-treated bovine preparations. Longer treatment with thrombin, or treatment with stronger thrombin, appears to shift much more of the procoagulant activity to the later eluting peak. Also, when the peak of factor VIII activity, found in the void volume after thrombin treatment, was again incubated with dilute thrombin, an increase in factor VIII activity occurred. Chromatography of this incubation mixture demonstrated only a small amount of activity in the void volume, while the bulk of the activity was present in the second peak. On the other hand, thrombin treatment of factor VIII activity from peak 2 caused a rapid decline of activity instead of a further increase. It is proposed that the residual factor VIII activity found in the void volume represents unreacted factor VIII, while the late eluting peak represents thrombin-activated material that is of smaller apparent size. The late eluting peak differs from the small active factor VIII fragment obtained by Ca^{2+} dissociation, as the latter can be activated by thrombin. A similar set of experiments was performed using ultracentrifugation of bovine factor VIII preparations on sucrose density gradients. Results of these experiments agreed completely with those obtained with gel chromatography. Preparations made from human hemophilic plasma, by the procedure employed in the purification of human factor VIII, were also incubated with thrombin and chromatographed. von Willebrand factor was again found only in the void volume fractions, but there was no factor VIII activity in any fractions eluted. In other control experiments, activated and unactivated factor VIII fractions did not clot fibrinogen and contained no assayable factor IX or X. The thrombin-modified factor VIII of small size was inactivated by both a naturally occurring human inhibitor to factor VIII and the gamma globulin fraction of a rabbit antisera produced against the calcium-dissociated small active factor VIII fragment.

INTRODUCTION

Investigators (1, 2) studying the inactivation of factor VIII with thrombin noted that factor VIII also experienced a brief period of activation when dilute preparations of thrombin were used. The concept of activation of factor VIII was further extended by controlled studies in which trace amounts of crude thrombin were shown to increase the factor VIII activity of test plasmas 5–40-fold (3). These studies were soon confirmed with the use of a modified thromboplastin generation test, and it was determined that the thrombin-activated form of factor VIII generated factor X

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activator only in the presence of factor IX. In the absence of factor IX, thrombin activated factor VIII and destroyed or rendered it unstable, but did not produce any factor X activator (4). In addition, the presence of phospholipid was shown to potentiate the activation effect of thrombin (5).

Activation studies have been reported recently using highly purified thrombin and preparations of human (6), bovine (7), and canine (8) factor VIII. Results obtained were similar to those found for factor VIII activated by crude thrombin in a plasma system.

This report will deal with the thrombin activation of preparations of human, canine, and bovine factor VIII and the demonstration, by agarose gel chromatography and sucrose density gradient ultracentrifugation, of the appearance of a thrombin-modified factor VIII of apparent small molecular size. Activity of thrombin-modified factor VIII was neutralized by a naturally occurring human inhibitor from an individual with hemophilia A. An antibody raised in rabbits to a preparation of the canine small active factor VIII fragment obtained by Ca^{2+} dissociation also neutralized the activity of thrombin modified factor VIII. Thrombin activation of the salt-dissociated fragments of factor VIII is also described. No discernable effect of thrombin on the platelet aggregating factor (PAF)[1] of bovine plasma fractions or on the von Willebrand factor (vWF) of normal or hemophilic human plasma fractions was found.

METHODS

Blood collection. Factor VIII, factor IX, and factor VIII–IX-deficient dogs (9) were bled by jugular venipuncture with a 16-gauge needle and a two-syringe technique into 1/8 vol of 0.11 M trisodium citrate. Plasma was obtained by centrifugation at 3,500 *g* for 30 min. Samples were frozen for use as substrate plasma. Normal human, canine, and bovine plasmas for factor VIII preparations were collected and used immediately, as previously described (10). Human hemophilic plasma was collected by plasmapheresis in 4% sodium citrate (10).

Storage and freezing of materials. Plasmas, plasma preparations, sera, and thrombin were frozen and stored at −20°C.

Factor VIII preparations. Preparations of canine, bovine, and human factor VIII were obtained with a sp act 3,000–5,000 times that of the initial plasmas. This was accomplished by concentration of fresh plasma, collection of the heavy phase at 0°C, and chromatography with 4% agarose as described (11, 12). The void volume fractions containing the peak factor VIII activity were pooled, precipitated with a final concentration of 30% wt/vol of polyethylene glycol (PEG) (mol wt 3,700, Matheson, Coleman, & Bell, Cincinnati, Ohio). The mixture was incubated for 1 h at 0°C and centrifuged at 10,000 *g* at 4°C for 20 min. The wet precipitates were frozen. A similar preparation was obtained from human hemophilic plasma, and the inactive void volume fractions were pooled, precipitated with PEG, and frozen.

The fractions containing carrier protein and fractions containing the small active fragment of factor VIII were prepared by dissociation with 0.25 M $CaCl_2$ (8). Specific activities of the latter fractions have been up to 4.5 U per μg of protein, as estimated by the Waddell technique (13). Recombination of these fragments in the absence of calcium was performed as before (10, 12).

