Activation of Bovine Factor IX (Christmas Factor) by Factor XIₐ (Activated Plasma Thromboplastin Antecedent) and a Protease from Russell's Viper Venom*

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Bovine factor IX (Christmas factor) is a coagulation protein present in plasma in a precursor or inactive form. It is a glycoprotein (M, = 55,000) composed of a single polypeptide chain. Factor IX in the presence of calcium ions is converted to factor IXₐ by factor XIₐ (activated plasma thromboplastin antecedent) or a protease present in Russell's viper venom. The activation of factor IX by the protease from Russell's viper venom is due to the cleavage of a single internal Arg-Val peptide bond in factor IX. This leads to the formation of factor IXₐ, a protein with the same molecular weight as the precursor. Factor IXₐ is composed of two polypeptide chains held together by a disulfide bond(s), and these two chains have an NH₂-terminal sequence of Tyr-Asn-Ser-Gly- and Val-Val-Gly-Gly-. The mechanism of activation of factor IX catalyzed by the protease from Russell's viper venom differs from that catalyzed by factor XIₐ. The latter enzyme activates factor IX in a two-step reaction. In the first step, an internal Arg-Ala bond is cleaved, and in the second step, an Arg-Val bond is cleaved. The second cleavage leads to the liberation of an activation peptide from the NH₂-terminal portion of the heavy chain to produce factor IXₐ. The critical step in the activation of factor IX by both pathways is the cleavage of the same Arg-Val peptide bond and the formation of a new NH₂-terminal sequence of Val-Val-Gly-Gly- in the heavy chain of the enzyme which contains the active site serine residue. Factor IXₐ has approximately 50% of the specific activity of factor IX in a coagulant or esterase assay, and both enzymes are inhibited by antithrombin III. A number of other purified plasma proteases were also examined for their ability to activate factor IX. Factor Xₐ (activated Stuart factor) catalyzed a slow but definite activation of factor IX, while the activation of factor IX by bovine kallikrein, thrombin, or factor XII, (activated Hageman factor) was negligible.

Factor IX (Christmas factor)¹ is a vitamin K-dependent glycoprotein which participates in the middle phase of blood coagulation (see Ref. 1 for a recent review). In individuals with hemophilia B, this protein is absent or inactive, resulting in a bleeding disorder with clinical symptoms essentially identical with classical hemophilia (hemophilia A). Factor IX circulates in the blood in an inactive or precursor form. During the coagulation process, it is converted to an enzyme by factor XIₐ (activated plasma thromboplastin antecedent) in the presence of calcium ions (3-7).

The activation of factor IX by several other proteases has also been reported. These include the activation of bovine factor IX by factor Xₐ (8) and thrombin (9) and the activation of human factor IX by trypsin and kallikrein (10). The mechanism by which these activation reactions occur was not defined.

The mechanism for the activation of bovine factor IX by factor XIₐ has been reported (7). In this reaction, factor XIₐ initially cleaves an internal peptide bond yielding an inactive, two-chain intermediate held together by a disulfide bond(s). In a second step, an activation peptide is split from the NH₂-terminal end of the heavy chain of the intermediate giving rise to factor IXₐ. The specific peptide bonds cleaved in these two steps, however, were not identified. Recently, a similar mechanism was reported for the activation of human factor IX by factor XIₐ (11, 12).

This manuscript describes the molecular events associated with the activation of bovine factor IX by a protease from Russell's viper venom. This activation mechanism is then compared with the activation of factor IX by factor XIₐ. The effect of bovine thrombin, factor Xₐ, factor XII, and plasma kallikrein on factor IX is also reported.

