Activation of Human Factor VII During Clotting in Vitro

By L.V.M. Rao, S.P. Bajaj, and S.I. Rapaport

We have studied factor VII activation by measuring the ratio of factor VII clotting to coupled amidolytic activity (VIIc/VIIam) and cleavage of 125I-factor VII. In purified systems, a low concentration of factor Xa or a higher concentration of factor IXa rapidly activated 125I-factor VII, yielding a VIIc/VIIam ratio of 25 and similar gel profiles of heavy and light chain peaks of VIIa. On further incubation, VIIa activity diminished and a third 125I-peak appeared. When normal blood containing added 125I-factor VII was clotted in a glass tube, the VIIc/VIIam ratio rose fivefold, and 20% of the 125I-factor VII was cleaved. Clotting normal plasma in an activated partial thromboplastin time (APTT) system yielded a VIIc/VIIam ratio of 25 and over 90% cleavage of 125I-factor VII. Clotting factor XII-deficient plasma preincubated with antibodies to factor X in an APTT system with added Xa yielded a VIIc/VIIam ratio of 19 and about 60% cleavage, which indicates that Xa, at a concentration achievable in plasma, can effectively activate factor VII. Clotting normal plasma with undiluted tissue factor yielded a VIIc/VIIam ratio of 15 to 20 and 60% cleavage of 125I-factor VII, whereas clotting plasma with diluted tissue factor activated factor VII only minimally. We conclude that both Xa and IXa can function as significant activators of factor VII in vivo clotting mixtures but believe that only small amounts of factor VII may be activated in vivo during hemostasis.

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From the Departments of Medicine and Pathology, University of California, San Diego.

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Address reprint requests to Dr Samuel I. Rapaport, UCSD Medical Center (H811K), 225 West Dickinson St, San Diego, CA 92103.

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HUMAN FACTOR VII is a single-chain glycoprotein whose enzymatic activity is increased 20- to 30-fold after limited proteolysis to a two-chain molecule, factor VIIa.1,2 In purified systems containing phospholipid and calcium, a low concentration of factor Xa3,4 or a higher concentration of factor IXa4 rapidly activates factor VII. Factor XIIa can also activate factor VII in a reaction independent of added phospholipid or calcium,5 which accounts for the long-known observation that plasma factor VII activity may increase several-fold after plasma is exposed to a glass surface.6 Thrombin has also been reported to activate factor VII.7

Over 20 years ago, Johnston and Hjort7 reported that factor VII activity increases two- to three-fold after blood coagulation in vitro. In studies from this laboratory, Seligsohn et al8 reported a four- to fivefold increase in factor VII activity when plasma was recalcified in glass tubes and incubated for 2 1/2 hours before assaying factor VII activity. From the pattern of factor VII activation of different hereditary clotting factor deficient plasmas, these investigators concluded that factor IXa functioned as a principal activator of factor VII.8

It is difficult to reconcile this conclusion with the knowledge that trace amounts of factor Xa can rapidly activate factor VII in purified systems. Further studies from this laboratory did not resolve the apparent discrepancy. Thus, Masys and co-workers4 found that factor Xa was from 20 to 800 times more efficient than factor IXa as an activator of human factor VII in different types of purified systems. Masys et al9 also found that factor VII activity, which had increased about fivefold when normal blood was clotted in a glass tube, failed to increase further when a low concentration of exogenous factor Xa was added to the serum. These studies left unanswered whether the fivefold activation of factor VII found in the serum reflected incomplete activation of factor VII or maximal activation of factor VII followed by partial inactivation of factor VIIa.

In the earlier studies, generation of factor VIIa was measured as an increase in the ratio of factor VII clotting activity to factor VII coupled amidolytic activity (VIIc/VIIam).9 We have since developed techniques to purify and radiolabel factor VII that allow measurement of activation of factor VII by molecular cleavage of 125I-factor VII. Using both the VIIc/VIIam ratio and the extent of cleavage of 125I-factor VII found on reduced sodium dodecyl sulfate (SDS) gels, we have examined further the activation of factor VII in both purified and plasma systems. These experiments, reported herein, establish that factor VII is only partially activated during clotting in vitro, unless a potent initiator of either the contact activation reactions or a high concentration of tissue factor is added to reaction mixtures. The experiments also clarify earlier differences between results from purified and plasma systems.