Assays. Assay for factor VIII was performed using a modification (14) of a partial thromboplastin time (PTT) method (15). Bovine and canine factor VIII fractions were assayed using canine hemophilic substrate activated by mixing with 4 mg/ml of kaolin (Fisher Scientific Corp., Pittsburgh, Pa.) at 28°C for 10 min. Thrombofax (Ortho Diagnostics, Raritan, N. J.) was used as the partial thromboplastin and was diluted 1:20 with 0.15 M NaCl for the canine assay or 1:40 for the bovine assay. Calcium at a concentration of 0.044 M was used for both species. Standard curves were obtained using dilutions of normal canine and bovine plasma, respectively. Human factor VIII was assayed using human hemophilic substrate activated with 10 mg/ml of kaolin for 10 min at 37°C. Thrombofax was diluted 1:10 with normal saline, and the calcium was the same solution used for assay of canine and bovine factor VIII. Standard curves for the human factor VIII assay were obtained using dilutions of a lyophilized human reference plasma (Q-Pak Lot No. 3403T001AI, Travenol Laboratories, Inc., Costa Mesa, Calif.). Factor IX assays were performed by a similar one-stage method. Human and canine factor IX-deficient plasma was activated with kaolin as described above. Thrombofax was used as described for the assay of factor VIII. Normal curves were again obtained using dilutions of species specific frozen normal plasmas. Factor X was estimated using a one-stage prothrombin time assay employing human factor X-deficient plasma. Thromboplastin (Simplastin, Warner-Lambert Pharmaceutical Co., Morris Plains, N. J.) was reconstituted and used without dilution. For assay of the chromatographic fractions obtained with buffered 0.25 M Ca^{2+} solutions, the procedure described by Owen and Wagner (8) was used. Eluates containing buffered 1.0 M NaCl were assayed after a fivefold dilution with distilled water to adjust the ionic strength. The presence of albumin diluent had no effect on the assays when compared with Tris-buffered saline.

Assay for PAF and vWF. A macroscopic platelet aggregation test system was used to measure PAF and vWF activity (16). The assay used formaldehyde fixed, washed platelets prepared by a modification of the technique described by Allain, Cooper, Wagner, and Brinkhous (16). The washed platelet button was suspended in 5 ml of a mixture containing nine parts of 2% formaldehyde in 0.135 M NaH_2PO_4 and one part acid citrate dextrose, final pH 6.9. After 2 h fixation at 23°C, the suspension was diluted with 5 ml of 0.15 M phosphate buffer, pH 6.4, to further dilute the formaldehyde, and the platelets were centrifuged at 900 *g* for 10 min. The supernate was discarded, and the fixed platelet button was washed three times in imidazole-buffered saline, pH 6.4. Stability and specificity of platelets prepared in this manner were the same as previously described (16), and the time of preparation was reduced to a few hours. The assay was sensitive enough to detect 3% of normal bovine plasma or normal human plasma.

Units of activity. 1 U of human, canine, or bovine factor VIII activity is defined as that amount found in 1 ml of

[1] *Abbreviations used in this paper:* PAF, platelet aggregating factor; PEG, polyethylene glycol; PTT, partial thromboplastin time; SDS, sodium dodecylsulfate; vWF, von Willebrand factor.

human, canine, and bovine plasma, respectively. 1 U of PAF is defined as that amount found in 1 ml of normal bovine plasma, and 1 U of vWF is defined as that amount found in 1 ml of standard normal human plasma.

Thrombin activation. Highly purified bovine thrombin (2,670 NIH U/mg)[2] was diluted to the appropriate concentration using an albumin diluent (2% bovine albumin, 0.15 M NaCl, 0.002 M trisodium citrate, 0.05 M Tris-HCL, pH 7.35). Albumin was used in the diluent in an attempt to minimize adsorptive losses when using dilute thrombin. Tris-buffered saline at room temperature was added to the frozen factor VIII precipitates that were to be activated. After the precipitates had dissolved, the solutions were assayed and thrombin was added to give the desired final concentration. Materials containing human factor VIII were incubated at 37°C, while those containing bovine and canine factor VIII were incubated at 28°C. Aliquots were removed at specified time intervals and were diluted before assay with the albumin diluent to give clotting times within the range of the standard plasma curves. Two controls were run: (*a*) thrombin was incubated with a volume of albumin diluent equivalent to the test material; (*b*) the test material containing factor VIII was incubated after the addition of albumin diluent instead of thrombin. Aliquots were removed from the control mixtures and assayed at specific intervals for up to 4 h. In those experiments which were to include chromatography or ultracentrifugation, however, the incubation mix was assayed for 25–45 min to insure that some activation had occurred and was then introduced immediately onto the column or the sucrose density gradient.

Column chromatography. All gel chromatography was performed with siliconized glass columns and 4% agarose (Bio-Gel A-15m, 200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.). Chromatography of the thrombin-activated preparations was performed on columns of 2.5×19.5 or 1.5×26 cm. The samples were eluted by upward flow at 23°C with Tris-buffered saline (0.15 M NaCl, 0.05 M Tris-HCL, pH 6.8). Fractions were monitored for factor VIII activity, vWF or PAF activity, and UV absorption at 280 nm.

Sedimentation on a sucrose density gradient. A 12-ml continuous sucrose gradient (10–40% wt/vol) was prepared in 13-ml cellulose nitrate tubes with Tris-buffered saline, pH 6.8. 1-ml test samples were applied to the gradient. The tubes were centrifuged in a Beckman L3-50 ultracentrifuge with swinging bucket head (SW-36, Beckman Instruments, Inc., Fullerton, Calif.) at 36,000 rpm (average *g* of 140,000) for 16 h at 15°C. Fractions 1–12, each containing about 1.1 ml of the gradient, were collected by gravity from the bottom of each tube. Assays for factor VIII procoagulant activity and PAF activity were performed on samples diluted at least 1:10 to reduce the sucrose concentration (17). Fractions that contained the peak procoagulant factor VIII activity required dilutions of 1:100 to 1:500 for assay.

