Purification of a Bovine Plasma Protein (Factor VII) Which Is Required for the Activity of Lung Microsomes in Blood Coagulation*

(Received for publication, September 29, 1965)

WILLIAM J. WILLIAMSF AND DONALD G. NORRISS
From the Hematology Section of the Department of Medicine, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

SUMMARY

1. Factor VII, essentially free of Factor X but containing traces of prothrombin, and Factor X, very low in Factor VII, have been prepared by a combination of adsorption to and elution from barium sulfate and chromatography on diethylaminoethyl cellulose.

2. The activation of Factor X by lung microsomes is due to contamination of Factor X with Factor VII rather than to a direct attack on the Factor X molecule by lung microsomes.

3. Factor VII, lung microsomes, and calcium react to form a complex which is able to act as enzyme in activating Factor X. Indirect evidence has been obtained indicating the enzymatic activity of the complex is due to activated Factor VII.

4. Coagulant activity in reaction mixtures consisting of the tissue preparations, a bovine serum fraction, and calcium. The microsomes, human placenta microsomes, and human brain particles appear to function as enzyme in catalyzing the development of coagulant activity in reaction mixtures containing Factor VII and X are involved in the initial reactions of blood coagulation induced by tissue preparations. In the present study these two proteins were separated and purified from bovine plasma and their reactions with lung microsomes were studied. It was found that lung microsomes, Factor VII, and calcium react to form a complex which is then able to function as enzyme in activating Factor X. No enzymatic activity of lung microsomes per se could be shown in these experiments. Thus it appears that the enzymatic activity previously described was due to the activity of the lung microsome-Factor VII complex.

5. Factor VII was originally described as a plasma or serum protein which accelerated the formation of thrombin in aged plasma or plasma from patients treated with coumarin-type anticoagulants (5-7). Subsequently a patient congenitally deficient in this factor was described (8). The term "Factor VII" is used in this report to designate the protein reacting with lung microsomes and calcium. The preparations of this factor are able to correct the coagulation defect of the plasma of a patient congenitally deficient in Factor VII and may be called Factor VII on this basis. This reasoning is circular, however, and it would be preferable to refer to the coagulant protein in terms of its biochemical function such as was previously done with the Russell's viper venom substrate or Factor X (9). Unfortunately, lung microsomes and Factor VII could not be finally defined as enzyme or substrate in the reactions leading to the formation of the complex and therefore the less precise and less satisfactory term "Factor VII" is used.

EXPERIMENTAL PROCEDURE

Purification of Plasma Proteins—Frozen, oxalated beef plasma obtained commercially was used as starting material. The plasma was thawed by heating with a coil of copper tubing through which tap water at 37° was run (10). The plasma was then treated with reagent grade barium sulfate and the proteins adsorbed to the barium sulfate were eluted with citrate buffer as previously described (9). The proteins eluted after dialysis against 0.02 M sodium phosphate buffer at pH 7.2 were further fractionated by column chromatography on DEAE-cellulose. Columns were prepared and the fractionation carried out as described by Williams and Esmouf (11). Details of individual experiments are presented in the text.

Preparation of Purified Lung Microsomes Purified lung microsomes were prepared exactly as previously reported (1).

Assay of Coagulant Activity—The reagents used for the coagulation experiments were prepared as reported previously (1). Optimal concentrations of phospholipid and calcium were employed.
in all of the experiments except those in which the effects of the concentration of these components were being studied. Coagulant activity was estimated from the ability of the material being tested to accelerate the coagulation of normal citrated platelet-poor human plasma or bovine fibrinogen. All of the reagents were warmed to 37° before use in the assay system.

Factor X activity was determined with the use of the simplified assay system described by Erenouf and Williams (9). Factor X activity was calculated as units by reference to a standard curve prepared as previously described. The units of Factor X activity are arbitrarily defined. Thus, 0.1 ml of the fully activated, purified Factor X solution used in preparing the standard curve was assigned an activity of 100 units.

Prothrombin activity was estimated by a system in which prothrombin was converted to thrombin and the thrombin activity determined by its ability to clot fibrinogen (bovine Fraction I, Armour). Factor X in 0.15 m NaCl-0.01 m Tris-chloride buffer (Tris-NaCl buffer) at pH 7.4 (0.1 ml of a solution of 150 μg per ml) was incubated for 5 min at 37° with 0.1 ml of Russell’s viper venom (Stypven), 5 μg per ml in Tris-NaCl buffer, and 0.1 ml of 0.025 M CaCl2 in 0.01 m Tris-chloride buffer at pH 7.4. This reaction mixture was then diluted 1:5 with Tris-NaCl buffer at pH 7.4, and kept in melting ice. A 0.1-ml aliquot of the diluted reaction mixture was then added to a mixture of 0.1 ml of Factor V in Tris-NaCl buffer, 0.1 ml of an emulsion of crude brain phospholipid, 0.5 mg per ml in 0.01 m Tris-chloride buffer at pH 7.4, 0.1 ml of 0.025 M CaCl2 and 0.1 ml of the fraction to be tested. This mixture was incubated at 37° for 3 min, and then 0.1 ml was added to 0.4 ml of bovine fibrinogen, 5 mg per ml in 0.20 m borate buffer at pH 7.75, and the coagulation time determined. Pool, normal beef plasma was used as a source of prothrombin for comparison with the fractions. The concentration of Factor X employed was not in excess with respect to normal plasma. However, high dilutions of plasma were used for comparison with Factor VII preparations, and the Factor X added with the plasma was therefore not significant in the assay.