MATERIALS AND METHODS

Reagents

Tissue factor (TF) was a saline extract of human brain tissue. It clotted normal pooled "non-contact-activated" plasma in 20 sec-
ond. Rabbit brain thromboplastin and rabbit brain cephalin were obtained from Sigma Chemical Co (St Louis). Hereditary clotting factor deficient plasmas were obtained from George King Biomedical (Overland Park, Kan) or from patients seen in this laboratory. Chromogenic substrate Bz-Ile-Gly-Glu-Arg-p-nitroanilide (S-2222) was obtained from Kabi Diagnostics (Stockholm). Activated partial thromboplastin time (APTT) reagent was from General Diagnostics (Raritan, N.J). Na\(^{251}\) and sodium \(^{3}H\) borohydride were purchased from Amersham Corporation (Arlington Heights, Ill). All reagents for electrophoresis were from Bio-Rad Laboratories (Richmond, Calif). All other chemicals were of the best grade available from commercial sources.

**Purified Human Clotting Factors**

Human factor VII was purified to homogeneity by a modification, described in detail elsewhere,\(^{10}\) of a previously developed method.\(^{2}\) Factor IX and factor X were purified to homogeneity, also as described earlier from this laboratory.\(^{11}\) Human factor XI was purified by a slight modification of the method of Kurachi and Davie.\(^{12}\)

**Factor XIa.** Factor XIa was prepared by activating factor XI with insolubilized trypsin as described earlier.\(^{13}\) Factor IXa was prepared by activating purified factor IX, final concentration 100 \(\mu\)g/mL, with purified factor XIa, final concentration 1 \(\mu\)g/mL, in the presence of 5mol/L calcium. Full activation was obtained within 30 minutes as demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence and absence of a reducing agent. Factor XIa was not removed from the factor IXa preparation, since factor XIa does not activate factor VIII.\(^{14}\)

**Factor Xa.** Factor Xa was prepared by incubating purified factor X (specific activity, 60 U/mg) with insolubilized Russell's viper venom in the presence of calcium as described previously.\(^{11}\) Full activation was documented by SDS-PAGE. The fully activated material gave a specific activity of 1,245 U/mg in our factor Xa assay.

**Concentrations.** Concentrations of purified clotting factors were determined spectrophotometrically at A280 nm using E\(_{280}\) of 13.9 for factor VII,\(^{2}\) 13.2 for factor IX,\(^{14}\) 11.6 for factor X,\(^{14}\) and 13.4 for factor XI.\(^{15}\)

**Radiolabeling of Proteins**

\(^{125}I\)-Factor VII. factor VII was labeled with Na\(^{125}I\) by using Iodogen (Pierce Chemical Co, Rockford, Ill) as described in the manufacturer's product technical bulletin. Ten micrograms of Iodogen in 100 \(\mu\)L of methylene chloride were coated onto the bottom of each 12 \(\times\) 75-mm plastic tube and thoroughly dried under a stream of nitrogen gas. The reaction vessel was washed with cold TRIS borohydride followed by 10 \(\mu\)L of Na\(^{125}I\) (1 mCi) were added to the reaction vessel. The reaction was allowed to proceed for 30 minutes at 4°C with occasional shaking of the tube. The sample was then removed from the reaction vessel and dialyzed against 0.02 mol/L TRIS - HCl, 0.15 mol/L NaCl, pH 7.5, for 24 hours, with four changes of dialysis buffer, to remove free \(^{125}I\). Factor VII was stored frozen at -80°C in small aliquots and used within 1 month of preparation. The specific activity of preparations varied between 1 and 2 \(\times\) 10\(^5\) cmol/cmol of protein. Nonlabeled factor VII contained 1,600 to 1,750 U of clotting activity per mg of protein. Radiolabeled preparations retained about 95% of the activity of nonlabeled preparations. Both nonlabeled and radiolabeled preparations lost biologic activity on repeated freezing and thawing.

Sialyl-\(^{3}H\) factor IX was prepared as described earlier.\(^{17}\) Its specific activity was 1.1 \(\times\) 10\(^5\) cmol/cmol of protein. Sialyl-\(^{3}H\) factor X was prepared by a slight modification of the same procedure\(^{18}\) in which oxidizing agent and reducing agent were added in equimolar concentration to the sialic acid concentration of factor X. Its specific activity was 5.8 \(\times\) 10\(^6\) cmol/cmol of protein. Labeled preparations of factor IX and factor X retained from 85% to 90% of their original biologic activity.

**Antifactor X Antiserum**

A goat was immunized with purified factor X that had been passed through antibody affinity columns to remove traces of protein C, protein S, factor IX, and prothrombin. The immunoglobulin fraction of the resultant antiserum gave a single precipitation line of identity on double immunodiffusion plates with purified factor X and normal plasma. A 100-fold dilution of the antibody preparation neutralized 60% of the factor X clotting activity in normal plasma. The antibody was added to plasma in a final 10% (vol/vol) concentration to neutralize plasma factor X activity.