Electrophoresis was performed essentially according to the method of Weber and Osborn (18) with the use of 5% polyacrylamide gels and sodium dodecylsulfate (SDS) (19). The samples were dissolved in Tris-buffered saline and were then mixed with an equal volume of incubation mixture (0.06 M PO_4, pH 7.0, 2% SDS, 8 M urea, and either 2% dithiothreitol, for reduction or 0.06 M iodoacetamide) and incubated for 16 h at 37°C.

One part of the incubated sample was then mixed with one part glycerol and one part tracking dye (0.1% Bromophenol Blue). Samples (50 μl) of the mixture were layered under the chamber buffer (0.1 M PO_4, pH 7.0, 0.1% SDS) and electrophoresed at an initial current of 0.8 mA/column for 3 h to minimize heating and then at 2.5 mA/column until the tracking dye had migrated 4.5 cm. The gels were stained overnight in a mixture containing 0.05% Coomasie Brilliant Blue, 5% methanol, and 7.5% glacial acetic acid and destained with gentle mechanical stirring in destaining solution (5% methanol, 7.5% glacial acetic acid). The gels were photographed and the R_f values of bands were compared to those of unreduced standards of assigned mol wt (chymotrypsinogen 25,000; ovalbumin 43,000; albumin monomer 67,000; transferrin 90,000; albumin dimer 134,000; β-galactosidase 135,000; and gamma globulin 160,000).

Preparation of an antibody to the small active factor VIII fragment. Concentrated canine small active factor VIII fragment was precipitated at 0°C for 1 h with 30% PEG, wt/vol without the use of albumin, collected by 20 min centrifugation at 10,000 *g* at 4°C, and frozen for immunization procedures. The precipitates, which were suspended in 4 ml Tris-buffered saline (1/10th the original volume), contained 30–50 U/ml of factor VIII activity. A New Zealand white rabbit was bled; incubation of its plasma with normal canine plasma demonstrated no inhibitors. 20 ml of rabbit blood was allowed to clot overnight at 23°C. The serum was obtained by centrifugation at 10,000 *g* for 20 min and adsorbed for 30 min with 100 mg/ml of $BaSO_4$. The adsorbed rabbit serum was then heated to a 56°C for 30 min and clarified by centrifugation at 10,000 *g* for 20 min. This adsorbed rabbit serum was frozen in small samples to be used as control nonimmune rabbit serum. The rabbit was then given a single intravenous injection of 1.0 ml of the small active factor VIII fragment. After 7 days, 0.5 ml was injected subcutaneously and 0.5 ml intramuscularly twice a week for a total of 3 wk. 7 days after the last injections blood was drawn, and the serum was processed as described for the control serum. This immune rabbit serum had a low titer inhibitor to the factor VIII activity present in normal canine plasma. Two additional 3-wk courses of subcutaneous and intramuscular injections were required to obtain a higher inhibitor titer. The immune rabbit serum was then frozen in samples for factor VIII neutralization studies. It gave no precipitin lines when tested by double immunodiffusion in 1% agarose against normal canine plasma, canine factor VIII concentrates, the carrier protein of canine factor VIII, or against the immunization material containing the small active factor VIII fragment. However, at a dilution of 1:32 it neutralized >90% of the factor VIII activity of normal canine plasma, but showed no ability to inactivate factor IX. The inhibitory activity was present in the gamma globulin fraction after ion exchange chromatography of the rabbit antisera on DEAE Sephadex A-50.

Inhibition of factor VIII activity was followed by mixing 1 vol of the immune or the nonimmune rabbit serum, or gamma globulin fraction, or buffer with 1 vol of a frozen normal canine plasma or canine test material and incubating the mixture at 28°C. At intervals, samples were removed from the incubation mixture and tested for their effect on a PTT mixture that included kaolin-activated hemophilic plasma as a substrate. Inhibition studies with plasma from a human subject with classical hemophilia and a naturally occurring inhibitor were performed similarly;

[2] This material was kindly provided by C. M. Jackson and W. G. Owen, Department of Biological Chemistry, Washington University, St. Louis, Mo.

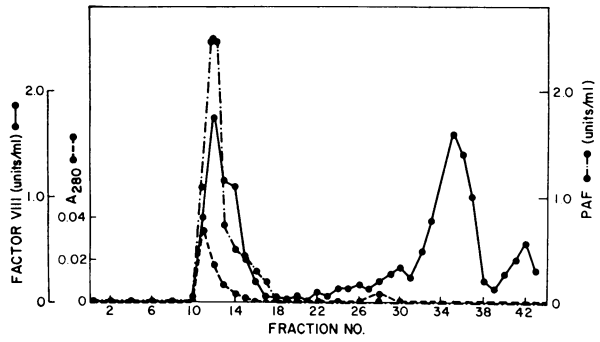

FIGURE 1 Chromatography after 25-min thrombin treatment of factor VIII. 3.9 ml bovine factor VIII preparation plus 0.1 ml (0.4 U/ml) thrombin incubated 25 min at 28°C. Sample (2.8 ml) applied to a 2.5 × 19.5-cm column of 4% agarose at 20°C. Flow rate 20 ml/h (4.1 ml·cm^{-2}·h^{-1}), Tris-buffered saline eluant. 3-ml fractions were collected.

human hemophilic plasma without an inhibitor served as the control.

