Activated Factor V (Va) was prepared by treating a high molecular weight form of Factor V with thrombin. The activated Factor V was isolated by ion exchange chromatography and was composed of two polypeptide chains ($M_w = 115,000$ and $73,000$). These chains were separated by ion exchange chromatography in the presence of EDTA. Biologically active Factor Va was restored from the inactive chains by incubation of the two chains in buffers containing MnCl$_2$. Restoration of biological activity was correlated with formation of a complex between the chains as monitored by either disc gel electrophoresis or gel filtration chromatography. The apparent molecular weight of the activated Factor V was 290,000.

Factor V was not dissociated in EDTA. However, this protein was split by thrombin to yield an activation intermediate composed of two chains ($M_w = 210,000$ and 115,000). Like activated Factor V, the two chains of the intermediate can be dissociated in EDTA and separated by gel filtration chromatography. The Factor V activity was restored by incubation of the two inactive chains in buffers containing MnCl$_2$. Like Factor Va, restoration of the biological activity corresponds to formation of a complex between the chains with a higher molecular weight than either of the isolated chains. Incubation of the activation intermediate with thrombin increased the specific activity 3- to 4-fold. This increase in specific activity resulted from cleavage of the heavy chain of the Factor V intermediate by thrombin.

Factor V is a high molecular weight glycoprotein which apparently functions nonenzymatically with the serine protease Factor Xa in the activation of prothrombin (1). Little is known about the mechanism by which Factor V functions; however, it does appear that the protein must be proteolytically activated by thrombin (1-5) or some other protease (1, 6) in order to become functional. One difficulty in studying Factor V arises with the apparent instability of the Factor Va form. However, the apparent instability could be caused by secondary proteolysis or failure to satisfy an ion requirement (7, 8). Specifically, Factor V activity is inhibited by chelating agents (9-14). Some investigators have been able to restore activity by addition of Ca$^{2+}$ (15) while others have failed (13, 14). Most investigators have concluded that divalent cations are required to maintain Factor V activity (7, 13). Greenquist and Colman (15) have proposed that Factor V is a metalloprotein with 1 g atom of Ca$^{2+}$ bound/300,000 g of Factor V.

A further complication in studying Factor V arises with the multiple structural forms in which Factor V appears to exist (1, 6, 16-19). Reported molecular weights for Factor V range from 1.2 million (20) to 38,000 (17). In addition, most preparations have contained multiple peptides when analyzed by SDS-gel electrophoresis (1). It appears likely that some of the molecular forms of Factor V are partial proteolysis products of the native protein (13, 20).

In this report, a method for isolating a high molecular weight form of Factor V is described. This form of Factor V can be cleaved by thrombin to form either an activation intermediate or a stable form of fully activated Factor V. Both the intermediate and the activated Factor V can be dissociated by chelating agents. A tentative scheme for the activation of Factor V is proposed based on the isolation of activation products from Factor V.

**MATERIALS AND METHODS**

*Reagents*-Thyroglobulin, P-galactosidase, phosphofructokinase, bovine plasma albumin, ovalbumin, catalase, aldolase, and QAE-Sephadex Q-50 were purchased from Sigma. Apoferritin was from Calbiochem. Ultrogel 22 was from LKB and Bio-Gel A-1.5m (200 to 400 mesh) from Bio-Rad. All chemicals were reagent grade or better.

*Proteins*-Bovine fibrinogen was prepared by the method of Straughn and Wagner (21) and was 96% clottable. Thrombin was assayed by its ability to clot fibrinogen. Activated Factor X (Xa) was prepared from purified Factor X (22) as described earlier (23). Prothrombin and thrombin were prepared as described previously (22).

*Polyacrylamide Gel Electrophoresis*-Sodium dodecyl sulfate-gel electrophoresis was carried out by the method of Laemmli (24). The acrylamide gels contained 6% acrylamide and 0.16% bisacrylamide (w/v). Acrylamide was electrophoresis grade and purchased from Eastman. Molecular weights were estimated from a plot of log molecular weight for standard proteins versus the relative mobility of the protein. The molecular weight standards employed were fibrinogen (unreduced) and reduced thyroglobulin, P-galactosidase, phosphofructokinase, and bovine plasma albumin. The interaction of the chains of activated Factor V was investigated by the acrylamide gel electrophoresis technique of Eisinger and Blumberg (25). Four percent acrylamide gels were prepared without a stacking gel and electrophoresis conditions were those of Davis (26). In some gels, MgCl$_2$ was included in both the separating gel and the electrophoresis buffers at a final concentration of 20 $\mu$M.

*The abbreviations used are: SDS, sodium dodecyl sulfate; DFP, diisopropyl fluorophosphosphate; H.C., heavy chain; L.C., light chain; QAE, quaternary aminoethyl.*
Estimation of Protein—Protein was monitored by absorbance at 280 nm. Approximate extinction coefficients were estimated by the dye-binding method of Bradford (27) and by the method of Lowry (28).

Factor V Assay—Factor V-deficient plasma was prepared from aged, oxalated human plasma. Factor V activity was assayed by the method of Kappeller (29). Standard curves were prepared with bovine plasma as the Factor V source. In our assay, bovine plasma contains 5 to 10 times the activity of human plasma. Therefore, the specific activities reported here are 5 to 10 times lower than if compared to a human standard. Bovine plasma is defined as containing 1 unit of Factor V/ml.