Factor VII was estimated as coagulant activity developing on incubation of purified lung microsomes and calcium with plasma fractions containing no Factor X. The reaction mixture consisted of 0.1 ml of purified lung microsomes, 0.1 ml of plasma fraction, and 0.1 ml of 0.025 M CaCl2. After the appropriate incubation period, 0.1 ml of the reaction mixture was tested for coagulant activity in either of two ways. (a) In the “complete” assay system, the reaction mixture was used either undiluted or after dilution with Tris-NaCl buffer at pH 7.4. The reaction mixture was added to 0.1 ml of Factor X in Tris-NaCl buffer simultaneously with 0.1 ml of 0.025 M CaCl2. This mixture was incubated at 37° for 1 min. A 0.1-ml aliquot was then mixed rapidly with 0.2 ml of phospholipid emulsion and 0.1 ml of Tris-NaCl buffer. After 30 and 60 sec, 0.1 ml of this mixture was added simultaneously with 0.1 ml of 0.025 M CaCl2 to plasma and the coagulation time determined. (b) In the “simplified” assay system, 0.1 ml of the reaction mixture was added simultaneously with 0.1 ml of 0.025 M CaCl2 to a mixture of 0.1 ml of plasma and 0.1 ml of phospholipid emulsion, and the coagulation time determined. In order to evaluate the presence of thrombin in the system, fibrinogen was substituted for plasma in the final step of either the complete or simplified assay.

With the complete system, the units of activity were calculated by reference to a standard curve. A reaction mixture containing 0.3 ml of the same Factor X solution used in the experiment was incubated at 37° for 5 min with 0.3 ml of a solution of 5 μg per ml of Russell’s viper venom in Tris-NaCl buffer and 0.3 ml of 0.025 M CaCl2. During this time, Factor X was fully activated. This mixture was then chilled in melting ice and a series of dilutions were prepared with Tris-NaCl buffer containing 1.67 μg per ml of Russell’s viper venom, in order to keep the venom concentration constant. Aliquots of the diluted reaction mixtures, 0.10 ml each, were then diluted 1:10 or 1:100. This solution, 0.10 ml, was then mixed with 0.2 ml of phospholipid emulsion and 0.1 ml of Tris-NaCl buffer and immediately 0.1 ml of this mixture was added simultaneously with 0.1 ml of 0.025 M CaCl2 to normal human plasma and the clot time determined. A standard curve was constructed and units of activity were estimated by comparison of the coagulation times of the experiments with those of the standard mixture (9).

This assay is based on the ability of mixtures of Factor VII, lung microsomes, and calcium to activate Factor X. Units of Factor VII activity are presented as the units of Factor X activated in 1 min by the reaction mixture containing Factor VII, lung microsomes, and calcium. With the Factor X preparation used, the coagulation time obtained without Factor VII in the system was greater than 30 sec. In the simplified system, Factor VII activity was estimated from standard curves obtained with reaction mixtures consisting of 0.4 ml of lung microsomes, 0.4 ml of Factor VII, and 0.4 ml of CaCl2. The reaction mixtures were incubated at 37° for 30 sec and then chilled in ice. These were diluted with ice-cold Tris-NaCl buffer at pH 7.4 and 0.1 ml of the diluted mixture was added simultaneously with 0.1 ml of 0.025 M CaCl2 to a mixture of 0.1 ml of normal human plasma and 0.1 ml of phospholipid emulsion. The undiluted reaction mixture was arbitrarily assigned an activity of 100 units, and the activity developing in the experimental vessels is expressed in terms of these units.

Estimation of Protein Concentration—As a routine, protein concentration was estimated from the absorption of solutions at 280 mg in a 1-cm cell. The factor necessary for conversion of A280 to milligrams of protein for Factor VII was determined as previously described (11) except protein concentration was measured in the analytical ultracentrifuge (12). The conversion factor for Factor X was determined previously (9).

RESULTS

Fractionation of Bovine Plasma Proteins—Attempts to isolate a component of beef plasma which would develop coagulant activity on incubation with lung microsomes were initiated with a modification of the chromatographic procedure previously employed for Factor X (9). Those bovine plasma proteins which were adsorbed to barium sulfate and eluted with 0.2 M sodium citrate buffer at pH 6.8 were used as starting material for the chromatogram illustrated in Fig. 1. Five peaks were collected as indicated in the figure. These peaks were assayed for Factor X activity, and this was found to be present only in Peak 4, that eluted with 0.4 M NaCl as previously reported (9). The peaks were then studied singly and in combination for their ability to be activated by lung microsomes. For this purpose the assay system was similar to that employed previously for assay of lung microsome activity (1). Lung microsomes were incubated with the fraction or fractions to be tested and calcium at 37° for 1 min. A 0.1-ml aliquot of this mixture was then mixed with 0.1 ml of phospholipid emulsion and 0.1 ml of Tris-NaCl buffer at pH 7.4. This mixture, 0.10 ml, was added simul-
Purification of Factor X Although the direct activation of Factor X by tissue factor has been previously reported (4), it appeared possible that the observed activation was due to contamination of Factor X with material from Peak 3 since there was considerable tailing of Peak 3 and possibly some material remained on the column to be eluted by the higher NaCl concentration used to elute Peak 4 (Fig. 1). In order to evaluate this possibility, the Factor X peak was rechromatographed on DEAE-cellulose as illustrated in Fig. 2A. As expected, the major peak was eluted with 0.4 M NaCl and contained the Factor X activity as is shown in Table I. In addition, there was a small peak eluted with 0.25 M NaCl which gave a small amount of coagulant activity by itself and which markedly accelerated the development of coagulant activity from reaction mixtures containing lung microsomes, Factor X, and CaCl₂. The specific activity of Factor X when tested with Russell's viper venom was increased by about 33% on rechromatography, while the activity which developed with lung microsomes and calcium was reduced about 3-fold. Factor X was rechromatographed a second time as is illustrated in Fig. 2B. Here a smaller peak was eluted with 0.25 M NaCl. This peak had only slight acceleratory activity in the reaction mixtures containing lung microsomes, Factor X, and CaCl₂. Factor X so prepared was activated by lung microsomes at a rate only one-eighteenth that of the starting material for this column, yet the specific activity as determined by the Russell's viper venom assay was increased by 10%. The overall reduction in activation of Factor X by lung microsomes accomplished by rechromatographing twice was 54-fold. This result indicates that the activation of Factor X by lung microsomes is not due to a direct attack on Factor X by lung microsomes but is due to contamination with another plasma protein which is essential for the coagulant activity of lung microsomes.