**Clotting Factor Assays**

**Assays of Factor VII and VIIa.** Factor VII clotting activity (VIIc) was measured by a one-stage clotting assay in which 50 \(\mu\)L of an equal part mixture of factor VII deficient plasma and BaSO\(_4\) adsorbed bovine plasma was incubated with 50 \(\mu\)L of rabbit brain thromboplastin in a glass tube (12 \(\times\) 75-mm) for three minutes, at 37°C. Then, 25 \(\mu\)L of test sample and 50 \(\mu\)L of 35 mmol/L CaCl\(_2\) were added and the clotting time noted. Test samples were diluted in 0.05 mol/L TRIS - HCl and 0.15 mol/L NaCl, pH 7.5, containing 1 mg/mL bovine serum albumin.

The coupled amidolytic assay for factor VII (VIIam) was carried out as described by Seligsohn et al,\(^{9}\) except that rabbit brain thromboplastin was used in place of human brain thromboplastin.\(^{16}\) In contrast to the clotting assay, the coupled amidolytic assay measures total factor VII level independent of the activity state of factor VII.\(^{9}\) Hence, the ratio of factor VII activity in the clotting assay to the factor VII activity in the coupled amidolytic assay (VIIc/VIIam) reflects the activation state of factor VII in a given test material.\(^{9}\)

**Assays of factor IXa and factor Xa.** A clotting assay for factor IXa was carried out by incubating 50 \(\mu\)L of APTT reagent and 50 \(\mu\)L hereditary factor IX-deficient plasma in a 12 \(\times\) 75-mm glass tube for five minutes at 37°C. Then, 50 \(\mu\)L of test sample and 50 \(\mu\)L of 25 mmol/L CaCl\(_2\) were added, and the clotting time noted. This assay measures existing factor IXa in a test sample plus any factor IXa formed from activation of factor IX in the test sample during the interval between its addition to the reaction mixture and the appearance of the fibrin clot end point.

Factor Xa activity was measured by incubating 50 \(\mu\)L of a mixture of equal parts of bentonite-adsorbed human plasma and BaSO\(_4\) adsorbed bovine plasma with 50 \(\mu\)L of half-diluted stock cephalin for three minutes. Then, 25 \(\mu\)L of test sample and 50 \(\mu\)L of 35 mmol/L CaCl\(_2\) were added, and the clotting time noted. The reference curve for the assay was prepared with our usual factor X assay method, which contains Russell's viper venom as an added reagent.\(^{17}\) Citrated pooled plasma from more than ten healthy donors was used as a reference standard that was defined as containing 1 unit of all clotting factor activities.

**Activation Peptide Release Assays**

Rates of factor IX and factor X activation were monitored by measuring \(^{3}H\)-activation peptide release from \(^{125}I\)-factor IX as described by Zur and Nemerson\(^{9}\) and from \(^{3}H\)-factor X as described by Silverberg et al.\(^{18}\) Details of our modifications of the technique for factor IX have been described earlier.\(^{19}\) One hundred percent
activation of factor IX or factor X yielded approximately 35% to 40% trichloracetic acid (TCA) soluble counts.

**Gel Electrophoresis**

SDS-gel electrophoresis was performed according to the method of Laemmli.  

**Measurement of Factor VII Activation From Radioactivity Gel Profiles**

Test samples from reaction mixtures were reduced with 5% 2-mercaptoethanol and subjected to SDS-gel electrophoresis. The gels were cut in 1-mm slices; the slices were counted in a Tracer (Elk Grove, Ill) analytic 1197 gamma counter, and the resultant radioactivity profiles were plotted with the aid of a computer program. The percentage of factor VII activation was calculated from the formula:

\[
100 \times \left( \frac{\text{CPM in native factor VII peak}}{\text{Total CPM}} \right)
\]

The percentage of the third peak was calculated from the formula:

\[
\frac{\text{CPM in third peak}}{\text{Total CPM}} \times 100.
\]

**Activation of Factor VII**

**Purified systems.** Purified \(^{125}\)I-factor VII was incubated at 37 °C with purified factor Xa or factor IXa, phospholipid (cephalin), and calcium at the final concentrations listed in the figure legends. The buffer used for diluting clotting proteins was 50 mmol/L TRIS·HCl, 0.15 mol/L NaCl, pH 7.5, containing 1 mg/mL bovine serum albumin. Ten-microliter test samples were removed at intervals and added to 190 μL of buffer containing 6 mmol/L Na\(_2\)EDTA. The samples were then diluted further as needed in buffer without EDTA and assayed in the factor VII clotting assay and the factor VII coupled amidolytic assay. A second aliquot was removed at the same incubation interval for gel electrophoresis. It was added to SDS-sample buffer containing 5% 2-mercaptoethanol in 6 mmol/L EDTA.

