Rabbit Plasma Inhibitor of the Activated Species of Blood Coagulation Factor X

PURIFICATION AND SOME PROPERTIES*

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SUMMARY

A naturally occurring inhibitor of activated blood coagulation Factor X has been isolated from pooled rabbit plasma and purified by the combination of Sephadex G-200 gel filtration, DEAE-Sephadex A-50, and DEAE-cellulose chromatography. On microzone electrophoresis the purified product traveled as an α2-globulin, and on 7.5% polyacrylamide gel disc electrophoresis at pH 9.5 it traveled as a single component close to transferrin. With exclusion chromatography on Sephadex G-75, G-100, and G-200 it emerged in the same elution volume as crystalline bovine serum albumin. On analytical disc electrophoresis, the inhibitor stained positive for glycoprotein with the periodic acid-Schiff technique. It contained 4.1% hexose, 4.6% sialic acid, and was soluble in 2.5% but not in 5% trichloroacetic acid. Greater than 80% of its original activity persisted at 56° in the 1st hour and gradually diminished to 20% by the 6th hour. The pH optimum of the inhibitor activity was between 7 and 9, and the activity was most stable at pH 6 to 8. Optimum inhibitor activity was at 37° and nondetectable at 1°. Preparative disc electrophoresis of the purified inhibitor on 15% polyacrylamide gel at pH 8.1 caused extensive aggregation of the eluted inhibitor protein with a concomitant total loss of biological activity within 48 hours of storage, even at -60°.

During the coagulation of mammalian blood in vitro some of the clotting factors essential for the formation of fibrin become activated. Two of these activated clotting factors, activated Factor X1 (autoprothrombin C) and thrombin, are absent from serum, while others, such as Factors XII and XI, persist in the serum in their activated form.

Activated Factor X (the enzyme responsible for the activation of prothrombin to thrombin (6)) does not clot fibrinogen, has not been shown to be adsorbed by fibrin, and does not lose its activity in the presence of thrombin. In fact, abundant activated Factor X is found in crude preparations of thrombin, and these two activities have been separated from each other (7, 8). Therefore, the rapid disappearance of activated Factor X, within a few minutes after plasma clots, may be attributed to the presence in blood of a naturally occurring blood clotting antagonist. The disappearance of activated Factor X upon its addition to bovine serum has been previously reported by others (9). This observation has been confirmed in our laboratory and the plasma fraction responsible for the action (activated Factor X inhibitor) has been isolated and partially purified from human blood (10).

In the present communication we describe a technique for the large scale isolation and purification from rabbit plasma of the inhibitor to activated Factor X together with data concerning some of its physicochemical properties.

EXPERIMENTAL PROCEDURE

Materials

Nonheemolyzed pooled rabbit plasma was obtained from Pel-Freez Biologicals, Rogers, Arkansas. The blood was collected in 0.1 M sodium oxalate, (9 parts blood to 1 part anticoagulant, v/v). Plasma was frozen in 1-liter plastic bottles and shipped to us in Dry Ice. Barium sulfate (x-ray diagnostic grade) was obtained from Merek and Company, Rahway, New Jersey, and used without further treatment. DEAE-cellulose (Selectaell), type 20, lot 1398, 1.17 meq per g of exchange capacity was a product of Carl Schleicher and Schuell Company, Keene, New Hampshire. Sephadex gels and six
different lots of DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey. Polyvinylpyrrolidone used as a 40% solution adjusted to pH 7.0 with 1 N NaOH, was purchased from Aloë Scientific Company, St. Louis, Missouri. Crystalline bovine serum albumin, cytochrome c (horse heart, type III), trichloroacetic acid, tris-(hydroxymethyl)aminomethane and Tris-nucleate were products of Sigma Chemical Company, St. Louis, Missouri. Bovine activated Factor X was purified, as described previously (8), from thrombin topical, Parke-Davis Company, Detroit, Michigan. Seitz filter pad, grade 7, was obtained from Carlson-Ford, Lancashire, England. Bovine Factor X-deficient plasma (Seitz) was prepared as described (11), and cephalin (crude phospholipids) was isolated from human brain (19). Visking dialysis tubing was boiled in 1% EDTA and rinsed in deionized water before use. The ultrafiltration units and the Dia-Flo membranes PM-10 lot 12, size 43 mm and lot 13, size 62 mm were obtained from Amicon Corporation, Lexington, Massachusetts. Trypsin, two times crystallized (TR 6187) was the product of Worthington Biochemical Corporation, Freehold, New Jersey.