Calcium oxalate was prepared at room temperature by addition of 500 ml of CaCl₂ (1 M) to 2 liters of 0.2 mM sodium oxalate with stirring. The precipitate was stored at 4°C for 2 days prior to use.

Isolation of Factor V—Factor V was isolated by a modification of a method described previously (22, 23). Slaughterhouse blood (bovine) was collected into one-tenth volume of 0.1 M sodium oxalate, 0.01 M benzamidine/HCl, and 0.04 mg/ml of soybean trypsin inhibitor. Plasma was prepared by removing the blood cells in a Westfalla centrifuge. The plasma was adsorbed with a final concentration of 25 mg/ml of BaSO₄. The BaSO₄ was stirred into the plasma at room temperature for 30 min and removed by centrifugation through the Westfalla centrifuge equipped to remove solids. The plasma was then adsorbed to QAE-Sephadex (53 g of QAE-Sephadex Q-50, swollen in 4 liters of 0.1 M NH₄, 0.02 M Tris-HCl, pH 7.5, and made to 1 mM in benzamidine/HCl, pH 7.5) for 45 min with gentle stirring. The QAE-Sephadex with Factor V adsorbed was allowed to settle for 10 min. The adsorbed plasma was decanted and the Sephadex was filtered on a Buchner funnel (25 x 9 cm) overlaid with nylon mesh.

The Sephadex was washed with 10 liters of 0.1 M NH₄Cl, 0.02 M Tris-HCl, 1 mM benzamidine/HCl, pH 7.5, at 4°C, and made into a 4-liter slurry of the same buffer. The QAE-Sephadex was packed into a column (10 x 60 cm), washed at 4°C with 4 liters of 0.02 M NH₄Cl, 0.02 M Tris-HCl, 1 mM benzamidine/HCl, pH 7.5. The Factor V activity was eluted at 4°C with 4 liters of 0.02 M NH₄Cl, 0.02 M Tris-HCl, 0.001 M benzamidine/HCl, pH 7.5. Approximately 500 ml samples were collected into plastic beakers and fractions with greater than 2 units/ml of Factor V activity were pooled into a 4-liter Nalgene beaker. The pooled fractions were made 1 mM in diisopropylfluorophosphate, 35 min later made 1 mM in phenylmethylsulfonyl fluoride, and then 30 min later made 0.1 mM in p-nitrophenylglycinobenzoate. The sample was stored with inhibitors at 4°C for 14 h before continuing the isolation. BaSO₄-adsorbed plasma was processed from whole blood within 3 h of collection. The samples were eluted from the ion exchange Sephadex within 6 h of the time of collection. Rapid handling appears to facilitate isolation of high molecular weight Factor V, and storage overnight in the presence of inhibitors helps prevent degradation at subsequent steps.

The pooled QAE-Sephadex eluate was made 0.01 M in benzamidine/HCl, 0.1 M in Tris-HCl, pH 7.5, by addition of a 2 M stock solution, 2 M in NH₄Cl and 0.1 M in CaCl₂, by addition of the solid reagents with stirring at 4°C. Calcium oxalate (0.4 mol) prepared as described above was added with stirring. The Factor V activity was adsorbed to the insoluble calcium oxalate within 30 min, and a flocculent precipitate was evident. The calcium oxalate was pelleted by centrifugation at 1200 x g for 5 min. The pellet was washed, and then eluted three times with 500 ml of 0.05 mM Na₂citrate, 0.1 M Tris-HCl, 0.1 mM p-nitrophenylglycinobenzoate, pH 7.5, at 4°C. The calcium oxalate was separated from Factor V by centrifugation at 4850 x g for 5 min.

The eluate was precipitated with (NH₄)₂S₂O₇ at 4°C (0 to 30% saturation) and the precipitate was removed by centrifugation at 4850 x g for 30 min. This precipitate contained material which was difficult to redissolve. Little Factor V was detected in this fraction. The 30% supernatant was maintained at 4°C and brought to 55% saturation, stirred for 45 min, and the precipitate was removed by centrifugation at 4850 x g for 45 min at 4°C. This pellet was resuspended in a minimal volume of 0.1 M Tris-HCl, 0.01 M benzamidine/HCl, pH 7.5. The final volume was usually 80 ml or less. This sample was chromatographed on Ultrogel 22 (Fig. 1).

Factor V was pooled as indicated in the legend to Fig. 2, brought to 1 mM in DFP, and then dialyzed against 0.15 M NH₄Cl, 0.02 M Tris-HCl, 1 mM benzamidine/HCl, 0.001 M CaCl₂, pH 7.5, for 18 h at 4°C. Factor V activity was applied to a column of QAE-Sephadex (1.5 x 60 cm) equilibrated in 0.4 M NH₄Cl, 0.02 M Tris-HCl, 0.001 M benzamidine/HCl, pH 7.5. Chromatography was performed at 4°C with a flow rate of 35 ml/h. Factor V activity was assayed as described under "Materials and Methods." Fraction volume was 26 ml.