**Plasma systems.** Activation of factor VII was studied in whole blood or recalcified plasma clotted in a glass tube as described further in Results; in plasma clotted by the addition of an activated partial thromboplastin time reagent (APTT system); and in plasma clotted by the addition of a tissue factor reagent (TF system). In the APTT system, 100 μL of plasma containing 6 μg/mL of \(^{125}\)I-factor VII was clotted in a 12 × 75-mm glass tube with 100 μL of APTT reagent and 5 μL of 0.5 mol/L CaCl\(_2\). Serum was obtained for analysis of factor VII activity after a further incubation of the clot at 37 °C. In experiments measuring the time course of activation, a series of tubes were set up and serum was removed for testing at increasing intervals beginning 2½ minutes after clotting. In the TF system, conditions were similar except that tissue factor reagent was used instead of APTT reagent, and the mixture was clotted and incubated in a plastic tube to avoid contact activation.

**RESULTS**

**\(^{125}\)I-Factor VII**

Factor VII was purified from human plasma to homogeneity as indicated by SDS-PAGE in the presence or absence of reducing agent (Fig 1 inset). \(^{125}\)I-factor VII, analysed in a similar manner, yielded a single radioactive peak with a migration coincident to that observed on stained gels (Fig 1). When factor Xa was added to factor VII in the presence of phospholipid and calcium, stained gels revealed cleavage of the zymogen to yield a two-chain factor VIIa molecule consisting of a heavy chain and a light chain doublet (Fig 1 inset). When similar activations were carried out with \(^{125}\)I-factor VII, radioactivity gel profiles (Fig 1) yielded a heavy chain peak and a single light chain peak instead of the light chain doublet seen on stained gels.

**Time Course of Factor VII Activation by Factor Xa and Factor IXa in Purified Systems**

The time course of \(^{125}\)I-factor VII activation by purified factor Xa, in the presence of calcium and phospholipid, is shown in Fig 2. Factor VII was activated maximally within 15 minutes as measured in the VIIc assay. However, radioactivity gel profiles revealed that only about 65% of the factor VII was cleaved into a two-chain molecule at 15 minutes. On stained gels this third peak was seen as a triplet. Appearance of the third peak was associated with a reduction in the heavy chain peak of activated factor VII and with a
loss of factor VII activity in both the clotting and coupled amidolytic assays.

Activation of factor VII with factor IXa, in the presence of calcium and phospholipid, yielded similar radioactivity profiles, although the third peak was less prominent. When a mixture of factor VII activated by factor Xa and of factor VII activated by factor IXa was subjected to reduced SDS-PAGE, the mobility of the resultant radioactivity peaks could not be distinguished from the mobility of the peaks obtained with the individual preparations. Thus, factor IXa and factor Xa appeared to induce the same cleavages in factor VII. However, in keeping with earlier results from this laboratory, a much higher concentration of factor IXa (200 nmol/L) than of factor Xa (1 nmol/L) was required to obtain similar time courses of activation. Therefore, antiserum to factor X was included in reaction mixtures with factor IXa to eliminate any possible effect of trace contamination of the factor IXa preparation with factor Xa.

Similar cleavage products were obtained when 125I-factor VII was incubated with thrombin. However, thrombin was only a weak activator of factor VII. A 400 nmol/L final concentration of thrombin cleaved only about 20% of native factor VII in one hour.

**Factor VII Activation During Clotting of Whole Blood**

125I-factor VII, in a final concentration of 5 μg/mL, was added to normal venous blood. One portion of the blood was placed in a plastic tube containing one-tenth volume of citrate anticoagulant; a second portion of blood was allowed to clot in a glass tube; and both samples were incubated for 2½ hours at 37 °C. Then, their VIIc and VIlam activities were assayed and the molecular cleavage of the 125I-factor VII was determined by SDS-PAGE.

In five experiments, the VIIc/VIlam ratios were: plasma, 1.0 to 1.2; serum, 3.5 to 5.4. Eighteen percent to 24% of the 125I-factor VII in serum was cleaved. Similar results were obtained with serum prepared by recalcifying normal platelet poor plasma in a glass tube.