Methods

A unit of activated Factor X is defined as the activity that would evolve from 1 ml of normal human plasma, when Factor X is fully activated by an optimum amount of Russell's viper venom. Accordingly, it was established that 1 unit of activated Factor X gave the same clotting time (in the absence of the venom) as 1 unit of plasma Factor X, when the latter was measured in the system containing venom. In the present study activated Factor X was determined by methods of a system identical with the Bachmann assay for plasma Factor X (11) with the same cephalin concentration except that the Russell's viper venom was omitted. In constructing the calibration curve for activated Factor X, however, the Bachmann Factor X assay method using the venom was employed. Normal human plasma served as a control. The 280-nm absorbing material was monitored either with a Beckman DB-G spectrophotometer at 25°C or at 4°C before packing of the columns.

It was brought to our attention a year ago by Dr. P. G. Barton, Department of Biochemistry, University of Alberta, Edmonton, Canada, that contrary to our previous experience, he was unable to obtain a high yield of activated Factor X with high specific activity from Parke Davis thrombin topical preparations. His observation was later confirmed in our laboratory. Correspondence with Mr. C. J. Campbell of Parke-Davis and Company, Detroit, Michigan, revealed that since 1968 all thrombin topical preparations have been prepared by an improved method that markedly reduced the amount of activated Factor X contaminating the thrombin products. It is estimated that the level of activated Factor X activity in the new thrombin topical products is approximately 25% of that used in our reported study. We wish to thank Mr. Campbell of Parke-Davis and Company for providing the generous supply of their out-dated thrombin topical preparations manufactured prior to 1968 for the purpose of isolating the activated Factor X with high specific activity used in the present study.

Microzone Electrophoresis—Electrophoresis on cellulose acetate strips was performed at pH 8.6 with a Beckman model R-101 apparatus, using barbital buffer and a constant voltage of 250 volts, at room temperature for 20 min. Ponceau S stain was employed. All reagents used in this technique were obtained from Beckman Instruments, Inc., Palo Alto, California.

Analytical Polyacrylamide Gel Disc Electrophoresis— Disc gel electrophoresis (16) was carried out with a Canaco Model 12 apparatus, and all the reagents used in this technique were supplied by Canal Industrial Corporation, Rockville, Maryland. In all experiments the protein samples were loaded in 40% sucrose. Amido-Schwarz was employed in the staining procedure and samples were left in it for 1 to 24 hours. Electrophoretic destaining was used. The periodic acid-Schiff staining procedures for glycoproteins were performed by two different methods (17, 18).

Preparative Polyacrylamide Gel Disc Electrophoresis—This was performed with the Canaco unit and all reagents used were from the same supplier. The following modified formulae for the preparation of the various reagents were kindly supplied by them. The nomenclature designated for each solution corresponds to that used in their operation manual for preparative disc electrophoresis. Reagent A (pH 7.6) was comprised of 11.7 g of imidazole, 48 ml of 1 N HCl, and 0.24 ml of N,N',N',N'-tetramethylethylenediamine. The mixture was dissolved and the volume brought to 100 ml with deionized water. Reagent B (pH 5.9) was comprised of 2.93 g of imidazole, 0.46 ml of N,N',N',N'-tetramethylethylenediamine, 48 ml of 1 N HCl. The mixture was dissolved and the volume brought to 100 ml with deionized water. Reagent CN was comprised of 20 g of Prep/Cryl (preparative grade acrylamide), 0.06 g of N,N'-methylenbisacrylamide dissolved and brought to 50 ml with deionized water. Reagent DN was comprised of 7 g of Prep/Cryl, 0.125 g of N,N'-methylenbisacrylamide, dissolved and brought to 50 ml with deionized water. Reagent E was comprised of 0.004 g of riboflavin dissolved in 100 ml of deionized water. Imidazole buffer 10 X (pH 7.8), was comprised of 2 g of imidazole, 8.5 g of glycine dissolved and brought to 1000 ml with deionized water. The addition of 2-mercaptoethanol is optional. Elution buffer was 1:8 dilution of Reagent A in deionized water.