Reproducibly emerged in the second peak. The trailing shoulder usually contained additional protein species when analyzed by gel electrophoresis. The initially confusing aspect was that the specific activity of the Factor V was usually higher on the trailing edge of the column. However, if the specific activities were compared following activation of the sample with thrombin, the specific activities were comparable. As will be seen later, the species present in the trailing edge corresponds to an apparent Factor V activation intermediate. Each of the 10 large scale preparations within the last 8 months has resulted in qualitatively similar elution profiles, comparable specific activities (20 to 40 units/Aₕₐₚ), and comparable recovery of activity.

![Fig. 1. Gel filtration of Factor V on Ultrogel 22. The NH₄SO₄ precipitate (30 to 55%) was dissolved in 50 ml of 0.1 M Tris-HCl, 0.001 M benzamidine/HCl, pH 7.5, and applied to a column of Ultrogel 22 (5 x 130 cm) equilibrated in 0.4 M NH₄Cl, 0.02 M Tris-HCl, 0.001 M benzamidine/HCl, pH 7.5. Chromatography was performed at 4°C with a flow rate of 35 ml/h. Factor V activity was assayed as described under "Materials and Methods." Fraction volume was 26 ml.](http://www.jbc.org/)
The major differences involve the relative amount of high molecular weight Factor V and Factor V intermediate present in the preparations. In all preparations, the leading edge of the ion exchange column eluate contains high molecular weight Factor V as judged by SDS-gel electrophoresis. The purification is summarized in Table I.

### RESULTS

The apparent molecular weight of Factor V was estimated by SDS-gel electrophoresis to be 320,000 before and 280,000 following disulfide bond reduction. In all preparations, Factor V has appeared as a tightly spaced doublet. The relative intensity of each component of the doublet has been variable.

Evidence that the protein observed is Factor V comes from the study of the activation of Factor V with thrombin (Fig. 3). Even at 4°C and in the presence of 1 mM benzamidine/HCl, the high molecular weight species were rapidly cleaved. No appreciable difference was observed in the apparent rate of disappearance of the proteins in the doublet. Within 12 min, the Factor V was completely cleaved and two new species were formed corresponding to a molecular weight of 210,000 and 115,000. Formation of these species corresponds to a 3- to 4-fold increase in specific activity. Evidence will be presented that these two bands correspond to the light and heavy chains of a Factor V activation intermediate. Both the activity and the gel pattern remained constant until the sample was shifted to 37°C. Following the temperature shift, there was an additional 3-fold increase in Factor V activity and the FV-H.C. completely disappeared. A new species with an apparent molecular weight of 73,000 appeared below the FV-L.C. This species corresponds to the light chain of Factor Va. A transient species located midway between the FV-H.C. and FV-L.C. was observed. This band has a mobility similar to an activation peptide formed from the FV-H.C. on incubation with thrombin.

### Table I

**Purification of Factor V**

|                | Volume | Total protein | Total activity | % Recovery | Specific activity | Purification |
|----------------|--------|---------------|----------------|------------|------------------|--------------|
| BaSO₄-adsorbed plasma | 1,600 | 104,000 | 16,000 | 100 | 0.0154 | 1 |
| QAE eluate | 2,500 | 15,750 | 7,500 | 46.8 | 0.476 | 30.9 |
| Calcium oxalate eluate | 1,300 | 4,940 | 9,360 | 58.5 | 1.89 | 122 |
| NH₄SO₄ (30 to 55%) | 95 | 2,983 | 7,980 | 50 | 2.7 | 175 |
| Ultrogel 22 | 440 | 346 | 5,200 | 33 | 15 | 974 |
| QAE-Sephadex Q-50 | 300 | 109 | 4,400 | 27 | 40 | 2,500 |

![Fig. 3. The activation time course of Factor V by thrombin. Factor V (1.8 units/ml) was incubated with thrombin (0.833 μg/ml, 2 units/ml) at 4°C in 0.1 M NH₄Cl, 0.02 M Tris-HCl, 0.001 M benzamidine/HCl. At 10 min (first arrow), additional thrombin was added (0.833 μg/ml). At 20 min (second arrow), the reaction mixture was shifted to 37°C. The incubation was performed in plastic tubes. A, samples were removed from the incubation mixture at the times indicated, diluted and assayed for Factor V activity. B, samples (0.1 ml) were removed at the times indicated and rapidly added to 0.02 ml of 10% SDS in 0.02 M EDTA at 100°C. Samples were electrophoresed on 6% SDS-acrylamide gels. The samples were treated with β-mercaptoethanol (10%, v/v) and heated at 100°C for 1 min immediately prior to electrophoresis. C, an identical sample was removed and treated as described in B except that no β-mercaptoethanol was added to the sample.](http://www.jbc.org/)

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bin. However, this band has not been unambiguously identified. Several other less intense bands are generated by thrombin proteolysis. Their relationship to Factor V or Factor Va is uncertain.

The time course study is compatible with the activation scheme: FV (Mn = 280,000) → FV-H.C. (Mn = 210,000) + FV-L.C. (Mn = 115,000) → FVα-H.C. (Mn = 115,000) + FVα-L.C. (Mn = 73,000) + activation peptide(s). Support for this model is presented below.