RESULTS

Activation of normal factor VIII preparations. Preliminary studies were carried out to investigate the optimal conditions for activation of factor VIII preparations to find a final concentration of thrombin which would give prompt activation and slow loss of activity. Some activation could be achieved with final concentrations of thrombin as low as 5×10^{-5} U/ml. It was found that a final concentration of 0.01 U/ml of bovine thrombin would activate factor VIII preparations from normal human, bovine, and canine plasma with a slow decay of the activity over a period of 3-4 h. This concentration of thrombin was routinely used for subsequent activation studies involving chromatography or ultracentrifugation. High concentrations of bovine thrombin (> 0.1 U/ml) resulted in rapid activation, but the levels of activity were lower than those obtained with lesser concentrations of thrombin (< 0.1 U/ml). Activation by the concentrated thrombin was followed by rapid decay of the activity over periods of 60–90 min.

Agarose gel chromatography of the thrombin-factor VIII incubation mixture. Factor VIII preparations were incubated with thrombin. Once some activation had occurred and a relatively stable activity level was achieved, the incubation mixtures were chromatographed. Fig. 1 shows the results obtained after a 25-min incubation of bovine factor VIII with thrombin. The factor VIII activity appeared in two distinct peaks. The first peak of factor VIII activity eluted at or near the void volume, while the second peak eluted in later fractions with significant tailing. Note that detectable protein was found only in the void volume fractions, except for a trace of albumin (tube 28) introduced in the mixture with the thrombin. A similar result was obtained with human and canine preparations.

The concentration of factor VIII activity in the first peak was found to be variable and appeared to be inversely related to the length of incubation with thrombin before chromatography. This is suggested by the results of a 45-min incubation with thrombin shown in Fig. 2. Most of the activity was present in the late fractions with little activity in the void volume. When void volume fractions, obtained after thrombin treatment, that contained measurable factor VIII activity were again incubated with thrombin, activation occurred similar to that observed with the original incubation mixture. Chromatography of this thrombin-

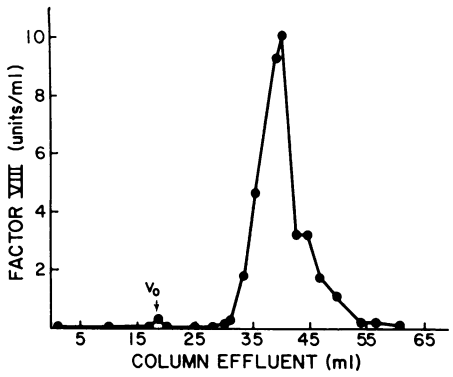

FIGURE 2 Chromatography after 45-min thrombin treatment of factor VIII. 3.9 ml bovine factor VIII preparation plus 0.1 ml (0.4 U/ml) thrombin incubated 45 min at 28°C. Sample (3.6 ml) applied to a 1.5 × 26-cm column of 4% agarose at 20°C. Flow rate 10 ml/h (5.6 ml·cm^{-2}·h^{-1}), Tris-buffered saline eluant. 1-ml fractions were collected.

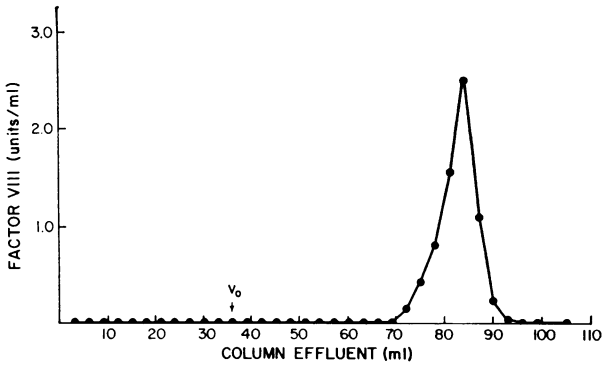

FIGURE 3 Chromatography of thrombin-modified factor VIII in 1.0 M NaCl. 2 ml of thrombin-modified factor VIII, obtained by chromatography, was applied to a 2.5 × 19.5-cm column of 4% agarose at 20°C. Flow rate 15 ml/h (4.9 ml·cm^{-2}·h^{-1}), eluant, 0.05 M Tris-HCl, 1.0 M NaCl, pH 6.8. 3-ml fractions were collected.

treated material showed almost all of the factor VIII activity in the late eluting peak.

The second peak of factor VIII activity had an elution behavior that suggested interaction with the gel. To assess whether this interaction was on the basis of charge, chromatography was carried out in the presence of 1.0 M NaCl, 0.05 M Tris, pH 6.8. The factor VIII activity eluted in fractions denoting small molecular size but with little or no tailing of the activity (Fig. 3).

The effect of thrombin on PAF and vWF activity was also studied. The activity of PAF and vWF was not increased or decreased by incubation with 0.01 U/ml thrombin during a period of 2 h. On chromatography of a thrombin-activated bovine factor VIII preparation, measurable PAF activity eluted only in the void volume associated with the first peak of factor VIII activity (Fig. 1). In comparable experiments with a normal human factor VIII preparation and a similar preparation from human hemophilic plasma, measurable vWF activity also eluted only in the void volume fractions. The vWF activity found in the void volume after chromatography of the thrombin-treated hemophilic preparation was comparable in amount to that found with preparations from normal human plasma.

Density gradient ultracentrifugation of the thrombin-factor VIII incubation mixture. The sedimentation behavior of the factor VIII procoagulant activity and the PAF activity was studied with a bovine factor VIII preparation that contained 29.5 U/ml of factor VIII. This material before reduction did not enter a 5% polyacrylamide gel after denaturation in SDS and 4 M urea. The same material, after reduction, showed a major protein band at about 270,000 mol wt and four faint minor bands. After ultracentrifugation for 16 h in the sucrose gradient, the absorbance at 280 nm, factor VIII activity, and PAF activity all appeared at the bottom of the gradient. The peak for each activity was in the first 1-ml fraction with diminishing activity in fractions 2 and 3. Fractions 4–12 did not have detectable PAF activity, factor VIII activity, or UV absorbance at 280 nm.