Because of the incomplete activation of factor VII found in these experiments, we measured the extent of generation of factor IXa and factor Xa when whole blood containing 3H-factor IX (final concentration 8 μg/mL) or 3H-factor X (final concentration 10 μg/mL) was clotted and incubated in a glass tube for 2½ hours at 37 °C. Under these conditions, approximately 45% of factor IX and 15% of factor X were activated, as measured by activation peptide release.

In a further experiment, blood was clotted and incubated for 2½ hours, and then exogenous factor Xa, in a final concentration of 5 μg/mL, was added to the serum together with calcium, final concentration 6 mmol/L, and phospholipid, final concentration 10% vol/vol. When examined after one hour of further incubation, the VIIc/VIlam ratio of the serum had increased to about 20, and radioactivity profiles revealed that about 75% of the labeled factor VII was in the activated form.

**Factor VII Activation in Normal Plasma Clotted in the APTT and TF Systems**

Normal plasma containing added 125I-factor VII, final concentration 6 μg/mL, was clotted in either the APTT or TF system. Samples removed from successive tubes at increasing intervals after clotting were assayed for VIIc and VIlam activities and subjected to reduced SDS-PAGE (Fig 4). In the APTT system, the VIIc/VIlam ratio increased about 25-fold within 15
minutes after clotting and did not change further over the next 45 minutes. Similar kinetics were observed in the TF system, but the VIIc/VIIam ratio rose only 15- to 20-fold. The extent of molecular cleavage paralleled the extent of increase in the VIIc/VIIam ratio. In contrast to purified systems, a third chain was not formed on incubation for up to one hour in these plasma systems.

**Activation of Factor VII in Hereditary Clotting Factor Deficient Plasmas After Clotting in the APTT and TF Systems**

Factor VII activity in normal and different hereditary clotting factor deficient plasma was measured before and one hour after clotting the plasma in the APTT and TF systems. In the APTT system, factor VII was activated 25-fold in normal plasma, 21- to 22-fold in factor VIII deficient plasma, and also in factor X-deficient plasma, but only two- to five-fold in factor XII-deficient plasma or factor IX-deficient plasma (Table 1). Adding factor X antibodies to the factor X-deficient plasma (to remove traces of factor X activity from the plasma) did not decrease activation of factor VII in this plasma. In all experiments, the extent of cleavage measured from radioactivity gel profiles paralleled the degree of activation determined by the VIIc/VIIam ratio.

In further experiments (Table 2), factor VII was found to activate fully in factor XII-deficient plasma when exogenous factor Xla, in a final concentration of 4 µg/mL, was added to the clotting mixture. Since factor IXa, factor Xa, or both activities could account for the activation of factor VII in this reaction mixture, factor XII-deficient plasma was incubated for 20 minutes with factor X antibodies. This inhibited measurable factor X activity in the plasma, which was then clotted in the APTT system with added factor Xla. Under these conditions, in which only very limited amounts of factors XIIa, Xa, or thrombin could form, factor IXa functioned as the major activator of factor VII. Factor VII activity increased 19-fold, and molecular cleavage of 125I-factor VII was 57%.

When plasma was clotted in the TF system with undiluted TF reagent (Table 3), VIIc/VIIam ratios of 15 to 20 were obtained with normal plasma, factor XII-deficient plasma, and factor VIII-deficient plasma. A VIIc/VIIam ratio of ten was obtained with factor IX-deficient plasma and of seven with factor X-deficient plasma. As in the APTT system, the degree of cleavage of radiolabeled factor VII paralleled the increase in VIIc/VIIam ratio.

The effect upon factor VII activation of clotting normal plasma in a plastic tube with decreasing concentrations of TF was also examined, and the data are

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**Table 1.** Activation of Factor VII in Different Clotting Factor-Deficient Plasmas Clotted in the APTT System

<table>
<thead>
<tr>
<th>Type of Plasma Clotted</th>
<th>VIIc/VIIam Ratio</th>
<th>Activation From Gel Profiles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal plasma</td>
<td>25.0</td>
<td>92</td>
</tr>
<tr>
<td>Factor XII-dp†</td>
<td>2.0</td>
<td>5</td>
</tr>
<tr>
<td>Factor IX-dp</td>
<td>5.0</td>
<td>13</td>
</tr>
<tr>
<td>Factor VIII-dp</td>
<td>21.0</td>
<td>67</td>
</tr>
<tr>
<td>Factor X-dp</td>
<td>21.0</td>
<td>67</td>
</tr>
<tr>
<td>Factor X-dp + anti-factor-X</td>
<td>19.0</td>
<td>67</td>
</tr>
</tbody>
</table>

*Mean values are shown for three to six experiments.
†dp, deficient plasma.