Isolation of Activated Factor V—Activated Factor V was obtained by incubating Factor V with thrombin. The activated Factor V was then separated from other activation products by ion exchange chromatography on QAE-Sephadex Q-50 (Fig. 4). SDS-gel electrophoresis was used to analyze the elution profile and revealed that two chains of Mn = 115,000 and 73,000 appeared in the fractions with the Factor V activity. Incubation of these fractions with thrombin failed to alter the Factor V activity. Visual inspection of the SDS-gels suggested that the relative concentration of two chains remained constant across the ion exchange peak which contained the Factor V activity. The activated Factor V was subjected to polyacrylamide gel electrophoresis in the absence of SDS, a single diffuse band was observed (Fig. 5, inset). Although diffuse, it is important to note that it has the appearance of a single component, as there are no sharp bands visible within the stainable band.

Evidence for a Two-chain Structure—Previous investigators (1, 13, 14) have demonstrated that Factor V and probably Factor Va activity are inhibited by EDTA. This experiment was repeated and the results analyzed both in terms of the biological activity and in terms of the Factor Va chain structure, as monitored by disc gel electrophoresis in the absence of detergent (Fig. 5). Once exposed to EDTA, the Factor Va activity falls rapidly. The starting material appeared as a single diffuse band. A sample removed at 30 min and subjected to electrophoresis revealed a slow and fast moving component (Fig. 5, inset). The sample maintained in CaCl₂ retained the same characteristics after a 4-h incubation as shown at zero time. No further changes were observed in the sample treated with EDTA after a 4-h incubation. Based on the similar molecular weights of the peptide components, this mobility difference was assumed to be due to different charged properties between the peptides, suggesting that the two species could be separated by ion exchange chromatography.
The incubation mixtures were also analyzed by disc gel electrophoresis. As the relative concentration of light chain to heavy chain is increased, a decrease in the amount of rapidly migrating heavy chain can be seen to occur on gel electrophoresis (Fig. 7b). At optimal activity, the chains form a single broad band with only a trace of rapidly migrating material remaining. When excess light chain is added, a tightly spaced doublet in the slow migrating region of the gel results; no rapidly migrating material is observed. From these data, it is clear that the heavy chain corresponds to the rapidly migrating material seen in Factor Va upon addition of EDTA (Fig. 5). The reassociation is slow at 4°C. As a control experiment, the chains were mixed and immediately electrophoresed under identical conditions with those in Gel D of Fig. 7b. No reconstitution was observed, indicating that the change in electrophoretic mobility is related to reassociation of the activity. Based on the data presented, it appears that both the heavy chain and the light chain comprise this form of Factor Va and that divalent cations are necessary for retention of the tight association between these subunits.

Ion Specificity—The relative ability of Ca$^{2+}$ to restore Factor Va activity from the isolated subunits of the Factor Va has been compared to that of Mn$^{2+}$ (Fig. 6). Mn$^{2+}$ appeared to be much more effective in causing the re-formation of Factor Va activity than Ca$^{2+}$. Several other ions were investigated under identical conditions and the rate of formation of Factor Va activity was measured after incubation for 10 min and 1 h (Table I). Sr$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, and Ba$^{2+}$ were relatively ineffective, while Mn$^{2+}$, Cd$^{2+}$, and Co$^{2+}$ were good effectors of the re-formation of Factor Va activity from the isolated chains.

Precursor-Product Relationships in the Activation of Factor V—As described above, the SDS-gel time course indicated the rapid formation of a two-chain intermediate from the Factor V precursor (Fig. 3B). This intermediate has been confirmed by incubating Factor V with a trace of thrombin, treating the reaction mixture with DFP, and isolating the intermediate by ion exchange chromatography. This intermediate was eluted at the same ionic strength as Factor V and could, upon addition of thrombin, increase from 3- to 4-fold in specific activity. This Factor V intermediate was also found in the trailing fractions of the QAE column used for preparation of the Factor V or, after prolonged storage of the Factor V, the high molecular weight Factor V has degraded into a species electrophoretically identical with the Factor V intermediate.

The activation time course suggested that the light chain of Factor Va (29.4 A$_{280}$) was dialyzed for 18 h against 0.05 M NH$_4$Cl, 0.02 M Tris-HCl, 0.001 M benzamidine/HCl, 0.001 M EDTA, pH 7.5, and applied to a QAE-Sephadex Q-50 column (1.5 X 30 cm) equilibrated in the same buffer. The column was developed with a linear gradient from 0.05 M NH$_4$Cl to 0.5 M NH$_4$Cl (135 ml/reservoir) with the other buffer components as stated above. The recovery of applied A$_{280}$ was 96%.

**Fig. 6. Chromatography of Factor Va in the presence of EDTA.** Factor Va (29.4 A$_{280}$) was dialyzed for 18 h against 0.05 M NH$_4$Cl, 0.02 M Tris-HCl, 0.001 M benzamidine/HCl, 0.001 M EDTA, pH 7.5, and applied to a QAE-Sephadex Q-50 column (1.5 X 30 cm) equilibrated in the same buffer. The column was developed with a linear gradient from 0.05 M NH$_4$Cl to 0.5 M NH$_4$Cl (135 ml/reservoir) with the other buffer components as stated above. The recovery of applied A$_{280}$ was 96%.