EXPERIMENTAL PROCEDURES

Materials—Bovine factor IX and factor Xₐ were purified to a homogeneous state as previously described (13, 14). Bovine thrombin and bovine plasma kallikrein were kindly provided by Drs. W. Kisiel and R. Heimark in our laboratory. Bovine factor XII, was purified by the method of Fujikawa et al. (15), and bovine factor XIₐ was kindly provided by Dr. K. Kurachi in our laboratory. Bovine thrombin, kallikrein, factor XII, and factor XIₐ were homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Russell's viper venom was obtained from the Miami Serpentarium, Miami, Florida, and was purified by the procedure of Schifflman et al. (16) or by the method of Kisiel et al. (17). The specific activity was about 5000 units/mg of protein (17) and both preparations gave identical results. Bovine antithrombin III was isolated by the method of Thaler and Schmer (18) and was kindly provided by Dr. K. Kurachi. Carboxypeptidase A and B were purchased from Worth-
Sephadex G-100, G-50, SP-Sephadex C-50, and Sepharose 4B were products of Pharmacia N.V.-methylenediamineylamide, 2-mercaptoethanol, and N-vinylmaleimide were purchased from Eastman. Acrylamide was obtained from Bio-Rad Laboratories. Baker Chemical Co. supplied 4-vinylpyridine which was distilled before use. Benzoyl-L-arginine ethyl ester labeled with $^{3}H$ was synthesized in our department by Eric Fodor, and tosyl-L-arginine methyl ester or 1 mM tosyl-L-arginine methyl ester solution in dry dimethylformamide was then added and mixed. Ten milliliters of Omnifluor scintillation fluid were added and counting was performed immediately in a Beckman model LS-100C scintillation counter at room temperature.

The protease from Russell's viper venom was coupled to Sepharose 4B by the method of Cuatrecasas (31). Activated Sepharose 4B was prepared by reacting 10 g of cyanogen bromide with 50 ml of washed, packed Sepharose at 25° for 15 min. Thirty milligrams of the partially purified protease from Russell's viper venom in 50 ml of 0.1 M NaHCO$_3$ (adjusted to pH 9.0) were added to the activated Sepharose 4B and the mixture was stirred slowly for 20 h at 4°. The RVV-X-Sepharose was then washed with 0.05 M Tris/HCl buffer (pH 8.0) containing 1 M NaCl, followed by 0.05 M Tris/HCl buffer at room temperature. This washing cycle was repeated three times.

Factor $X_{I}$ was prepared by activating bovine factor X with RVV-X-Sepharose. In these experiments, factor X (2 mg) was incubated with 5 ml of 0.1 M Tris/HCl buffer (pH 8.0) and 0.15 M NaCl in the presence of 0.05 M CaCl$_2$. At the end of the reaction, 20 μl of 0.1 M EDTA were added and the RVV-X-Sepharose was removed by centrifugation. The activation of factor X went to completion as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15).

The separation of the two chains of factor $X_{I}$ was carried out in the following manner. A reaction mixture containing 12 mg of factor IX, 0.5 mg of Russell's viper venom protease, 5 mm CaCl$_2$, and 50 mm Tris/HCl (pH 8.0) in a total volume of 10 ml was incubated at 37° for 30 min. The reaction was stopped by the addition of 1 ml of 0.1 M EDTA and the reaction mixture was lyophilized. The lyophilized sample was then reduced and pyridylated by the method of Friedman et al. (24), and salt and excess reagent were removed by gel filtration on a Sephadex G-50 column (1.9 x 50 cm) previously calibrated with 0.01 M NaCl and 100 units of heparin as previously described (30). The mixture was incubated at 37° for 90 min and assayed for activity by the esterase method.

**RESULTS**

Activation of Bovine Factor IX by a Protease from Russell's Viper Venom—A time curve for the activation of factor IX by the Sepharose-bound protease of Russell's viper venom is shown in Fig. 1. In these experiments, factor IX was activated in the presence of 0.01 M CaCl$_2$ and 0.05 M Tris/HCl buffer (pH 8.0). Factor $X_{I}$ was measured by its coagulant activity (open circles) and its esterase activity toward benzoyl-L-arginine ethyl ester (closed circles). These two activities increased concurrently and reached a maximum after 10 min incubation.

Similar experiments employing the soluble protease from Russell's viper venom showed that the same rate of reaction, as measured by esterase activity, could be obtained when the weight ratio of enzyme to factor IX was 1 to 20. In the absence of CaCl$_2$ or when Sepharose 4B was added alone, no increase in either coagulant or esterase activity was observed.