When a sample of the same bovine preparation was studied after incubation with thrombin (0.01 U/ml final concentration) for 1 h at 28°C, there was a 21-fold increase in factor VIII activity with no change in the level of PAF activity. SDS polyacrylamide gel electrophoresis of the thrombin-treated material, after reduction, revealed no recognizable differences from the results obtained before thrombin treatment. Duplicate 1-ml samples of the incubation mixture were applied immediately to each of two sucrose density gradients and centrifuged for 16 h. The factor VIII activity was

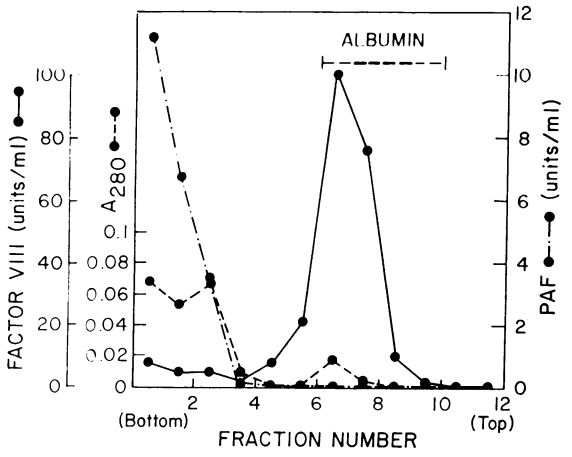

FIGURE 4 Sedimentation of thrombin-treated factor VIII. A sample of bovine factor VIII preparation (29.5 U/ml) was incubated for 1 h at pH 6.8, 28°C, with thrombin (final concentration 0.01 U/ml). 1-ml aliquots were layered on 12 ml buffered pH 6.8 sucrose gradients (10–40%) and centrifuged at 15°C for 16 h at 140,000 average *g*. 12 1.1-ml fractions were collected by gravity and assayed.

now found in a single peak with the maximal concentration in fraction 7, thus sedimenting somewhat faster than crystalline bovine albumin (Fig. 4). Total recovery of factor VIII activity was about 36% (225 U). The protein and PAF activity were still found in the bottom fractions (1–3) together with 18.0 U of factor VIII activity. Fraction 7, which contained 100 U/ml of factor VIII procoagulant activity, had less than 0.06 U/ml PAF activity even when tested undiluted and a small amount of absorbance at 280 nm. Sucrose, at the concentrations used in the assay, had little effect on the detection of PAF activity. Similar results were obtained in a total of six experiments. The factor VIII sedimenting to the bottom after thrombin treatment could be activated by incubation with additional thrombin, but the factor VIII activity sedimenting in fraction 7 could not.

In a different type of experiment, the thrombin-modified factor VIII eluting in the second peak after 4% agarose gel chromatography was pooled, brought to a concentration of 0.1% albumin (wt/vol), and precipitated in 30% PEG (wt/vol). The precipitate was redissolved in Tris-buffered saline, and 1-ml samples were applied to duplicate gradients and centrifuged for 16 h. The factor VIII activity peak appeared in fraction 7. The albumin peaked in fractions 8 and 9.

Experiments to establish that activity measured after thrombin treatment is factor VIII. Preparations were made from human hemophilic plasma in an identical fashion to that used for obtaining factor VIII from normal plasma. When these hemophilic preparations

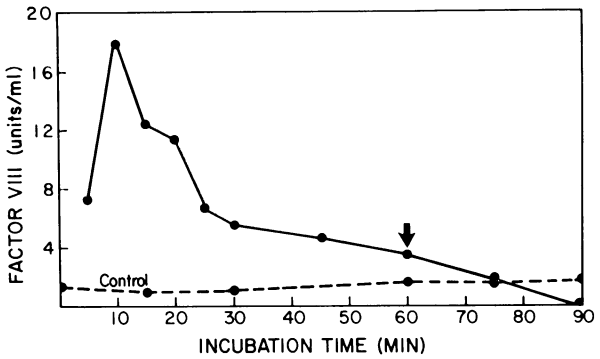

FIGURE 5 Thrombin activation of the Ca^{2+}-dissociated small active factor VIII fragment. Small active fragment (1.5 U/ml) was incubated with thrombin at a final concentration of 0.01 U/ml. Incubation mix (28°C) assayed at intervals. Arrow indicates 0.01 U/ml of additional thrombin. Control is small active fragment (1.5 U/ml) incubated (28°C) with buffer and assayed at intervals for factor VIII activity.

were incubated with a final concentration of 0.01 U/ml of thrombin and the mixture assayed at intervals for up to 4 h, no factor VIII activity was detected.

When the albumin diluent was incubated with 0.01 U/ml of thrombin and the mixture assayed at intervals, there was no change from the blank clotting time obtained with albumin diluent plus Tris-buffered saline. In the other control, the incubation for 2–3 h of factor VIII preparations with albumin diluent alone showed no activation of the factor VIII.

Assay of the factor VIII preparation for factor IX and factor X both before and after the addition of thrombin revealed no detectable factor IX activity (< 0.01 U/ml) and no factor X activity (< 0.003 U/ml). The same preparations showed levels of > 10 U/ml of factor VIII activity after the addition of thrombin.