**Fig. 7. Reconstitution of Factor Va activity from isolated Factor Va subunits.** a, Factor Va heavy chain in 0.1 M NH$_4$Cl, 0.002 M Tris-HCl, 0.001 M benzamidine/HCl, 0.001 M EDTA, pH 7.5, and applied to a QAE-Sephadex Q-50 column (1.5 X 30 cm) equilibrated in the same buffer. The column was developed with a linear gradient from 0.05 M NH$_4$Cl to 0.5 M NH$_4$Cl (135 ml/reservoir) with the other buffer components as stated above. The recovery of applied A$_{280}$ was 96%.

The extinction coefficients were also estimated by the dye binding method of Bradford (27). The extinction coefficient for the heavy chain to be 9.8 and for the light chain to be 15.8. The extinction coefficients were also estimated by the dye binding method of Bradford (27). The extinction coefficient for the heavy chain to be 9.8 and for the light chain to be 15.8. The extinction coefficients were also estimated by the dye binding method of Bradford (27). The extinction coefficient for the heavy chain to be 9.8 and for the light chain to be 15.8. The extinction coefficients were also estimated by the dye binding method of Bradford (27). The extinction coefficient for the heavy chain to be 9.8 and for the light chain to be 15.8.
The Subunit Structure of Thrombin-activated Factor V

Like activated Factor V, Factor V can be inactivated by incubation with EDTA (data not shown). Gel filtration in buffer containing EDTA resulted in separation of the Factor V-H.C. from the Factor V-L.C. (Fig. 9). Neither the isolated heavy chain nor the isolated light chain had Factor V activity either in the presence or absence of Ca\(^{2+}\), Mn\(^{2+}\), or Cd\(^{2+}\) at a final concentration of 100 mM. However, as in the case with the Factor Va subunits, the activity could be regained by mixing the heavy chain with the light chain in the presence of MnCl\(_2\) (Table III).

The sample applied to the column contained a trace of high molecular weight Factor V and this high molecular weight Factor V did not dissociate but rather retained limited activity even in the presence of the EDTA. Addition of Ca\(^{2+}\) to this protein species resulted in a specific activity increase of about 10-fold.

The ability to separate the chains allowed a direct test of the origin of each of the components of the Factor Va and Factor V intermediate. The time course study suggested that the Factor V-L.C. is formed concomitantly with the Factor V-H.C. The Factor V-L.C. appeared to ultimately become the Factor V-H.C. while the Factor V-L.C. appeared to be derived from the Factor V-H.C. As seen in Table III, incubation of the Factor V-L.C. with the Factor V-H.C. results in biological activity comparable to that obtained by incubation of the Factor V-H.C. with the Factor V-L.C. In contrast, the Factor V-L.C. is not biologically active when combined with the Factor V-H.C. None of the chains enhanced the activity of the high molecular weight Factor V.

Addition of the Factor V-L.C. to the Factor V-H.C. resulted in biological activity comparable to that of the Factor Va. Thus, in addition to the similarity in molecular weight, the light chain of the intermediate can be functionally interchanged with the heavy chain of the Factor Va.

Subunits stored in EDTA have always retained greater than 50% of their biological activity for in excess of 2 months at 4°C.

![Fig. 8. Reconstitution of Factor Va activity with Ca\(^{2+}\) and with Mn\(^{2+}\). Factor Va heavy chain in 0.1 M NH\(_4\)Cl, 0.02 M Tris-HCl, 0.001 M EDTA, 0.001 M benzamidine/HCl, pH 7.5, at a final concentration of 0.0469 A\(_{280}\)/ml and light chain in the same buffer at a final concentration of 0.0469 A\(_{280}\)/ml were incubated with MnCl\(_2\) at a final concentration of 25 mM MnCl\(_2\), 2.5 mM MnCl\(_2\), or 25 mM CaCl\(_2\). Incubations were performed at 23°C. Samples were removed and assayed at the times indicated.](http://www.jbc.org/)

![Fig. 9. Isolation of the heavy chain and light chain of the Factor V intermediate. Factor V containing lower molecular weight species was pooled from the back half of the Factor V peak of a preparative QAE-Sephadex Q-50 column and precipitated with 80% saturation NH\(_4\)SO\(_4\) at 4°C. The fraction chosen corresponds to Fractions 35 to 41 of Fig. 2 but was taken from a preparation which contained Factor V. The precipitate was dissolved in 0.1 M Tris-HCl, 0.001 M benzamidine/HCl, pH 7.5, and applied to an Ultrogel 22 column (2.5 x 160 cm), equilibrated in 0.1 M NH\(_4\)Cl, 0.02 M Tris-HCl, 0.001 M EDTA, 0.001 M benzamidine/HCl. The recovery of applied material was 86%, the flow rate was 16 ml/h, and the column was developed at 4°C. Insert from the left is an SDS-gel of the sample applied to the column and the other gels are located above the fraction from which the sample was removed.](http://www.jbc.org/)
The Subunit Structure of Thrombin-activated Factor V