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The protease from Russell's viper venom results from the activation of factor IX by the protease from Russell's viper venom. Factor IX (2.7 mg) was incubated with gentle shaking at 37°C with 1.0 ml (settled volume) RVV-X-Sepharose in the presence of 0.05 M Tris/HCl (pH 8.0) and 10 mM CaCl₂ in a final volume of 8.0 ml. At various times, 1 ml of the reaction mixture was withdrawn into 0.2 ml of 0.1 M EDTA and centrifuged for 5 min. The supernatant was removed and assayed for factor IX esterase activity with benzoyl-l-arginine ethyl ester as substrate or diluted 100-fold with Michaelis buffer and assayed for coagulant activity as described under "Methods." The activity of the 30-min sample was employed as maximal activity, and the percentage of activity of each sample was determined from a standard curve employing the 30-min sample.

A possible cleavage of a peptide bond in factor IX after activation by the protease from Russell's viper venom was tested by carrying out sodium dodecyl sulfate-polycrylamide gel electrophoresis experiments on aliquots of the reaction mixture. Fig. 2 shows the gel pattern for factor IX before and after activation. Factor IX migrates as a single polypeptide chain in the absence or presence of 2-mercaptoethanol (Gels 1 and 2, respectively). After activation, a protein band is observed with the same migration as factor IX (Gel 3). These experiments indicate that the activated factor IX (referred to as factor IX₃₃) has essentially the same molecular weight as its precursor. In the presence of 2-mercaptoethanol, factor IX₃₃ appears as two bands of smaller molecular weight (Gel 4). These experiments suggest that the activation of factor IX by the protease from Russell's viper venom results from the hydrolysis of a single internal peptide bond. Factor IX₃₃ migrates at a rate identical with factor IX (Mₚ = 55,400), but contains two chains of similar size held together by a disulfide bond(s). The migration of the faster moving band (referred to as the valine chain) is the same as the heavy chain of factor IX₃₃ formed in the presence of factor XI₃₃ (Gel 6). The molecular weight of this polypeptide chain was found to be 27,300 by sedimentation equilibrium (7).

In addition to the major protein band seen in the gel of factor IX₃₃, a very minor band is evident with an apparent lower molecular weight (Gel 3, Fig. 2). This minor band migrates in the same position as factor IX₃₃ formed in the presence of factor XI₃₃ (Gel 5) and has been called factor IX₃₃. Factor IX₃₃ and factor IX₃₃ can be separated by gel filtration on a column of Sephadex G-100, and both possess esterase activity. Factor IX₃₃ was observed in all preparations of factor IX₃₃, but accounted for less than 5% of the total factor IX₃₃ observed on gels after activation with the protease from Russell's viper venom.

**Characterization of Two Chains of Factor IX₃₃**—The above experiments indicate that the protease from Russell's viper venom activates factor IX by a mechanism different from that catalyzed by factor XI₃₃. Thus, it was important to characterize the two chains of factor IX₃₃ in order to identify the specific peptide bond which was split during the activation reaction. Accordingly, factor IX₃₃ was reduced, pyridylethylated, and chromatographed on SP-Sephadex (Fig. 3). Two major peaks were observed. The protein fractions in each peak region were pooled separately, desalted on a Sephadex G-50 column, and lyophilized. The sodium dodecyl sulfate-polycrylamide gel electrophoresis patterns of the two peaks are shown in Fig. 4. Gel 1 is Peak II from the SP-Sepahdex column and corresponds to the valine chain or faster moving component of reduced factor IX₃₃. Gel 2 is a mixture of Peak I and Peak II and corresponds to the doublet seen for reduced factor IX₃₃ (Gel 4, Fig. 2). Gel 3 is Peak I and corresponds to the tyrosine chain or slower moving component of reduced factor IX₃₃.

Each chain from factor IX₃₃ was then subjected to automated Edman degradation in order to determine its NH₂-terminal sequence. Peak I contained an NH₂-terminal tyrosine residue, and no other amino acids were detected. The NH₂-terminal sequence and equivalents per 25,500 g of glycoprotein were as follows: tyrosine (0.3), asparagine (0.1), serine (not quantitated), and glycine (0.4). This sequence is identical with that of factor IX (7) and indicates that this polypeptide chain is derived from the NH₂-terminal end of the precursor molecule. Peak II contained an NH₂-terminal valine residue, and no other amino acids were detected. The NH₂-terminal sequence and equivalents per 27,000 g of glycoprotein were as follows: valine (0.6), valine (0.8), glycine (0.7), and glycine (0.3). This sequence is identical with that of the heavy chain of factor IX₃₃ formed by factor XI₃₃ (7) and indicates that this polypeptide chain is derived from the COOH-terminal end of the precursor molecule.