Assay of Plasma Protein Reacting with Lung Microsomes—Data on the assay systems employed for study of the plasma protein reacting with lung microsomes are presented in Table II.
made to elute all of the protein removable with 0.25 M NaCl and the 0.4 M NaCl was started while significant protein was still being eluted. The protein eluted with 0.15 M NaCl was divided into two parts, 1A and 1B, and that eluted with 0.25 M NaCl was divided into four parts, 3A, 3B, 3C, and 3D. The distribution of Factor VII activity among the fractions is presented in Table III. The recovery of Factor VII activity was only about 50% of the total added to the column. The major activity was

Table I

Rechromatography of Factor X

The assay system for Factor X employing Russell’s viper venom was previously described (9) and measures total activated Factor X. The reaction mixture consisted of 0.1 ml of lung microsomes, 1.2 ng of phosphorus per ml in 0.01 M Tris-Cl buffer at pH 7.4, and 0.1 ml of Tris-C1 buffer at pH 7.4. It was incubated at 37°C for 1 min. Stage 1 consisted of 0.1 ml of Factor VII, 120 ng of protein per ml, in Tris-Cl buffer at pH 7.4, and 0.1 ml of 0.025 M CaCl2. It was incubated at 37°C for 30 sec. Stage 2 consisted of 0.1 ml of Stage 1, 0.1 ml of Factor X, 120 pg of protein per ml, in Tris-Cl buffer at pH 7.4, and 0.1 ml of 0.025 M CaCl2. It was incubated at 37°C for 1 min. Stage 3 consisted of 0.1 ml of Stage 2, 0.2 ml of an emulsion of 0.5 M of phospholipid per ml of 0.01 M Tris-chloride buffer at pH 7.4, and 0.1 ml of 0.025 M CaCl2. It was incubated at 37°C for 30 sec. Stage 2 consisted of 0.1 ml of Stage 1, 0.1 ml of phospholipid emulsion, 0.5 mg per ml in 0.01 M Tris-chloride buffer, 0.1 ml of 0.025 M CaCl2, and 0.1 ml of plasma. The plasma and phospholipid were mixed and warmed to 37°C; the Stage 1 and CaCl2 were added simultaneously and the clotting time determined.

Table II

Effect of omissions in assay system

In the complete system, Stage 1 consisted of 0.1 ml of lung microsomes, 1.2 ng of phosphorus per ml in 0.01 M Tris-chloride buffer, 0.1 ml of Factor VII, 67 ng per ml, in Tris-Cl buffer at pH 7.4, and 0.1 ml of 0.025 M CaCl2. It was incubated at 37°C for 30 sec. Stage 2 consisted of 0.1 ml of Stage 1, 0.1 ml of Factor X, 120 ng of protein per ml, in Tris-Cl buffer at pH 7.4, and 0.1 ml of 0.025 M CaCl2. It was incubated at 37°C for 1 min. Stage 3 consisted of 0.1 ml of Stage 2, 0.2 ml of an emulsion of 0.5 M of phospholipid per ml of 0.01 M Tris-chloride buffer at pH 7.4, and 0.1 ml of 0.025 M CaCl2. It was incubated at 37°C for 30 sec. Stage 2 consisted of 0.1 ml of Stage 1, 0.1 ml of phospholipid emulsion, 0.5 mg per ml in 0.01 M Tris-chloride buffer, 0.1 ml of 0.025 M CaCl2, and 0.1 ml of plasma. The plasma and phospholipid were mixed and warmed to 37°C; the Stage 1 and CaCl2 were added simultaneously and the clotting time determined.

Table III

Purification of Plasma Protein Reacting with Lung Microsomes

In the experiments with reaction mixtures consisting of lung microsomes, calcium, Factor X, and the plasma protein reacting with lung microsomes, coagulation times of 12 sec were regularly achieved by using plasma. The coagulation times with fibrinogen were greater than 10 min, indicating that there was very little prothrombin activation in the system. However, when the peak containing the protein reacting with lung microsomes was tested for prothrombin by the method outlined above, considerable prothrombin was found to be present. Accordingly this peak was rechromatographed to attempt to remove the contaminating prothrombin. Preliminary experiments suggested that prothrombin was eluted in the first part of the peak which contained the protein reacting with lung microsomes. The rechromatography procedure was based on this observation as illustrated in Fig. 3. The chromatogram was developed with 0.15 M NaCl until the major peak was eluted. Then 0.18 M NaCl was used and this solvent was continued for a prolonged period. A small amount of protein was eluted slowly with this solvent, and a large volume was required to reduce the absorbance of the effluent nearly to zero. Subsequent elution with 0.25 M NaCl and 0.4 M NaCl yielded two additional peaks. In order to shorten the time required for chromatography, no attempt was

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Coagulant activity</th>
<th>Coagulant activity of mixtures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
<td>Specific activity with Factor VII venom</td>
</tr>
<tr>
<td>Fig. 2A</td>
<td></td>
<td>mg</td>
</tr>
<tr>
<td>Peak 1</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>Peak 2</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>Peak 3</td>
<td>24</td>
<td>12</td>
</tr>
<tr>
<td>Peak 4a</td>
<td>110</td>
<td>32</td>
</tr>
<tr>
<td>Peak 4b</td>
<td>36</td>
<td>29</td>
</tr>
<tr>
<td>Peak 5</td>
<td>12</td>
<td>84</td>
</tr>
<tr>
<td>Fig. 2B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 3a</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Peak 3b</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Peak 4a</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>Peak 4b</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Peak 5</td>
<td>12</td>
<td>84</td>
</tr>
<tr>
<td>Fig. 2A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 3a</td>
<td>126</td>
<td>320</td>
</tr>
<tr>
<td>Peak 3b</td>
<td>126</td>
<td>320</td>
</tr>
<tr>
<td>Peak 4a</td>
<td>126</td>
<td>320</td>
</tr>
<tr>
<td>Peak 4b</td>
<td>126</td>
<td>320</td>
</tr>
</tbody>
</table>