Samples were incubated at 23°C for 1 h in the presence of 0.05 M MnCl₂ and assayed for Factor V activity. The incubation mixtures contained protein at the concentrations indicated in parentheses (Am/ml) expressed as the final concentration of that species. The light chain derived from the heavy chain of the Factor V intermediate corresponds to Fraction 15 with the Factor Va-H.C. This was tested directly by chromatography on QAE-Sephadex (Fig. 10). As anticipated, no Factor Va-L.C. regenerated activity but reconstitution with the Factor Va-H.C. activation peptide (0.11) demonstrated that the chains of the intermediate do interact with concomitant disappearance of the light chain of the intermediate from its usual elution position. This experiment suggested that the cationic light chain of the Factor Va was derived from the Factor Vi-H.C. This was tested directly by incubating the heavy chain of the Factor V intermediate with thrombin and subjecting the incubation mixture to chromatography on QAE-Sephadex (Fig. 10). As anticipated, no biological activity was eluted from the column. Combination of Fraction 15 with the Factor Va-H.C. resulted in formation of Factor V activity (Table III). Reconstitution of the Factor V intermediate (0.44 A₅₆₀) was chromatographed on the above column. Recovery = 79%; C, the heavy chain of the Factor V intermediate (0.96 A₅₆₀) was combined with the light chain of the intermediate (0.44 A₅₆₀) and chromatographed on the above column. Recovery A₅₆₀ = 82%; Recovery Factor V activity = 90%.

### Table III
Reconstitution of Factor V activity from isolated chains

| Incubation mixture | Specific activity |
|-------------------|------------------|
| Factor V-H.C. (0.252) | 2.1 ± 0.2 |
| Factor V-H.C. (0.252) + Factor V-L.C. (0.11) | 78 ± 11 |
| Factor V-H.C. (0.252) + Factor VH.C. (0.11) | 85 ± 7 |
| Factor V-H.C. (0.252) + Factor V-L.C. (0.11) | 3.1 ± 0.2 |
| Factor V-H.C. (0.252) + Factor V-L.C. from Factor V-H.C. (0.11) | 3.0 ± 0.3 |
| Factor V-H.C. (0.252) + Factor V-H.C. activation peptide (0.11) | 2.2 ± 0.1 |
| Factor V-L.C. (0.11) | 4.0 ± 0.4 |
| Factor V-L.C. (0.11) + Factor V-H.C. (0.11) | 4.2 ± 0.4 |
| Factor V-L.C. (0.11) + Factor V-H.C. (0.11) | 362 ± 35 |
| Factor V-L.C. (0.11) + Factor V-L.C. from Factor V-H.C. (0.11) | 243 ± 24 |
| Factor V-L.C. (0.11) + Factor V-H.C. activation peptide (0.11) | 8.1 ± 2.5 |
| Factor V-H.C. (0.11) | 6.1 ± 0.8 |
| Factor V-H.C. (0.11) + Factor V-L.C. (0.11) | 407 ± 22 |
| Factor V-H.C. (0.11) + Factor V-L.C. from Factor V-H.C. (0.11) | 227 ± 16 |
| Factor V-H.C. (0.11) + Factor activation peptide (0.11) | 6.1 ± 0.5 |
| Factor V-L.C. (0.11) | 1.5 ± 0.4 |
| Factor V-L.C. (0.11) + Factor V-L.C. from Factor V-H.C. (0.11) | 1.1 ± 0.6 |
| Factor V-L.C. (0.11) + Factor V-H.C. activation peptide (0.11) | 2.1 ± 0.2 |
| Factor V-L.C. from Factor V-H.C. (0.11) | 1.1 ± 0.2 |
| Factor V-H.C. activation peptide (0.11) | <0.5 |
| Factor V-L.C. (0.11) + Factor V-H.C. activation peptide (0.11) | <0.5 |

### Fig. 10.
Formation of the Factor Va light chain from the heavy chain of the Factor V intermediate. The heavy chain of the Factor V intermediate (6.36 A₅₆₀/12 ml) in 0.05 M NH₄Cl, 0.02 M Tris-HCl, 0.001 M CaCl₂, pH 7.5, was digested with thrombin (0.21 mg). The sample was digested for 30 min at room temperature before chromatography on a QAE-Sephadex column (0.6 × 30 ml). The column was developed with a linear gradient in NH₄Cl, 0.05 to 1.0 M in 0.02 M Tris-HCl, 0.01 M CaCl₂, 0.001 M benzamidine/HCl, pH 7.5. The recovery of A₅₆₀ absorbing material was 74.6%. The gels shown in the inset are 6% SDS-gels.

### Fig. 11.
Reconstitution of Factor V intermediate from isolated subunits. Samples (1.0 ml) were chromatographed on a column (0.9 × 94 cm) of Bio-Gel A-1.5m (200 to 400 mesh) equilibrated in 0.4 M NH₄Cl, 0.02 M Tris-HCl, 0.01 M CaCl₂, 0.001 M benzamidine/HCl, pH 7.5, at 23°C. A, the heavy chain of the Factor V intermediate (0.96 A₅₆₀) was chromatographed on the above column. Recovery = 89%; B, the light chain of the Factor V intermediate (0.44 A₅₆₀) was chromatographed on the above column. Recovery = 79%; C, the heavy chain of the Factor V intermediate (0.96 A₅₆₀) was combined with the light chain of the intermediate (0.44 A₅₆₀) and chromatographed on the above column. Recovery A₅₆₀ = 82%. Recovery Factor V activity = 90%.