**Table 2.** Generation of Factor VIIa on Clotting Factor XII-Deficient Plasma With Factor Xla in the APTT System in the Presence or Absence of Antibodies to Factor X

<table>
<thead>
<tr>
<th>Clotting Mixture</th>
<th>VIIc/VIIam Ratio</th>
<th>Activation From Gel Profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal plasma</td>
<td>25.0</td>
<td>92</td>
</tr>
<tr>
<td>Factor XII-dp†</td>
<td>2.0</td>
<td>5</td>
</tr>
<tr>
<td>Factor XII-dp + factor Xla†</td>
<td>21.0</td>
<td>67</td>
</tr>
<tr>
<td>Factor XII-dp + anti-factor-X</td>
<td>19.0</td>
<td>67</td>
</tr>
</tbody>
</table>

*Mean values are shown for four to six experiments.
†dp, deficient plasma.

**Table 3.** Activation of Factor VII in Different Clotting Factor-Deficient Plasmas Clotted With Undiluted TF

<table>
<thead>
<tr>
<th>Type of Plasma Clotted</th>
<th>VIIc/VIIam Ratio</th>
<th>Activation From Gel Profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal plasma</td>
<td>18.0</td>
<td>57</td>
</tr>
<tr>
<td>Factor XII-dp†</td>
<td>15.0</td>
<td>50</td>
</tr>
<tr>
<td>Factor IX-dp</td>
<td>10.0</td>
<td>30</td>
</tr>
<tr>
<td>Factor VIII-dp</td>
<td>16.0</td>
<td>58</td>
</tr>
<tr>
<td>Factor X-dp</td>
<td>7.0</td>
<td>21</td>
</tr>
</tbody>
</table>

*Mean value of three experiments.
†dp, deficient plasma.
Table 4. Limited Factor VII Activation on Clotting Normal Plasma
With Dilutions of TF

<table>
<thead>
<tr>
<th>Dilution of Tissue Factor</th>
<th>Prothrombin Time* (s)</th>
<th>VIIc/VIIam Profiles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>20</td>
<td>15.0 50</td>
</tr>
<tr>
<td>1/10</td>
<td>40</td>
<td>3.5  7</td>
</tr>
<tr>
<td>1/20</td>
<td>49</td>
<td>1.4  NM†</td>
</tr>
<tr>
<td>1/40</td>
<td>59</td>
<td>1.2  NM</td>
</tr>
<tr>
<td>1/80</td>
<td>71</td>
<td>0.8  NM</td>
</tr>
</tbody>
</table>

*Performed in a plastic tube by clotting 50 µL of normal plasma with 50 µL of TF at the initial dilution listed and 50 µL of 25 mmol/L CaCl₂.
†NM, not measurable.

summarized in Table 4. Diluting the tissue factor preparation markedly diminished factor VII activation. With a concentration of TF that clotted normal plasma in about 40 seconds, the VIIc/VIIam ratio increased only about two- to threefold, and cleavage of [¹²⁵I]-factor VII was below 10%.

Time Course of Generation of Factor IXa in Factor X-Deficient Plasma Clotted in the APTT and TF Systems

Because factor VII in factor X-deficient plasma was activated to a greater degree in the APTT system (21-fold) than in the TF system (sevenfold), we measured the time course of generation of factor IXa in factor X-deficient plasma clotted in both systems (Fig 5). In the APTT system, factor IXa was generated in factor X-deficient plasma as rapidly as it was generated in normal plasma. In contrast, in the TF system, generation of factor IXa in factor X-deficient plasma lagged behind generation of factor IXa in normal plasma. However, by 20 minutes after clotting, the extent of factor IXa generation in factor X-deficient plasma was the same for both clotting systems.

Residual Factor VII Activation in Serum

As already described, adding a final concentration of 5 µg/mL of purified factor Xa with calcium and phospholipid to serum prepared by clotting normal blood in a glass tube fully activated factor VII. In further experiments, we varied the final concentration of added factor Xa from 0.02 to 10.00 µg/mL and measured VIIc/VIIam ratios after one hour incubation at 37 °C. At final concentrations between 0.02 and 0.20 µg/mL, added factor Xa did not increase the VIIc/VIIam ratio. Only with higher concentrations of factor Xa could substantial activation of factor VII be demonstrated: a VIIc/VIIam ratio of 15 with 1 µg/mL Xa; a VIIc/VIIam ratio of 20 to 25 with 2 µg/mL Xa.