Clot time (sec) | Units
--- | ---
Stage 1 | 42-42 | 1
Omit lung microsomes | 32-32 | 2
Omit Factor VII | 13-13 | 20
Stage 2 | 48-48 | 1
Omit Stage 1 | 25-26 | 4
Omit Factor X | 11.5-12 | 18
Stage 3 | 64-66 | 1
Stage 2 | 20-30 | 3
Omit phospholipid | 190-190 | 1
Omit Stage 3 | 50-50 | 1
Omit calcium | >3000 | 1
Fibrinogen substituted for plasma | 12-12 | 100
Simplified system | 50-50 | 1
Omit lung microsomes | 31-32 | 3
Omit Factor VII | 28-27.5 | 4
Stage 2 | 80-84 | 1
Omit phospholipid | 15.5-15 | 35
Omit calcium | 52-53 | 1
Fibrinogen substitute for plasma | >3000 | 1

Downloaded from http://www.jbc.org/ by guest on October 29, 2017
present in the material eluted with 0.25 M NaCl as expected. The specific activity of the most active portion of the Factor VII peak was increased about 2-fold by the rechromatography. The prothrombin activity appeared primarily in Peaks 1A and 1B. The prothrombin activity of the four fractions of Peak 3 and in Peak 4 was quite low, giving clotting times of fibrinogen of 10 min to greater than 25 min. The prothrombin activity present in Peak 3 is the equivalent of 1:1000 dilution of pooled beef plasma, while that in Peaks 3C and 3D is equal to 1:2000 dilution or more. As shown in Table II, there was essentially no thrombin formed in the reaction mixtures used for assay of Factor VII.

It should be emphasized that the removal of prothrombin from Factor VII was a difficult procedure, and the prolonged elution of the column with 0.18 M NaCl is essential. The prothrombin content of the starting material is variable, and prothrombin may not be removed completely from those preparations rich in prothrombin. It is desirable to attempt to purify those Factor VII preparations already low in prothrombin.

Fractionation of Factor VII on Sephadex—When the Factor VII preparations were examined in the analytical ultracentrifuge, two components were present, a smaller one with $s_{20}$ about 12, and a larger one with $s_{20}$ about 4.8. Attempts were made to separate these two components by gel filtration on Sephadex G-200 as is shown in Fig. 4. The two components could also be separated by preparative ultracentrifugation by using the procedure described previously (9). For these studies, 2 ml of Factor VII preparations were layered over 2.5 ml of a sucrose density gradient of concentration from 2 to 10% in a tube (1.2 X 5 cm) and centrifuged at 40,000 rpm for 8 hours in the SW 39L rotor of the Spinco model L ultracentrifuge. Fractions from the Sephadex column and the density gradient tube were assayed for Factor VII activity by the simplified system. Nearly all of the activity was present in the smaller component. The specific activity of the component with $s_{20}$ of 12 was only 3% of that of the component with $s_{20}$ of 4.8.

Relationship of Plasma Protein Reacting with Lung Microsomes to Known Coagulation Factors—The rechromatographed fraction reacting with lung microsomes was free of Factor X and contained very little prothrombin. It was found to correct the one-stage prothrombin time of plasma from a patient congenitally deficient in Factor VII, and therefore is presumed to have the activity of Factor VII.

Degree of Puriﬁcation of Factor VII—Estimation of the extent of the purification of Factor VII was complicated by the lack of an assay system suitable for use with plasma or the starting material for column chromatography because of the presence of large amounts of prothrombin in these materials. In addition, the presence of Factor X in serum and the reported increase of Factor VII activity in serum over that in plasma (13) makes serum unsatisfactory also. However, attempts were made to compare rechromatographed Factor VII and bovine serum in the complete assay system containing sufficient Factor X to permit maximal reaction rate and thus negate the effect of Factor X contained in the serum. In this system, the specific activity (units per mg of protein) of the Factor VII preparation was 100 times that of pooled bovine serum. With the use of the ability to correct

IRA 0.25 M NaCl from DEAE-cellulose. A solution containing 117 mg of protein in 6 ml of 0.02 M sodium phosphate buffer at pH 7.2 was chromatographed at 4° on a column (2.5 X 30 cm) of DEAE-cellulose as described in Fig. 2. Fractions were 10 ml. The yield of protein was 98%.