Formation of the Factor Va Light Chain from the Isolated Heavy Chain of the Intermediate—As seen in Table III, the Factor V-H.C. would combine with the Factor Va-H.C. to give biological activity. This, coupled with the time course and the fact that specific activity of the reconstituted intermediate could still be increased by incubation with thrombin, suggested that the cationic light chain of the Factor Va was derived from the Factor Vi-H.C. This was tested directly by incubating the heavy chain of the Factor V intermediate with thrombin and subjecting the incubation mixture to chromatography on QAE-Sephadex (Fig. 10). As anticipated, no biological activity was eluted from the column. Combination of Fraction 15 with the Factor Va-H.C. resulted in formation of Factor V activity (Table III). Reconstitution with Factor Vi-L.C. regenerated activity but reconstitution with the Factor Vi-H.C. failed to generate activity. Prior to incubation with thrombin, the heavy chain was eluted from the QAE-Sephadex as a single peak at approximately the same ionic strength as the material in the second peak of the digest. The material in the second peak (Fraction 40) was not active with any isolated peptide and may correspond to the difference between the Factor Vi-H.C. and Factor Va-L.C.

Reconstitution of the Subunits of Factor V, and Factor Va—As shown previously, separately, the Factor Vi-H.C. and Factor Vi-L.C. were without biological activity. Reformation of the Factor V activity should correspond to the formation of a complex between the heavy chain and the light chain of the intermediate. In Fig. 11, the elution profiles of the Factor Vi-H.C., the Factor Vi-L.C. and the recombined chains were analyzed by gel filtration chromatography. The heavy chain (Fig. 11A) and the light chain (Fig. 11B) were both devoid of biological activity. Recombination of the heavy chain with the
presence of EDTA, two separate peaks are observed (data not shown). Since these chains were originally separated by gel filtration in the presence of EDTA, it would appear that the Factor Vr also requires divalent ions to retain tight association and biological activity.

The interaction of the chains of Factor Va has also been investigated by gel filtration. The Factor Va-H.C., the Factor Va-L.C., and the reconstituted Factor Va heavy and light chains were subjected to gel filtration in the presence of Ca\(^{2+}\). Preincubation of the heavy chain and the light chain in the presence of Mn\(^{2+}\) resulted in formation of a higher molecular weight species with biological activity (Fig. 12, A to C). Preincubation as described above followed by chromatography in buffer containing EDTA resulted in separation of the two species (Fig. 12, D). Reconstitution of the first (elution volume, 41 ml) and second (elution volume, 47 ml) peak material in the presence of 100 mM Mn\(^{2+}\) resulted in a species with a specific activity of 180 units/A\(^{280}\). Neither of the peaks were active by themselves (specific activity, <5). The results confirm the dissociable nature of the two chains observed previously.

**Molecular Weight Estimation**—The molecular weights of the Factor V and the partial proteolysis products were estimated by gel filtration chromatography on Bio-Gel A-1.5m. All proteins were eluted as single essentially symmetrical peaks. The apparent Stokes radius for Factor V and other chains are reported in Table IV. The elution volume of the isolated chains was essentially unaltered by the presence of Ca\(^{2+}\) or EDTA in the buffers.

**DISCUSSION**

Factor V appears to be composed of a very high molecular weight polypeptide. Thrombin can cleave the polypeptide with concomitant increase in biological activity. Once split, the chains are held together by noncovalent interaction, and the tight association between chains is inhibited by EDTA and restored by Mn\(^{2+}\) or Ca\(^{2+}\). Current results indicate that two nonidentical peptides derived from the high molecular weight precursor protein are required for biological function. These studies, however, do not determine the mechanism of divalent ion involvement in maintaining the association between the peptides. One possibility is that Ca\(^{2+}\) or Mn\(^{2+}\) forms a bridge between the molecules, much as the postulated bridge between Ca\(^{2+}\) and phospholipid in the case of prothrombin (30). Greenquist and Colman (15) have suggested that EDTA covers the Ca\(^{2+}\) bound in the molecule. These authors postulated that the Ca\(^{2+}\) was involved in phospholipid interaction. If that is the case, then it must also be involved in subunit-subunit interaction. Alternatively, the divalent ions could be involved in maintaining some conformational state in one or both subunits, making them capable of binding.

Previous investigations have suggested a variety of structures for Factor V and Factor Va. Only a few studies have employed gel electrophoresis (1, 5, 20, 31, 32) and these have given conflicting results. The apparent polypeptide size of the Factor V described here is approximately 280,000 based on SDS-gel electrophoresis. Our gels have always shown a tightly spaced doublet in this region. Both species are rapidly cleaved by thrombin and no appreciable difference in the rate of cleavage has been observed. The basis for this doublet remains enigmatic but may be related to differences in carbohydrate content or minor proteolysis occurring in vivo or during isolation. The possibility of two nonidentical chains cannot be totally excluded, but this would seem difficult to reconcile with the activation patterns observed in this study.
Attempts to isolate Factor V from slaughterhouse blood sometimes result in a preparation which contains predominantly the Factor V activation intermediate. The intermediate and other degradation products have been difficult to separate except by gel filtration in buffers containing EDTA. This method allows the isolation of the high molecular weight Factor V even from preparations showing considerable degradation. Of importance is that the activated Factor V species characterized here can be obtained even from such degraded Factor V preparations. The activated Factor V prepared as described here is stable at 4°C maintaining greater than 70% of its initial activity for 1 month. Once the activated Factor V is separated into chains, the activity seems even more stable. Greater than 80% of the original activity has been recovered from the isolated chains stored for 3 months. These data indicate that Factor Va is not an intrinsically unstable protein. Several lines of evidence support the conclusion that the Factor Va preparation is essentially homogeneous and composed of two polypeptide chains. Although the Factor Va does not migrate on gels as a sharp band, it does split into a cationic and anionic component upon incubation with EDTA. The correlation between the re-formation of the isolated chains into a single band with the re-formation of the Factor V activity provides further evidence for the two-chain structure of the activated Factor V and the purity of the preparation. Despite their different charge properties, these chains appear to co-chromatograph on QAE-Sephadex. Once treated with EDTA, the two inactive chains can be separated by either gel filtration or ion exchange chromatography. If the chains are allowed to reconstitute, then the Factor Va which is formed chromatographs with a higher apparent molecular weight than either chain, and the biological activity co-chromatographs with the newly formed protein species. Factor Va formed from isolated chains has the same apparent molecular weight as the Factor Va from which the chains were prepared. Thus, gel electrophoresis, gel filtration, and co-chromatography of the two chains constitute the evidence that the Factor Va is composed of two polypeptide chains and that the preparation is essentially homogeneous.