1 The method for the separation of the two chains of factor IX₃₃ on SP-Sephadex was initially developed by Dr. Koiti Titani in our department.
FIG. 3. Elution profile for the S-pyridylethyl chains of factor IX, from an SP-Sephadex column. Factor IX (10 mg) was activated with the protease from Russell's viper venom and the S-pyridylethyl derivatives were prepared as described under "Methods." The S-pyridylethyl peptides were then separated by chromatography on a SP-Sephadex C-50 column (1.5 x 30 cm) equilibrated in 9% formic acid, 0.2 M NaOH, and 7 M urea. Elution was carried out by a gradient formed from 150 ml of equilibration buffer and 150 ml of 9% formic acid, 0.8 M NaOH, and 7 M urea (pH 3.8) at a flow rate of 12 ml/h. Peaks I and II were pooled as shown by the bars.

The amino acid compositions of the tyrosine and valine chains of factor IX, were then determined (Table I). The amino acid composition of the tyrosine chain is shown in Column 1 and is essentially identical with the summation of the tyrosine chain of factor IX, and the activation peptide (Column 2). The amino acid composition of the valine chain is shown in Column 3 and is essentially identical with that of the valine chain of factor IX, (Column 4) (7). Furthermore, a summation of the amino acids in the tyrosine chain and the valine chain of factor IX, (Column 5) agrees well with the amino acid composition of the precursor factor IX molecule (Column 6). These data support the conclusion that no activation peptide is split from factor IX when it is converted to factor IX, by the protease from Russell's viper venom. These data also support the conclusion that the activation of factor IX results from the cleavage of a single internal peptide bond in the precursor protein.

The COOH-terminal residue of the tyrosine chain of factor IX, was then examined after incubating this polypeptide with carboxypeptidase A or B. The amino acids released by these two enzymes were then analyzed by the amino acid analyzer. Arginine (1.05 mol/mol of tyrosine chain) and small amounts of serine and lysine were found after incubation with carboxypeptidase B for 24 h at 37°. Only traces of amino acids were released by carboxypeptidase A treatment under essentially identical conditions. These data indicate that the tyrosine chain of factor IX, has an arginine as its COOH-terminal residue. From these data and the NH2-terminal analysis of the valine chain of factor IX, it is evident that the protease from Russell's viper venom hydrolyzes a single, specific arginine-valine peptide bond during the conversion of factor IX to factor IX.

4 This conclusion has been confirmed by the amino acid sequence data of Drs. K. Katayama and K. Titani who have shown the COOH-terminal sequence of the tyrosine chain of factor IX, to be Ser-Arg (personal communication).

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the S-pyridylethyl fragments of factor IX, isolated by chromatography on SP-Sephadex. The two S-pyridylethyl chains were isolated as shown in Fig. 3 and incubated at 37° for 4 h with 2% sodium dodecyl sulfate containing 5% 2-mercaptoethanol and 3 M urea and then applied to 8.5% polyacrylamide gels. Gels 1 and 2 contain 15 μg of Peaks II and I, respectively. Gel 2 contains 15 μg of both Peaks I and II. The anode was at the bottom of the gels.