---

### Table III

Rechromatography of fraction eluted with 0.085 M NaCl

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Protein concentration used for assays (µg/ml)</th>
<th>Factor VII assay, coagulation time (sec)</th>
<th>Specific activity (units/mg protein)</th>
<th>Total activity (units)</th>
<th>Coagulation time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>3.4</td>
<td>100</td>
<td>25-25</td>
<td>30</td>
<td>100</td>
<td>55-50</td>
</tr>
<tr>
<td>1B</td>
<td>40.0</td>
<td>340</td>
<td>25-25</td>
<td>30</td>
<td>100</td>
<td>120-125</td>
</tr>
<tr>
<td>2</td>
<td>1.7</td>
<td>95</td>
<td>20-19.5</td>
<td>140</td>
<td>240</td>
<td>1,200-1,200</td>
</tr>
<tr>
<td>3A</td>
<td>4.3</td>
<td>720</td>
<td>13-13</td>
<td>15</td>
<td>410</td>
<td>600-600</td>
</tr>
<tr>
<td>3B</td>
<td>3.2</td>
<td>610</td>
<td>12-12</td>
<td>140</td>
<td>730</td>
<td>840-840</td>
</tr>
<tr>
<td>3C</td>
<td>8.6</td>
<td>200</td>
<td>12-12</td>
<td>450</td>
<td>3,900</td>
<td>1,400-1,400</td>
</tr>
<tr>
<td>3D</td>
<td>3.4</td>
<td>190</td>
<td>13-13</td>
<td>175</td>
<td>1,600</td>
<td>&gt;1,500</td>
</tr>
<tr>
<td>4</td>
<td>8.6</td>
<td>240</td>
<td>13-13</td>
<td>270</td>
<td>2,300</td>
<td>&gt;1,500</td>
</tr>
<tr>
<td>Total</td>
<td>75.2</td>
<td></td>
<td></td>
<td>610</td>
<td>9,880</td>
<td></td>
</tr>
</tbody>
</table>

---

**Fig. 2.** Rechromatography of those proteins eluted with 0.25 M NaCl from DEAE-cellulose. A solution containing 117 mg of protein in 6 ml of 0.02 M sodium phosphate buffer at pH 7.2 was chromatographed at 4° on a column (2.5 X 30 cm) of DEAE-cellulose as described in Fig. 2. Fractions were 10 ml. The yield of protein was 98%.

**Fig. 3.** Rechromatography of those proteins eluted with 0.25 M NaCl from DEAE-cellulose. A solution containing 117 mg of protein in 6 ml of 0.02 M sodium phosphate buffer at pH 7.2 was chromatographed at 4° on a column (2.5 X 30 cm) of DEAE-cellulose as described in Fig. 2. Fractions were 10 ml. The yield of protein was 98%.

**Fig. 4.** Fractionation on Sephadex G-200 of the proteins eluted from DEAE-cellulose with 0.25 M NaCl. A solution of 19 mg of protein in 3.8 ml of 0.1 M NaCl-0.01 M Tris-chloride at pH 7.4 was fractionated on a column (1.4 X 80 cm) of Sephadex G-200 equilibrated with 0.1 M NaCl in 0.01 M Tris-chloride buffer at pH 7.4. This buffer was used as solvent for the fractionation. Recovery of protein was 98%.
the one-stage prothrombin time of plasma from a patient congenitally deficient in Factor VII, the specific activity of another Factor VII preparation was 490 times that of serum.

**TABLE IV**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Coagulant activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung microsomes</td>
<td>Factor VII</td>
</tr>
<tr>
<td>µg phosphorus/ml</td>
<td>µg per cm/ml</td>
</tr>
<tr>
<td>Sediment</td>
<td>Supernatant</td>
</tr>
<tr>
<td></td>
<td>Reacted with</td>
</tr>
<tr>
<td></td>
<td>Lung microsomes</td>
</tr>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>Relative activity</td>
</tr>
<tr>
<td></td>
<td>in sediment*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>µg phosphorus/ml</th>
<th>µg per cm/ml</th>
<th>units</th>
<th>units</th>
<th>units</th>
<th>units</th>
<th>Expected</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>19</td>
<td>50</td>
<td>0</td>
<td>7</td>
<td>57</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>38</td>
<td>130</td>
<td>2</td>
<td>30</td>
<td>100</td>
<td>2</td>
<td>2.0</td>
</tr>
<tr>
<td>10</td>
<td>75</td>
<td>170</td>
<td>12</td>
<td>77</td>
<td>247</td>
<td>3</td>
<td>3.4</td>
</tr>
<tr>
<td>20</td>
<td>76</td>
<td>240</td>
<td>10</td>
<td>250</td>
<td>10</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>24</td>
<td>1</td>
<td>150</td>
<td>174</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

the one-stage prothrombin time of plasma from a patient congenitally deficient in Factor VII, the specific activity of another Factor VII preparation was 400 times that of serum.

**Reaction of Lung Microsomes with Factor VII**—The results obtained with the assay system employed for the purification procedures showed that lung microsomes reacted with Factor VII to yield a substance active in blood coagulation. The next experiments were designed to study the details of these reactions. Attempts were made to study the rate of development of coagulant activity in reaction mixtures containing lung microsomes, Factor VII, and calcium, with the complete system described above. It was found that even with dilute reagents the activity which developed was the same whether the reaction mixture was incubated for 30 sec or 5 min. With the use of the simplified system described above, it was possible to use incubation periods as short as 5 sec. Data obtained with this system are presented in Fig. 5. The coagulant activity developed rapidly and was complete in 10 sec. Further, the rate of development of coagulant activity and the yield of activity was dependent on the concentration of either reactant, whichever was present in limiting quantity. These data are not compatible with an enzyme-substrate reaction, but suggest that a complex might be formed between lung microsomes and Factor VII. This concept is supported by the following additional evidence.

Experiments are presented in Table IV which show that the coagulant activity which develops in the reaction mixture is sedimented with the lung microsomes; that the activity which can be recovered in the sediment depends on the concentration of both reactants; and that the Factor VII remaining in the supernatant fluid is less with higher concentrations of lung microsomes or lower concentrations of Factor VII in the reaction mixtures. In all of the reaction mixtures, essentially all of the coagulant activity was found in the sediment. However, when the supernatant fluid was incubated with lung microsomes and CaCl₂, further coagulant activity could be detected. Measurement of the activity in this sediment plus that which could be developed in the supernatants permitted estimates of the recovery of total activity. In the experiment shown in the top half of Table IV, the amount of Factor VII in the original reaction mixture was varied while the amount of lung microsomes was held constant. The activity in the sediment was nearly proportional to the amount of Factor VII added, while the residual Factor VII in the supernatant fluid was greater in the vessels containing larger quantities of Factor VII. Residual Factor VII was nearly 0 in the vessel containing the smallest amount of Factor VII, and increased in proportion to the amount of Factor VII in the reaction mixtures of the other two vessels.