The immune rabbit serum was tested for its ability to inactivate the thrombin-modified factor VIII activity. This antibody, which neutralized the factor VIII activity of plasma, factor VIII preparations, and the calcium-dissociated small active fragment of factor VIII, also neutralized the activity of the thrombin-modified factor VIII by $> 90\%$ within 60 min of incubation. In a control study, nonimmune rabbit serum incubated with the thrombin-modified factor VIII over a similar time-course resulted in no difference from values obtained with a buffer control.

The human inhibitor in the plasma of a subject with classical hemophilia was also tested as follows. 0.1 ml of thrombin modified bovine factor VIII was mixed with 0.9 ml of plasma from a human hemophiliac without an inhibitor. The clotting time in the activated assay was 76 s, and after 15 min at 23°C, the clotting time was 75 s. No factor VIII activity could be detected immediately after a comparable addition of the inhibitor plasma (166 s).

Thrombin activation of the calcium-dissociated fragments of factor VIII. The carrier protein fractions after dissociation of human and bovine factor VIII preparations in 0.25 M $CaCl_2$ were not completely devoid of factor VIII activity even after recycling through a calcium dissociation column. These fractions were incubated with 0.01 U/ml of thrombin, and aliquots of the mixture were removed and assayed at intervals for factor VIII activity. The residual activity after dissociation increased after thrombin treatment. Agarose gel chromatography of the mixture at room temperature again demonstrated two peaks of factor VIII activity, a small peak at the void volume, and a larger peak of activity that eluted later with tailing.

The small active factor VIII fragment (1.5 U of factor VIII activity/ml) from calcium dissociation of a canine factor VIII preparation was incubated with thrombin at a final concentration of 0.01 U/ml. Assay of the incubation mixture at intervals demonstrated a 12-fold activation after 10 min. The activity declined over a period of 60 min, and addition of more thrombin at that point failed to elicit any further activation (Fig. 5). Incubation of a low concentration of factor VIII activity (0.2 U/ml final concentration) and a very dilute thrombin (0.001 U/ml final concentration) resulted in a sluggish reaction with indication of only slight activation after 75 min (Fig. 6). If a sample containing the same amount of factor VIII activity (0.2 U/ml) was incubated with a slightly more concentrated thrombin (0.005 U/ml), definite activation was demonstrated and was maximal in about 15 min. When the

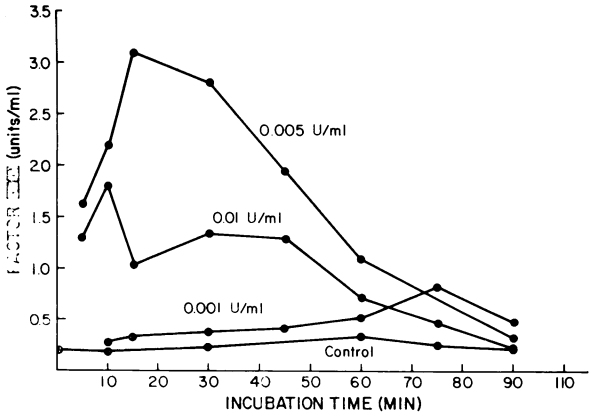

FIGURE 6 Effect of thrombin concentration on activation of dilute (0.2 U/ml) Ca^{2+}-dissociated small active factor VIII fragment. Samples of the small fragment were incubated at 28°C with thrombin to give 0.001, 0.005, and 0.01 U/ml final thrombin concentration and were assayed at intervals. Control was incubated in buffer without thrombin.

amount of thrombin was further increased to a final concentration of 0.01 U/ml, even more prompt activation occurred reaching maximal activation in 10 min, but the degree of activation was less. If, on the other hand, the concentraiton of factor VIII activity was increased to 4.0 U/ml and incubated with that amount of thrombin (0.001 U/ml) shown to have little effect on 0.2 U/ml of factor VIII activity, activation was again easily demonstrated. Increasing the thrombin concentration to 0.005 U/ml caused even more pronounced activation. Similar results were obtained with the small active fragments from human and bovine preparations.

The effect of thrombin on recombined factor VIII fragments. The small fragment obtained from human, bovine, and canine factor VIII after Ca^{2+} dissociation was recombined with its respective carrier protein, as previously described (13, 15). In each of the three species, the recombined factor VIII, when incubated with thrombin at a final concentration of 0.01 U/ml, activated in a manner indistinguishable from that seen with the undissociated factor VIII preparations.

Components needed for manifestation on factor VIII activity. The availability of dogs congenitally deficient in factor VIII, factor IX, and factors VIII and IX offered an opportunity to test the hypothesis that thrombin-treated factor VIII required the presence of factor IX to manifest its activity. A bovine factor VIII preparation was incubated with a final concentration of 0.1 U/ml of thrombin, and aliquots were removed at intervals and tested with a PTT using kaolin-activated factor VIII, factor IX, and factor VIII–IX-deficient plasmas as substrates (Table I). The incubation mixture when assayed in factor VIII-deficient plasma required a 5,000-fold dilution to reach a value on the standard curve. Aliquots of the same mixture, at a 1:10 dilution, did not change the clotting time significantly from the blank when added to factor IX-deficient plasma and showed only minimal correction of the VIII–IX-deficient plasma.

Further characteristics of the thrombin-modified factor VIII. The thrombin-modified factor VIII obtained after chromatography on 4% agarose in Tris-buffered saline was studied for its stability at 4 and 23°C. The material stored at 23°C was relatively stable for several hours but lost 97% of its activity in 24 h and had no detectable activity by 72 h. At 4°C, however, the same sample lost only 6% of its activity in 24 h and still retained 40% of its activity at 72 h. After 1 wk at 4°C only 2% of the original activity remained.