Comparison of Activation of Factor IX by the Protease from Russell's Viper Venom and Factor XI, - A time curve for the activation of factor IX by factor XI, employing an enzyme:substrate ratio of 1:50 is shown in Fig. 5 (open circles). In this experiment, the activation of factor IX leveled off in approximately 25 min. Factor IX, was the only product formed in this reaction as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Gel 5, Fig. 2). The specific activity of factor IX, in the clotting assay, however, was approximately twice that obtained after activation of factor IX by the protease from Russell's viper venom (open triangles). As previously noted, factor IX, was the predominant product (>95% by sodium dodecyl sulfate-polyacrylamide gels) formed in the activation of factor IX by the protease from Russell's viper venom. The addition of factor XI, to an aliquot of the latter reaction mixture (shown by the arrow) resulted in an immediate increase in clotting activity (solid triangles), and this clotting activity was now essentially identical with that obtained by the activation of factor IX by factor XI,. The conversion of factor IX, to factor IX, in the last experiment was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Factor IX, formed by the protease from Russell's viper venom was also separated from traces of factor IX, by gel filtration on a Sephadex G-100 column (1.6 x 80 cm). This preparation of factor IX, had a specific activity in the clotting
assay of approximately one-half of that obtained by factor IX, plus activation peptide. Furthermore, the coagulant activity doubled upon incubation with factor XI, calcium ions. Esterase activities of each of the purified factor IX, and factor IX, preparations were determined by the method of Roffman et al. (29) as described under "Methods." Factor IX, was hydrolyzed 0.15 μmol of tosyl-L-arginine methyl ester/mg/h and factor IX, hydrolyzed 0.32 μmol/mg/h. Thus, the specific esterase activity of factor IX, was approximately 2 times higher than that of factor IX, plus activation peptide. The addition of 5 × 10⁻³ m CaCl₂ had no influence on the esterase activity. These data indicate that the cleavage of a specific arginyl-valine bond in factor IX by the protease from Russell's viper venom gives rise to factor IX, an enzyme with esterase and coagulant activity. The coagulant and esterase activities, however, are doubled by the cleavage of a second polypeptide bond in the light chain by factor XI, giving rise to factor IX, and the activation peptide.

Identification of COOH-terminal Amino Acid in Tyrosine Chain of Factor IX, and Activation Peptide—In our previous studies on the activation of factor IX with factor XI, two cleavage sites in the precursor molecule were reported (7). In this reaction, factor XI, first hydrolyzed an internal peptide bond yielding an inactive factor IX intermediate (factor IX,). Containing two chains held together by a disulfide bond(s). The heavy chain contained an NH₂-terminal alanine residue. In the second step, an activation peptide was split from the NH₂-terminal end of this chain giving rise to factor IX, plus arginine (0.52 mol/mol of tyrosine chain) was the only amino acid found after incubation with carboxypeptidase B for 24 h at 37°C. Only traces of amino acids were released by carboxypeptidase A. This indicates that the tyrosine chain of factor IX, has an arginine as the COOH-terminal residue. Thus, the conversion of factor IX, to factor IX, is due to the cleavage of a specific arginyl-alanine peptide bond.

In order to further identify the specific bond split in the second reaction, the activation peptide was incubated with carboxypeptidase A or B and the amino acids released were again analyzed by the amino acid analyzer. Arginine (0.31 mol/mol of activation peptide) was the only amino acid found after incubation with carboxypeptidase B for 24 h at 37°C. Only traces of amino acids were released by carboxypeptidase A. These data indicate that the activation peptide has an arginine as the COOH-terminal residue. This is the same COOH-terminal residue found earlier in the tyrosine chain of factor IX, and indicates that the conversion of factor IX, to factor IX, or factor IX to factor IX, is due to the cleavage of a specific arginyl-valine peptide bond.

Effect of Other Serine Proteases on Factor IX—The activation of bovine factor IX by homogeneous preparations of thrombin, factor X, plasma kallikrein, and factor XII, was also examined to compare these enzymes with factor XI, and the protease from Russell's viper venom. In these experiments, a high enzyme:substrate ratio (1:20) was employed. In order to assay for factor IX, in the presence of thrombin or factor X, aliquots of the reaction mixtures were applied to small DEAE-Sephadex columns to separate factor IX, from thrombin or factor X, prior to assay (see "Methods"). As shown in Fig. 6, this conclusion has been confirmed by the amino acid sequence data of Drs. K. Katayama and K. Titani who have shown the COOH-terminal sequence of the tyrosine chain of factor IX, to be Lys-Lys-Leu-Thr-Arg (personal communication).
factor IX, like factor IX$_{a}$, was also readily inhibited by antithrombin III. In these experiments, factor IX$_{a}$ (3.9 x $10^{-4}$ M) was incubated with increasing concentrations of antithrombin III at 37°C for 90 min in the presence of heparin and then assayed for esterase activity. Extrapolation of the inhibition curve to zero activity gave an intercept at approximately 5 x $10^{-4}$ M antithrombin III. These data were essentially identical with analogous experiments carried out with factor IX$_{a}$ (30). These studies provide good evidence for the conclusion that factor IX$_{a}$, like factor IX$_{a}$, forms an equimolar complex with antithrombin III.