In experiment shown in the lower half of Table IV, the quantity of Factor VII was held constant while that of lung microsomes was varied over a 10-fold range. The activity in the sediment was proportional to the quantity of lung microsomes added, and the residual Factor VII in the supernatant fluid was much greater in the vessel containing the smaller amount of lung microsomes. In this experiment the total activity recovered in the vessel containing the smaller quantity of lung microsomes was low, but the point that only a small amount of the Factor VII was bound to the lung microsomes is clearly illustrated.

If a complex is formed involving lung microsomes and Factor VII, then the nitrogen content of the sediment should be increased. In order to determine this, three reaction mixtures were incubated at 37° for 1 min. The first consisted of lung microsomes containing 58 µg of phosphorus, 4.4 mg of Factor VII, 140 µmole of CaCl₂, and 43 µmole of Tris-chloride buffer at pH 7.4 in a total volume of 13 ml. The second vessel was the same as the first except that CaCl₂ was omitted, and the third was the same as the first, except that Factor VII was omitted. At the end of the incubation period the reaction mixtures were chilled in ice and then centrifuged at 105,000 × g for 30 min.

**Fig. 5.** Rate of development of coagulant activity from reaction mixtures containing lung microsomes (LM), Factor VII (VII), and CaCl₂. The reaction mixture contained 0.1 ml of lung microsomes in 0.01 M Tris-chloride buffer at pH 7.4 at the final concentration noted on the figure, 0.1 ml of Factor VII in Tris-NaCl buffer at pH 7.4 at the final concentration noted on the figure, and 0.1 ml of 0.025 M CaCl₂. The reaction mixtures were incubated at 37° for the times noted, and then 0.1 ml was tested for coagulant activity in the simplified system. Zero time activity was estimated by using reaction mixtures in which 0.01 M Tris-chloride buffer at pH 7.4 at the final concentration noted on the figure, 0.1 ml of Factor VII in Tris-NaCl buffer at pH 7.4 was substituted for CaCl₂.
The supernatant fluid was decanted and the sediments were suspended in 2 ml of water. The coagulant activity was assayed in the complete system employing incubation of the supernatant fluid or sediment with Factor X. Lung microsomes alone did not activate Factor X significantly in this system. The total coagulant activity present in the sediment of Vessels 1, 2, and 3 was 1,000, 0, and 0 units, respectively. The supernatant fluids contained 20, 20, and 0 units, respectively. The nitrogen to phosphorus molar ratios found in the sediment from Vessels 1, 2, and 3 were 13.3, 11.7, and 11.1, respectively. The increased nitrogen to phosphorus molar ratio of the sediment from the complete system indicates binding of protein to the lung microsomes. There was also a slight increase in the nitrogen content of the lung microsomes in the vessel containing Factor VII but no CaCl₂, suggesting a small amount of protein may be bound nonspecifically.

Further evidence for binding of protein to lung microsomes was obtained from experiments in which the lung microsomes were allowed to react with Factor VII in CaCl₂ and then examined by density gradient centrifugation. Again, three reaction mixtures were prepared. Vessel 1 contained lung microsomes, 100 µg of phosphorus, 1.2 mg of Factor VII, 50 µmoles of CaCl₂, and 50 µmoles of Tris-chloride buffer at pH 7.4 in a total volume of 5 ml of 0.12 m NaCl. Vessel 2 contained the same reaction mixture except for the omission of CaCl₂ while Factor VII was omitted from Vessel 3. The vessels were incubated at 37°C for 1 min, chilled in ice, and centrifuged at 40,000 rpm for 30 min in the SW 39L rotor of the Spinco model L centrifuge.

Fig. 6. Density gradient centrifuge patterns of lung microsomes reacted with Factor VII and CaCl₂. These are negative images of centrifuge tubes illuminated from above. The density marker is at 1.15 g per ml. Tube 1 is from Vessel 1 and Tube 3 is from Vessel 3 of the experiment described in the text.

Fig. 7. The effect of pH on the rate of formation of coagulant activity. ---, the development of coagulant activity in reaction mixtures containing 0.1 ml of lung microsomes containing 3 µg of phosphorus in 0.01 M buffer, 0.1 ml of Factor VII containing 225 µg per ml and 0.025 M CaCl₂ and 0.1 ml of 0.025 M CaCl₂ in 0.025 M buffer. The pH values are noted on the figure. After 10-min incubation, 0.1 ml of the reaction mixture was added simultaneously with 0.1 ml of 0.025 M CaCl₂ in 0.1 M Tris-chloride buffer at pH 7.4 to a mixture of 0.1 ml of plasma and 0.1 ml of an emulsion of 0.5 mg of phospholipid per ml of 0.1 M Tris-chloride buffer at pH 7.4 and the coagulation time was determined. ---, the development of coagulant activity in reaction mixtures consisting of lung microsomes-Factor VII complex, Factor X, and CaCl₂. The lung microsome-Factor VII complex was prepared by incubating 4.5 ml of lung microsomes containing 3 µg of phosphorus per ml, 4.5 ml of Factor VII containing 225 µg of protein per ml, and 4.5 ml of 0.025 M CaCl₂. After 1-min incubation at 37°C, the reaction mixture was chilled in ice and centrifuged at 105,000 × g for 30 min. The supernatant fluid was decanted and the sediment suspended in 0.5 ml of Tris-NaCl buffer at pH 7.4. As expected, the supernatant fluids were essentially devoid of coagulant activity, and only the sediment from Vessel 1 had significant activity. The sediments were then subjected to density gradient centrifugation exactly as previously described (1). Fig. 6 illustrates the pattern observed in Vessels 1 and 3. It can be seen that, in the tube containing the sediment from Vessel 1, there is a band of density of about 1.13 g per ml which is not apparent in the sediment from Vessel 3. The tube from Vessel 2 is not illustrated, but showed a faint band at a density of 1.13 g per ml, much less prominent than that in Vessel 1. This experiment shows increased density of lung microsomes after reaction with Factor VII and CaCl₂ and supports the concept of formation of a complex involving lung microsomes, Factor VII, and CaCl₂.