The time courses of activation of factor VII in serum after adding factor Xa in a final concentration of 1.0 or 2.0 µg/mL are shown in Fig 6. The time courses of inactivation of the added factor Xa are also plotted in this figure. Both concentrations of factor Xa exerted their full effect upon the VIIc/VIIam ratio within five minutes. By this time, more than 80% of the factor Xa clotting activity added to the serum had been neutralized.

Since a high concentration of factor IXa is required to activate factor VII in purified systems, we added factor IXa to serum only at final factor IXa concentrations of 7.5 and 15.0 µg/mL. The time courses of the increase in the VIIc/VIIam ratio and of loss of factor IXa clotting activity obtained in these experiments are shown in Fig 7. Again, factor VII was activated primarily within the first five minutes, and the same as for factor Xa clotting activity, factor IXa clotting activity was also rapidly neutralized in the serum.

The increase in VIIc/VIIam ratio observed with the higher concentrations of factor Xa or factor IXa used in these experiments did not stem from error due to carry-over of factor Xa or factor IXa into our factor VII assay systems. When similar concentrations of factor Xa or factor IXa were added to buffer, and the mixtures—at the same dilutions as used for the serum...
samples were used as the test samples in our factor VII assays, the values obtained were almost the same as blank times. Moreover, as can be noted in Figs 6 and 7, high VIIc/VIIa ratios persisted in subsamples taken long after essentially all factor Xa or factor IXa activity added to serum had been inhibited.

**DISCUSSION**

In earlier work from this laboratory, both factor Xa and factor IXa in the presence of calcium and phospholipid, were shown to activate human factor VII about 25-fold in purified systems. Now we have shown that proteolysis of 125I-factor VII by either factor Xa or factor IXa yields cleavage products with identical mobilities on reduced SDS-PAGE. From this we conclude that activation of factor VII by either enzyme stems from cleavage of the same bond.

Radcliffe and Nemerson reported that prolonged incubation of bovine factor VII with factor Xa resulted in a loss of factor VII protease activity associated with cleavage of the heavy chain of factor VIIa and the appearance of two lower molecular weight chains. We have now found that continued incubation of human factor VII in purified systems with either factor Xa or factor IXa also leads to a loss of factor VIIa activity associated with proteolysis of the heavy chain of factor VIIa. On radioactivity gel profiles, a broad, low molecular weight, third peak appears; on stained gels this can be seen as a triplet.

Although readily demonstrable on gels from purified systems, this third peak was either not seen or only very minimally demonstrable on gels from plasma systems. This probably reflects the rapid loss of factor Xa or factor IXa enzymatic activity in serum (Figs 6 and 7). We therefore believe it unlikely that proteolysis of the heavy chain of factor VIIa by either factor Xa or factor IXa plays a physiologically significant role in the regulation of plasma factor VIIa activity in vivo.

Whereas factor VII activity rapidly increases 25-fold when factor VII is incubated with factor Xa or factor IXa in purified systems, factor VII activity increases only fivefold when blood is clotted and incubated in a glass tube. We could not resolve previously whether this difference reflects only partial activation of factor VII during the clotting of blood in a glass tube or, alternatively, a more complete activation followed by partial inactivation of factor VIIa in serum. We have shown herein that only 20% of 125I-factor VII added to blood or plasma is cleaved during clotting in a glass tube. Thus, incomplete activation accounts for the limited increase in factor VII activity observed when whole blood or plasma is clotted and incubated in a glass tube.

The limited activation of factor VII reflects both incomplete activation of factors IX and X during clotting under this circumstance plus rapid inactivation of factors IXa and Xa in serum. In experiments in which increasing amounts of exogenous factor Xa with calcium and phospholipid were added to serum, a final concentration of 2 \( \mu \text{g/mL} \) of factor Xa was needed to obtain the same rapid, maximal activation of factor VII found with trace amounts of factor Xa in both bovine and human purified systems. These results explain the earlier negative data of Masys et al., who were unable further to activate factor VII in serum on addition of calcium, phospholipid, and exogenous factor Xa in a final concentration of 0.3 \( \mu \text{g/mL} \) (5 nmol/L). Whether factors other than the rapid inactivation of factor Xa in serum contribute to the need for relatively high concentrations of factor Xa to activate factor VII in serum is not yet known.