When the thrombin-modified factor VIII was again incubated with thrombin at a final concentration of 0.01 U/ml, no further activation was noted. In fact, the factor VIII activity of the incubation mixture rapidly declined over a period of 60–90 min.

TABLE I
Assay of Thrombin-Treated Factor VIII

Incubation time	Dilution of incubation mix*	PTT with VIII deficient plasma	PTT with IX deficient plasma	PTT with VIII–IX deficient plasma
min				
0	—	102.8	120.0	166.8
0.5	1:10	47.5	117.8	141.3
	1:100	66.2		
	1:1000	70.2		
15	1:10	45.4	110.7	119.3
	1:1000	55.8		
	1:5000	68.5		
30	1:10		112.5	123.0
	1:1000	56.7		
	1:5000	67.1		
60	1:10		126.7	136.8
	1:5000	76.5		
60	—	106.3	125.9	169.7

* Bovine factor VIII preparation was incubated with thrombin (0.01 U/ml final concn.). Samples were removed, diluted, and the PTT was determined with the use of kaolin-activated substrate plasmas deficient in specific factors.

Treatment with kaolin of the hemophilic plasma used in the factor VIII assay was not a necessary requirement for the detection of the thrombin-modified factor VIII activity. The small molecular weight material obtained after the incubation of the intact factor VIII complex with thrombin was found to be equally active in the unactivated and activated PTT assay systems.

DISCUSSION

A previous report has suggested that the factor VIII molecule undergoes a change in molecular size upon incubation with thrombin. Weiss and Kochwa (20) showed that treatment of normal plasma with crude thrombin (0.35 U/ml) in the presence of phospholipid resulted in a slower sedimentation of the factor VIII activity in a sucrose gradient than that noted for untreated plasma. The modified-factor VIII sedimented at about the same rate as fibrinogen.

On the other hand, studies have also been reported that thrombin activation of factor VIII results in no detectable change in the molecule. Shapiro, Andersen, Pizzo, and McKee (21) have reported no detectable effect of thrombin on the molecular weight of a major band obtained with SDS polyacrylamide gel electro-

phoresis of factor VIII preparations in the presence of sulfhydryl reducing agents. In addition, they noted no change in the intensity of protein or carbohydrate staining of the major band nor the appearance of any new bands after treatment with thrombin. Based on the known proteolytic activity of thrombin on other substrates, they postulated that thrombin could produce specific modifications of the factor VIII molecule by cleaving a peptide too small to be detected by this method. We were also unable to show, by polyacrylamide gel electrophoresis after reduction, any change produced by thrombin treatment of a bovine factor VIII preparation. However, changes were observed in the sedimentation and chromatographic behavior of factor VIII activity after thrombin treatment. This suggests that thrombin is capable of modifying the factor VIII in such a way that an active molecule of smaller size is produced that contains only a small fraction of the initial protein. Changes were not observed in the sedimentation and chromatographic behavior of PAF or vWF activity after thrombin treatment.

Incubation for 25 min on bovine factor VIII preparations with 0.01 U/ml of thrombin, followed by chromatography on 4% agarose, resulted in the appearance of two peaks of factor VIII activity (Fig. 1). Several observations support the idea that the first peak of activity represented merely unmodified factor VIII rather than factor VIII resistant to thrombin. First, the activity eluted at or near the void volume, a behavior similar to that of untreated factor VIII. Secondly, if the fractions containing the activity in peak one were again incubated with 0.01 U/ml thrombin, prompt activation occurred, and chromatography of this mixture showed almost all of the factor VIII activity in a late eluting peak. Thirdly, a single long incubation with 0.01 U/ml thrombin before chromatography resulted in almost complete conversion to a molecule of apparently smaller size, i.e, activated, thrombin-modified factor VIII (Fig. 2).

The tailing of factor VIII activity in the second peak may be explained on the basis of interaction with the gel (Fig. 3). Here, conditions chosen to minimize interactions due to charge resulted in sharp elution of the activity and a shift of the elution volume of the peak from 105 ml (tube 35) in Fig. 1 to 84 ml in Fig. 4. Identical columns were used for these two figures.

The sedimentation experiments agreed with the results obtained by agarose gel chromatography. After thrombin treatment the bulk of the factor VIII sedimented as a smaller or less dense material while a small fraction of the total activity sediment rapidly, like the activity in the untreated control (Fig. 4). The fast sedimenting factor VIII activity like that found in the void volume fractions after gel chromatography could be further activated by thrombin. The thrombin-modified factor VIII, sedimenting slowly in the gradient (fraction 7), could not be further activated by thrombin but was neutralized by a specific human inhibitor to factor VIII.

Control studies were carried out to determine whether or not the activity being measured after thrombin activation was related to factor VIII. The concentrations of thrombin used in these experiments when tested without factor VIII were shown not to interfere with the PTT assay. Also, incubation of factor VIII with albumin diluent alone gave no activation. Treatment of a hemophilic preparation of factor VIII with thrombin did not result in the appearance of any activity. A similar study by Shapiro et al. resulted in only slight activation (21). The thrombin-modified factor VIII was shown by specific factor assay not to be factors IX or X. The activity of the thrombin-modified factor VIII was neutralized by a rabbit antiserum raised to the purified small active factor VIII fragment as well as by a human inhibitor in the plasma of a subject with classical hemophilia.