The preparative electrophoresis was carried out at 20°C using a PD2/320 upper column with a 4-em long 15% spacer gel and a 2-em 15% separating gel. The protein samples for electrophoresis were loaded in 40% sucrose.

Measurement of Activated Factor X Inhibitor Activity—Unless otherwise specified, the activity of activated Factor X inhibitor was determined as follows. A test fraction of 0.2 ml (appropriate dilution) and 0.2 ml of buffer (0.1 M NaCl in 0.01 M Tris-HCl, pH 8) were warmed in a 37°C water bath for 30 s. To this mixture was added 0.1 ml of activated Factor X in bovine serum albumin and mixed immediately. At 15 s after the addition of activated Factor X, an initial activated Factor X value was determined by removing 0.1 ml of the reaction mixture and adding it to another test tube containing 0.1 ml of Seitz plasma, 0.1 ml of cephalin (0.2 µg of lipid phosphorus), followed immediately by the addition of 0.1 ml of CaCl2 (0.025 M). Another subaliquot of the reaction mixture was tested for residual activated Factor X activity 10 min later. The clotting times plotted in Figs. 1 through 5 represent the differences between the initial and final sample values. In most instances, test fractions containing high activity required dilution in 0.1 m...
Activated Factor X Inhibitor

Purification of Inhibitor

**Step I: Preparation of Plasma Concentrate**

One-liter batches of freshly frozen rabbit plasma in polystyrene bottles were allowed to thaw overnight at 4°C. The plasma was clarified by centrifugation at 20,000 × g for 15 min at 4°C. It was then treated with barium sulfate (100 mg of BaSO₄ per ml of plasma) at room temperature with continuous mechanical stirring for 30 min. The BaSO₄ was removed by centrifugation at 20,000 × g for 10 min at 4°C, the plasma decanted, and the BaSO₄ treatment repeated once more. The plasma was then rapidly passed through an asbestos filter pad in a Whatman No. 1 filter paper. The filtered plasma, containing about 60 g of protein, was brought up to 400 ml with 0.145 M NaCl and after having warmed to room temperature it was then placed on a column (10 × 100 cm) of Sephadex (Pharmacia K100/100) previously equilibrated with the eluting solution of 0.145 M NaCl at room temperature (26°C). The flow rate was controlled by a hydrostatic head at 150 ml per hour and fractions of 25 ml each were collected.

**Concentration of Crude Inhibitor**—The inhibitor fractions were pooled, then dialyzed against three changes of 4 liters of 0.04 M NaCl at 4°C for 24 hours. The dialyzed fraction was then concentrated by lyophilization.

**Step II: Gel Filtration on Sephadex G-200**

**Chromatographic Procedure**—The plasma concentrate equivalent to 1 liter of the original material was reconstituted to 250 ml with deionized water and dialyzed against two changes of 4 liters of 0.04 M NaCl at 4°C over a period of 24 hours. The dialyzed plasma concentrate was clarified by centrifugation at 20,000 × g for 45 min at 4°C. Usually a small fatty layer floated on top of the centrifuged plasma. This layer was discarded by carefully decanting the plasma and filtering it through Whatman No. 1 filter paper. The filtered plasma, containing about 40 g of protein, was brought up to 400 ml with 0.145 M NaCl and after having warmed to room temperature it was then placed on a column (10 × 100 cm) of Sephadex (Pharmacia K100/100) previously equilibrated with the eluting solution of 0.145 M NaCl at room temperature (26°C). The flow rate was controlled by a hydrostatic head at 150 ml per hour and fractions of 25 ml each were collected.

**Concentration of Purified Inhibitor**—The inhibitor fractions were pooled and concentrated at 4°C by dialysis against 40% polyvinylpyrrolidone, pH 7.0.