In contrast to its fivefold activation when plasma was clotted in a glass tube, factor VII was activated 25-fold when normal plasma was clotted by extensive contact activation in the APTT system. Moreover, in this system, factor VII was activated about 20-fold both in factor VIII deficient plasma and in factor X-deficient plasma, whereas it was activated only two- to fivefold in factor XII-deficient plasma and in factor IX-deficient plasma. Adding factor XIa to factor XII-deficient plasma corrected the incomplete activation of factor VII in this plasma, which rules out an essential role for factor XIa as a direct activator of factor VII in the APTT system. Moreover, when factor XII-deficient plasma was incubated with antibodies to factor X to deplete the plasma of factor X activity, clotting induced by adding factor XIa to the APTT reaction mixture still resulted in an 18- to 20-fold activation of factor VII. Since plasma depleted of factor X can generate only traces of thrombin and since thrombin was found to be only a weak activator of factor VII, only factor IXa can account for the
activation of factor VII in this reaction mixture. These results confirm and strengthen earlier data from this laboratory from which Seligsohn et al. concluded that factor IXa can function as a significant activator of factor VII when plasma is clotted in vitro.

However, an inconsistency in our results deserves comment. In experiments in which we added exogenous factor IXa to serum prepared by clotting plasma in a glass tube, a final concentration of 7.5 μg/mL of factor IXa increased the VIIc/VIIam ratio to 14. Yet, a VIIc/VIIam ratio of 19 and 57% cleavage of added [125I]-factor VII was observed when factor XII-deficient plasma containing factor X antibodies and factor Xa was clotted in the APTT system. Even if all of the factor IX in the plasma were converted to IXa in this experiment, only about 4 μg/mL of factor IXa would be generated. Conceivably, this apparent discrepancy reflects a continuing availability of factor IXa when it is generated over ten minutes in the APTT system (Fig 5) in contrast to its 90% inactivation within five minutes of being added as a bolus to serum (Fig 7).

Factor VII was activated 15- to 20-fold but not maximally (Fig 4) when normal blood was clotted in plastic tubes with an undiluted TF preparation system. Moreover, in contrast to the 20-fold increase in VIIc/VIIam ratio observed when factor X-deficient plasma was clotted in the APTT system, the VIIc/VIIam ratio increased only seven-fold when factor X-deficient plasma was clotted in the TF system. This could mean that factor IXa cannot fully substitute for factor Xa in activating factor VII in the TF system; alternatively, it could reflect delayed activation of factor IXa in factor X-deficient plasma in the TF system (Fig 5). (The latter could, in turn, reflect possible inhibition by native factor VII of TF/factor VIIa activation of factor IX in the presence of less than saturating tissue factor.) Yet another difference between factor VII activation in the APTT and TF clotting systems—the more complete activation in the TF system of factor VII in factor XII-deficient plasma and in factor IX-deficient plasma—is readily explained by the known normal generation of factor Xa in these plasmas in clotting mixtures containing a high concentration of tissue factor.

When normal plasma was clotted in a plastic tube with a low concentration of tissue factor, only minimal amounts of factor VII were activated (Table 4). Thus, in our experiments, either a high concentration of tissue factor or maximal contact activation in the APTT system was required for substantial activation of factor VII during clotting of plasma in vitro. The rapid, complete activation of factor VII in purified systems induced by a very low concentration of factor Xa, in the presence of calcium and phospholipid, should not mislead one into thinking that the same phenomenon takes place in plasma systems.

Our results should be interpreted with the caveat that the addition of radiolabeled factor VII increased the concentration of factor VII in most reaction mixtures to 6.5 μg/mL, more than tenfold its physiologic concentration in plasma of about 0.5 μg/mL. This large increase in substrate concentration could have affected the kinetics of activation of factor VII. For example, the addition of radiolabeled factor VII could have affected the initial rates of activation of factor VII in the APTT and TF systems shown in Fig 4. However, we do not believe that increased substrate concentration affected the extent of factor VII activation that we observed. We found the same five-fold activation of factor VII—measured as an increase in VIIc/VIIam ratio—when whole blood was clotted and incubated in a glass tube whether or not radiolabeled factor VII was added to the blood. Moreover, in a supplemental experiment (data not shown), normal or hereditary clotting factor deficient plasmas were clotted in either the APTT or TF system without added radiolabeled factor VII, and factor VII activation was measured as an increase in VII clotting activity. No difference in the pattern or degree of factor VII activation was observed from that shown in the data of Tables 1 and 3, which were obtained from reaction mixtures containing radiolabeled factor VII.

If, as we believe, blood coagulation after tissue injury is initiated by contact of blood with a limited number of tissue factor sites expressed on cells of the vascular wall, then only small amounts of factor VII may be activated during normal hemostasis. Nevertheless, even relatively low concentrations of factor VIIa could exert a major effect on the initiation of blood coagulation, and we are currently studying the effect of small amounts of factor VIIa on the kinetics of activation of the tissue factor pathway of blood coagulation.

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LV Rao, SP Bajaj and SI Rapaport