The effect of pH on the rate of formation of the complex is shown in Fig. 7. Activity increased sharply with increase in pH from 6.0 to 9.0, and no optimum was found. The effect of calcium concentration on the rate of formation of the complex was also studied. Optimal rates were found with concentrations from 0.007 to 0.017 M, the highest concentration tested.

The formation of a complex involving lung microsomes, Factor VII, and CaCl₂ was studied.
Fig. 8. The effect of concentration of lung microsome-Factor VII complex and Factor X on the rate of formation of coagulant activity. The lung microsome-Factor VII complex was prepared as described in Fig. 7 except that the lung microsome preparation contained 1 μg of phosphorus per ml and the complex was suspended in 6 ml of Tris-NaCl buffer at pH 7.4. For Curve A, the reaction mixture consisted of 0.1 ml of the lung microsome-Factor VII complex at the concentration given on the figure, 0.1 ml of Factor X, 216 μg of protein per ml in Tris-NaCl buffer at pH 7.4, and 0.1 ml of 0.025 M CaCl₂. After 1-min incubation, 0.1 ml of the reaction mixture was added to a mixture of 0.2 ml of phospholipid emulsion, 0.5 mg per ml in 0.01 M Tris-chloride buffer at pH 7.4, and 0.1 ml of Tris-NaCl buffer at pH 7.4. This mixture, 0.1 ml, was then added simultaneously with 0.1 ml of 0.025 M CaCl₂ to 0.1 ml of plasma and the coagulation time was determined. For Curve B, the conditions were identical to A except that the concentration of the lung microsome-Factor VII complex was 0.22 μg of phosphorus per ml and the Factor X concentration was varied as shown on the figure.

VII, and calcium suggested the possibility that the reaction might occur as a result of binding of Factor VII to the surface of the lung microsomes. Latex particles of proper size have been shown to function as phospholipid in some coagulation systems (14). Since lung microsomes are also able to function as phospholipid in coagulation systems (1), latex particles were examined to determine whether or not coagulant activity would develop in reaction mixtures containing latex particles, Factor VII, and calcium. Latex particles of the following sizes were tested: 0.088 μm, 0.126 μm, 0.264 μm, 0.36 μm, 0.557 μm, 0.796 μm, and 1.305 μm. These were substituted for lung microsomes in the simplified assay system by adding suspensions of the following concentrations: 0.01, 0.1, 1.0, and 2.0 g per 100 ml. Those reaction mixtures containing lung microsomes gave coagulation times of 12 sec, while all of those containing latex particles gave coagulation times ranging from 46 to 80 sec. Substituting 0.01 M Tris-chloride buffer for lung microsomes resulted in coagulation times of 46 sec.

Lung microsomes and the lung microsome-Factor VII complex were examined for their ability to hydrolyze the synthetic amino acid esters tosyl-L-arginine methyl ester and L-lysine methyl ester by the titrametric method previously used (11). No esterase activity was found for either preparation.

Activation of Factor X by Complex Involving Microsomes and Factor VII—The rate of development of coagulant activity was studied in reaction mixtures containing Factor X, CaCl₂, and the lung microsome-Factor VII complex. It was found that the amount of Factor X activated increased with time in a manner consistent with an enzymatic reaction. In order to evaluate the roles of Factor X and the lung microsome-Factor VII complex as enzyme or substrate in the reaction, the effect of concentration of these two reactants on the initial rate of formation of coagulant activity was studied. In Fig. B the effect of concentration of lung microsome-Factor VII complex on the initial rate of formation of coagulant activity is shown. The initial rate varied directly with the concentration of lung microsome-Factor VII complex, indicating the complex was functioning as enzyme. In Fig. B the effect of concentration of Factor X on the initial rate of formation of coagulant activity is presented. This curve has the configuration of a rectangular hyperbola, indicating that Factor X was functioning as substrate.

The effect of calcium concentration on the initial rate of formation of coagulant activity in reaction mixtures containing lung microsome-Factor VII complex, Factor X, and CaCl₂ was studied. Maximal rates were obtained with concentrations from 0.007 M to 0.017 M, the highest concentration tested. The effect of pH is shown in Fig. B. Maximal rates were obtained from pH 7.2 to 9.0.

Nature of Coagulant Activity of Lung Microsome-Factor VII Complex—Attempts have been made to dissociate the lung microsome-Factor VII complex and show coagulant activity in a soluble component. The complex has been treated with EDTA, sodium citrate, sodium oxalate, and magnesium chloride. These studies have resulted either in loss of activity or no dissociation of the complex, so that no definite evidence bearing on the component of the complex responsible for its activity has been obtained.

Indirect evidence suggests that the activity is due to activated Factor VII. This came from the chance observation that one of our preparations of Factor VII developed activity spontaneously on storage at -15°C. This preparation had been chromatographed once on DEAE-cellulose and further purified by gel filtration. The Factor VII peak obtained by column chromatography was kindly supplied by L. J. Lippie of the Dow Chemical Company.
tography on DEAE-cellulose was concentrated by drying from the frozen state and then dissolved in 0.1 M NaCl-0.01 M Tris-chloride buffer at pH 7.2. It was then subjected to gel filtration on Sephadex G-200 by using a column (1.4 × 80 cm). The fractions containing Factor VII were pooled and stored at −15°C. After 1 month, this preparation was found to be able to activate Factor X without prior incubation with lung microsomes and calcium, if calcium was present in the reaction mixture. The preparation was fully active in that prior incubation with lung microsomes and calcium did not increase the activity. Omission of CaCl₂ from the reaction mixtures or of phospholipid from the assay system caused a marked decrease in coagulant activity. Reaction mixtures which would clot plasma in 11 sec failed to clot fibrinogen in 20 min.