A stable enzyme-inhibitor complex was also demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Factor IX$_{a}$ migrates with a molecular weight of 64,000, while antithrombin III has a mobility corresponding to a molecular weight of 55,000. When the two were preincubated employing a small molar excess of inhibitor, the factor IX$_{a}$ band disappeared and a new band with a molecular weight of approximately 100,000 was formed. These data provide additional evidence that a stable enzyme-inhibitor complex is formed and that it is composed of 1 mol of factor IX$_{a}$ and 1 mol of antithrombin III.

**DISCUSSION**

The data presented in this manuscript indicate that bovine factor IX is converted to an enzyme by the cleavage of a specific internal Arg-Val peptide bond. This bond is readily cleaved by the protease from Russell's viper venom, leading to factor IX$_{a}$ (lower pathway, Fig. 6). This reaction is followed by a very slow conversion of factor IX$_{a}$ to factor IX$_{a}$-$\alpha$. Whether the latter reaction is catalyzed by the protease from Russell's viper venom or by factor IX$_{a}$-$\alpha$ is not known. It does occur readily, however, in the presence of factor XI$_{a}$ and calcium ions.

The activation of factor IX by factor XI$_{a}$ and calcium ions involves a two-step mechanism (upper pathway, Fig. 6). In the first step, an Arg-Ala bond is cleaved giving rise to factor IX$_{a}$. This protein has no enzymatic activity. In a second slower step, factor IX$_{a}$ is converted to factor IX$_{a}$-$\alpha$ in the presence of factor XI$_{a}$ and calcium ions. In this reaction, an Arg-Val bond is cleaved giving rise to factor IX$_{a}$-$\alpha$ and an activation peptide. This reduces the molecular weight of the precursor from 55,000 to approximately 45,000. The clotting and esterase activities of factor IX$_{a}$-$\alpha$ and factor IX$_{a}$-$\alpha$-$\alpha$ differ in that factor IX$_{a}$-$\alpha$ has about 2-fold the specific activity of factor IX$_{a}$. Thus, the presence of the activation peptide on the COOH-terminal end of the tyrosine chain of factor IX$_{a}$ decreases the biological activity of this enzyme by approximately 50%. Both factor IX$_{a}$-$\alpha$ and factor IX$_{a}$-$\alpha$-$\alpha$, however, are readily inhibited by antithrombin III forming a 1:1 molar complex of enzyme and inhibitor.

The sequence of the active site of factor IX is homologous with many other serine proteases, including pancreatic trypsin (32). Thus, it seems probable that the basic mechanism for the proteolytic activation of bovine factor IX involves an ion pair formation between the newly formed NH$_{2}$-terminal valine residue and the carboxyl group of the aspartic acid residue adjacent to the active site serine. This activation mechanism would be analogous to that of the pancreatic enzymes (33, 34) which involve a charge relay network (33, 35-37) or a proton relay system (38) in their active sites.

The activation of factor IX by the protease from Russell's viper venom represents the third plasma protein activated by RVV-X. The other two plasma proteins known to be activated by this protease are human and bovine factor X (7, 13, 39-42) and bovine protein C (17). With factor X, a specific Arg-Ile bond is cleaved, and with protein C, a specific Arg-Val bond is cleaved during the activation reaction. RVV-X is different from a second protease in Russell's viper venom which activates factor V (16).
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In the present experiments, no activation of bovine factor IX was observed by thrombin. These results are in contrast to those published recently by Irvin and Mammen (9). The reasons for these differences are not known. Also, essentially no activation of bovine factor IX was observed by factor X, IX was observed by thrombin. These results are in contrast to the earlier data of Kalousek et al. (8). In this reaction, factor IX was separated from factor X before assaying for factor IX, coagulant activity. This was essential since factor X has a specific activity approximately 300 times greater than factor IX.

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