The apparent molecular weight of Factor Va by gel filtration is 290,000 which is in reasonable agreement with the molecular weight for Factor Va of 240,000 obtained by Smith and Hannah (6). This value is also in reasonable agreement with the sum of the molecular weights of the Factor Va chains estimated by SDS-gel electrophoresis (188,000) with molecular asymmetry probably accounting for the discrepancies. The observation of much lower molecular weight forms of Factor V are difficult to explain based on these studies (1, 16, 17). The "dissociated" forms could be adsorbing to the columns. Alternatively, additional proteolytic cleavages may be possible. Once additional proteolysis occurs, the new species may be unstable.

The time course of the Factor V activation by thrombin suggests that thrombin cleaves Factor V to form two noncovalently associated peptide chains which correspond to an activation intermediate with increased biological activity. The Factor V-H.C. is then cleaved to yield the Factor Va-L.C. The assignment of the heavy chain of the intermediate as the precursor to the light chain of the Factor Va was confirmed by isolating the Factor Va light chain from a thrombin digest of the heavy chain of the intermediate. Based on the activation time course, formation of the Factor V intermediate from Factor Va appears to correspond to an increase of 3- to 4-fold in specific activity. Formation of Factor Va from the intermediate appears to correspond to an additional 3- to 4-fold increase in specific activity. These interpretations of the time course data are entirely consistent with the observation that the intermediate, reconstituted from isolated chains, is activated 3 to 4-fold by thrombin. A transient band is observed (M, \( \approx 140,000 \)). Its presence is a reproducible feature of the activation process, but its identity remains uncertain. It has a mobility similar to the activation peptide isolated from the thrombin digest of the heavy chain of the intermediate (Fig. 10, Peak 2).

Molecular weight estimations by SDS-gel electrophoresis and gel filtration chromatography are subject to considerable error, especially for glycoproteins. However, the assignments of precursor-product relationships presented here are only secondarily dependent on these molecular weight estimates, since each of the chains has been isolated and since biological activity can be restored only by reconstitution of the proper chains. Thus, reconstitution and limited proteolysis of the isolated chains provide the evidence for the precursor-product relationship presented here. A tentative model for Factor V activation is presented below:

\[
\text{Factor V} \xrightarrow{\text{thrombin}} \text{Factor Va} + \text{activation peptide(s)}
\]

A curious feature of the overall activation process is that all of the original polypeptide material appeared to migrate on SDS-gel electrophoresis in positions corresponding to Factor Va. However, during chromatography, material co-electrophoresing with the light chain was separated in the first peak of the ion exchange column while material co-electrophoresing with the heavy chain of the Factor Va was separated on the trailing side of the Factor Va peak. To date, neither of these species has been reconstituted successfully with any isolated chain. Chemical, immunological, and additional isolation studies will be necessary to clarify the fate of the activation peptides.

The Factor V preparation described here is rapidly and completely cleaved by thrombin under mild conditions. Sarawathi et al. (20) have isolated Factor V from venipuncture blood. They propose that Factor V contains a disulfide-linked light chain which is not cleaved by thrombin and a heavy chain which is thrombin-sensitive. Upon activation, their model proposes a disulfide-linked form of Factor Va. Neither the Factor V activation intermediate nor the Factor Va described here is covalently attached. It is possible that the light chain proposed by Sarawathi et al. (20) alters the specificity of the thrombin such that cleavage occurs within a disulfide loop. Many additional studies will be necessary to establish the association of the light chain with the heavy chain and what role the light chain may have in altering the cleavage of the Factor V by thrombin.

For most mechanistic studies of Factor V function, it is essential to have a highly purified and stable preparation. The preparation described here shows excellent stability characteristics and therefore provides a useful reagent. It is possible that the stability seen for our activated Factor V preparation is due to the complete removal of thrombin from the Factor Va afforded by ion exchange chromatography. The fact that the subunits can be stored for at least 2 months at 4°C and recombined with regain of most of their biological activity makes this separation technique useful for analysis of Factor V functions. The ability to separate regions of the Factor V molecule and recombine them to regain biological activity should provide a useful probe into the mechanism by which Factor V participates in prothrombin activation.

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