The effect of concentration of the spontaneously activated Factor VII on the initial rate of formation of coagulant activity in reaction mixtures containing activated Factor VII, Factor X, and CaCl₂ is shown in Fig. 9A. The initial rate varied directly with activated Factor VII concentration, indicating this component was functioning as enzyme. In Fig. 9B the Factor X concentration was varied, and here the curve has the configuration of a rectangular hyperbola, indicating Factor X was functioning as substrate.

**DISCUSSION**

The data presented show that in order to develop coagulant activity, lung microsomes require at least two plasma proteins which are both adsorbed to barium sulfate but which may be separated by column chromatography on DEAE-cellulose. One of these appears to be Factor X (9), while the other is referred to as Factor VII because it corrects the coagulation defect of plasma from a patient congenitally deficient in Factor VII.

Factor X isolated from plasma by column chromatography is readily activated by lung microsomes, but after rechromatography twice, Factor X is only slowly activated by lung microsomes. It was further shown that on rechromatography a protein is removed from Factor X which is able to accelerate the activation of Factor X by lung microsomes. This property is characteristic of Factor VII, as determined in the present study. It is concluded that lung microsomes activate Factor X only if Factor VII is present, and therefore the activation of Factor X preparations by lung microsomes is due to contaminating Factor VII rather than to any direct attack on the Factor X molecule.

Straub and Duckert (4) reported activation of Factor X by brain extracts, but their Factor X contained some Factor VII as well, and this would account for the observed reaction. Nemerson and Spaet (15) considered Factor VII to be an accelerator of Factor X activation but the present results support direct participation of Factor VII as an obligatory reagent rather than functioning only as an accelerator. Lechner and Deutsch (16) have recently suggested that probably Factor VII is an obligatory reagent in Factor X activation by tissue thromboplastin.

The Factor VII preparation contained essentially no Factor X and only traces of prothrombin. No thrombin could be detected in the assay systems used, and the significance, if any, of traces of prothrombin in the Factor VII cannot be determined. Attempts to estimate the degree of purification of Factor VII required the use of serum instead of plasma. Purification of Factor VII from 100- to 500-fold as compared with serum was obtained. It is known that the apparent Factor VII activity of serum is about 3 times that of plasma (13). Since serum was used as the reference standard, the degree of purification would be underestimated. Factor VII has been prepared from serum by Duckert, Yin, and Straub (17) and Prydz (18). These investigators employed column chromatography. Duckert, Yin, and Straub (17) reported purification of Factor VII of 2200-fold from serum, while Prydz (18) attained from 400- to 1300-fold purification.

The evidence presented indicates a reaction occurs among Factor VII, tissue factor, and calcium, and it appears that this reaction leads to the formation of a complex between Factor VII and the lung microsomes. The complex functions as enzyme in activating Factor X, but no esterase activity on the synthetic amino acid esters, tosyl-l-arginine methyl ester, or t-lysine methyl ester could be shown. No direct evidence was obtained to indicate the component of the complex which is responsible for the enzymatic activity. However, the finding that one of our Factor VII preparations developed activity spontaneously and then was able to activate Factor X without prior incubation with lung microsomes and calcium suggests that activated Factor VII was the enzymatically active component. It seems unlikely from the method of preparation of the Factor VII that there were other coagulant proteins present which could activate Factor X, such as activated Factor VIII. The reason for the "spontaneous" activation of Factor VII is not clear, but the phenomenon is similar to that which has been observed with Factor X (9, 19).

No evidence was obtained which bears on the nature of the reaction among lung microsomes, Factor VII, and calcium. One possibility is that Factor VII is attacked enzymatically by the protein portion of the microsomes and the product is then bound to the phospholipid portion of the microsomes. Calcium may be involved either in the enzymatic activation of Factor VII or in the binding reaction. Alternately, it could be that the Factor VII is activated as a result of adsorption to the surface of the microsomes. No esterase activity could be shown for the lung microsomes, and latex particles of varying size did not activate Factor VII so neither of these possibilities can be supported at the present time. In favor of the concept of enzymatic activation of Factor VII by brain preparations are the experiments of Nemerson and Spaet (15) who extracted a soluble protein from brain which was able to function enzymatically in the activation of mixtures of Factor VII and X. Nemerson and Spaet (15) believed their preparations represented the protein component of brain thromboplastin.

In previous work, evidence was obtained to indicate that lung microsomes function as enzyme in blood coagulation. The present experiments indicate that the apparent enzymatic activity of lung microsomes is due to the lung microsome-Factor VII complex rather than to the microsomes themselves. The precise role of lung microsomes remains to be elucidated.

Recently Biggs et al. (20) have suggested that the tissue factor system may have a physiological role in blood coagulation in vivo by providing a means for the rapid activation of small amounts of thrombin. The present results are consistent with this possibility since the reaction between tissue factor (lung microsomes) and Factor VII is extremely fast, and could initiate rapid thrombin formation.

**Acknowledgment**—We are indebted to Mrs. Karen M. Dechter for excellent technical assistance.
REFERENCES

Purification of a Bovine Plasma Protein (Factor VII) Which Is Required for the Activity of Lung Microsomes in Blood Coagulation

William J. Williams and Donald G. Norris


Access the most updated version of this article at [http://www.jbc.org/content/241/8/1847](http://www.jbc.org/content/241/8/1847)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/241/8/1847.full.html#ref-list-1](http://www.jbc.org/content/241/8/1847.full.html#ref-list-1)