The effect of thrombin on the calcium-dissociated fragments of factor VIII was also studied. The normal carrier protein fractions retained low but detectable factor VIII activity even after recycling through a calcium dissociation column. Thus, dissociation under the conditions of 0.25 M calcium is not complete. Incubation of these carrier protein fractions with thrombin, followed by chromatography, demonstrated two peaks of factor VIII activity.

The small active fragment produced by calcium dissociation can be readily activated by thrombin (Fig. 5). It was previously reported that thrombin (0.001 U/ml final concentration) did not activate 0.2 U/ml of the calcium-dissociated small active fragment (8). An explanation for this apparent discrepancy is found in Fig. 6. Duplication of the original conditions used resulted in insignificant activation. However, activation was readily obtained with the use of more concentrated thrombin. Activation also occurred if an increased concentration of the small active fragment was incubated with dilute thrombin (0.001 U/ml). Thus, the demonstration of prompt activation depends upon the concentration of both reactants. It should be emphasized that the thrombin activation studies in this report were performed without added phospholipid. It was also not unexpected that recombined factor VIII was activated with thrombin, as no other differences have been found between factor VIII before dissociation and after recombination.

Thrombin-modified factor VIII corrected an VIII-deficient substrate (Table I). It has no factor IX activity, and the minimal effect seen with VIII–IX-de-

ficient substrate agrees with earlier reports that factor IX_a is a requisite for thrombin-modified VIII to generate factor X activator. Factor VIII, IX_a, phospholipid, and calcium in the absence of thrombin have been reported to produce only an insignificant amount of factor X activator in 15 min (*22*). Whether this demonstrates an absolute requirement of thrombin for the completion of the intrinsic pathway in vivo must await further investigation.

Three observations were made concerning the thrombin-modified factor VIII obtained after 4% agarose chromatography: (*a*) The activity was stable for up to 72 h at 4°C and was fairly stable for a few hours at 23°C. (*b*) Addition of thrombin to this material did not result in further activation, in fact, activity was lost rapidly. This suggests that the decay of activity of the thrombin incubation mixtures was primarily caused by further inactivation by thrombin, rather than inherent lability of the thrombin-activated material. (*c*) Thrombin-modified factor VIII, like the calcium-dissociated small active fragment (8), was active in both the activated and unactivated PTT assay system. This is in contrast to other factor VIII-like preparations of small molecular size in which the omission of kaolin results in the demonstration of no factor VIII activity (*23*, *24*).

PAF activity of bovine factor VIII preparations did not increase after incubation with 0.01 U/ml of thrombin. vWF, as previously reported (*25*), was also insensitive to this concentration of thrombin. No change in size of the PAF or vWF was demonstrated by gel chromatography after thrombin treatment (Fig. 1). In addition, fractions containing the small active fragment after thrombin treatment had no detectable vWF or PAF. The lack of PAF or vWF activity in these fractions cannot be explained as a failure of detection because of a lack of sensitivity in the assay. For example in Fig. 4, even if the small active peak, 100 U/ml of factor VIII, represents material activated 60-fold, it should still correspond to 1.7 U/ml of factor VIII of normal bovine plasma. The assay can easily detect 0.03 U/ml of PAF. Therefore, thrombin treatment followed by gel chromatography or sucrose density gradient ultracentrifugation is another way to separate factor VIII activity from the vWF.

Activation of factor VIII strikingly resembles the activation of factor V. Factor V is also activated with trace amounts of thrombin (*26*, *27*) and is changed to a smaller sized active molecule (*27*, *28*). Unlike native factor V, the activated form binds to prothrombin-Sepharose (Pharmacia Fine Chemicals Inc., Piscataway, N. J.), and an interesting suggestion has been made that activated factor VIII, in an analogous fashion, should bind to factor X (*29*).

It can be speculated that factor VIII consists of a large carrier protein to which is bound by noncovalent bonds an unknown number of small active factor VIII fragments. The large carrier protein possesses several types of functional sites, three of which have been described: (*a*) immunologic sites which participate in the immunoprecipitin reaction with heterologous antibodies raised to the partially purified factor VIII complex; (*b*) sites which participate in the aggregation of platelets; and (*c*) sites which bind noncovalently the small active factor VIII fragment. Factor VIII-like antigen (site 1) and PAF or vWF (site 2) have been reported to be unaffected by thrombin (*25*).

The small active factor VIII fragment must have four types of functional sites: (*a*) the sites that participate in the noncovalent binding to the carrier protein; (*b*) a site associated with factor VIII activity; (*c*) sites associated with neutralization by an antibody; and (*d*) sites sensitive to the proteolytic action of thrombin. The action of thrombin on site *d* results in enhancement and subsequent loss of factor VIII activity.

Thrombin treatment of large molecular weight factor VIII results in two observable events: activation and production of factor VIII activity of smaller size. If these are two separate and distinct events at different sites, then an additional thrombin sensitive site must also be postulated either on the carrier molecule or on the small active fragment. If cleavage at this site alone could be obtained with thrombin, a small but nonactivated factor VIII fragment should be produced.

A decrease in the apparent molecular size of factor VIII has recently been shown to accompany activation with trypsin (*30*). The enzymatic activation of factor VIII is probably complex and numerous speculations are possible. The relevance of these in vitro activation studies to the role that factor VIII plays in in vivo coagulation remains to be elucidated.

Addendum. Since the submission of this manuscript, Rick and Hoyer (*31*) have reported thrombin activation of the low molecular weight fragment of antihemophilic factor (factor VIII). Their low molecular weight fragment was obtained by Ca^{2+} dissociation and agarose gel chromatography. Their results are in complete agreement with ours.

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