**Step III: Chromatography on DEAE-Sephadex A-50**

**Preparation of Column**—The DEAE-Sephadex A-50 was allowed to swell for 48 hours at room temperature in 0.05 M NaCl-0.1 M Tris-HCl, pH 8.32, and then was packed in a Lucite column with a polyethylene disc base. A layer of preswollen Sephadex G-25 about 1 cm thick was first poured into the column, allowed to settle, and then followed by the DEAE-Sephadex. Another 1 cm layer of Sephadex G-25 was stacked on top of the DEAE-Sephadex bed. The Sephadex G-25 at the bottom of the bed facilitated a good flow rate and the top layer prevented disturbance of the DEAE Sephadex bed when the protein fraction was being applied. Fresh DEAE-Sephadex was used for each chromatographic procedure in the present study.

**Chromatographic Procedure**—For each DEAE-Sephadex chromatograph, lyophilized material from two separate gel filtration runs as in Step II were pooled and reconstituted to 80 ml with 0.05 M NaCl in 0.1 M Tris-HCl, pH 8.32. This material was then dialyzed against three changes of 4 liters of the same buffer at 4°C for 48 hours. The entire fraction, containing approximately 4 to 6 g of total protein in less than 100 ml, was chromatographed on a DEAE-Sephadex A-50 column (4 × 50 cm) previously equilibrated with 0.05 M NaCl in 0.1 M Tris-HCl, pH 8.32, at room temperature. The flow rate was 60 ml per hour under hydrostatic pressure and fractions of 12 ml were collected. Chromatography was performed by stepwise increase in ionic strength of the buffer.

**Concentration of Partially Purified Inhibitor**—The fractions with inhibitor activity were pooled, dialyzed against six changes of 4 liters of deionized water during a 3-hour period at room temperature, and lyophilized.

**Step IV: Chromatography on DEAE-cellulose**

**Preparation of Column**—The dry cellulose was sieved to obtain the 60 to 100 mesh for packing the column. It was pretreated as described (8) and then equilibrated with the initial buffer to be used for the chromatographic procedure. Packing of the cellulose in a glass column was performed at room temperature.

**Chromatographic Procedure**—The entire lyophilized, partially purified inhibitor fraction from Step III was dissolved in less than 5 ml of deionized water and dialyzed for 24 hours against two changes of 4 liters of the buffer used for conditioning the DEAE-cellulose. The dialyzed fraction was centrifuged at 35,000 × g at 4°C for 1 hour. For each chromatograph to 3 ml of the fraction, containing 35 to 70 mg of protein, were applied on a DEAE-cellulose column, 1 × 35 cm, previously equilibrated at 4°C with 0.02 M NaCl in 0.1 M Tris-HCl, pH 8.32. A linear salt gradient elution technique was adopted with the reservoir containing 150 ml of 0.10 M NaCl in 0.1 M Tris-HCl, pH 8.32, and the mixing chamber containing 150 ml of 0.02 M NaCl in 0.1 M Tris-HCl, pH 8.32. The flow rate was maintained at 7 to 10 ml per hour with a hydrostatic head, and fractions of 3 ml each were collected.

**Concentration of Purified Inhibitor**—The inhibitor fractions were pooled and concentrated at 4°C by dialysis against 40% polyvinylpyrrolidone, pH 7.0.

**Step V: Gel Filtration on Sephadex G-200**

**Chromatographic Procedure**—Of the concentrated fraction (3 ml) were chromatographed on a Sephadex G-200 (100 to 200 mesh size) column, 2.5 × 50 cm, at 4°C previously equilibrated with the eluting buffer, 0.10 M NaCl in 0.05 M Tris-HCl, pH 8.32. The elution flow rate was controlled by a hydrostatic head at 9 ml per hour, and fractions of 3 ml each were collected.

RESULTS

In principle, the activated Factor X inhibitor was purified from BaSO₄-treated rabbit plasma by the combination of gel filtration on Sephadex G-200, DEAE-Sephadex, and DEAE-cellulose chromatography.

**Preparation of Plasma Concentrate**—The slow thawing of the frozen rabbit plasma at 4°C was highly effective in removing an cryoprecipitable fibrinogen fraction occasionally encountered that would precipitate out during gel filtration. Passing the BaSO₄-treated plasma through an asbestos (Seitz) filter helped to eliminate some existing bacteria, since the blood was collected under nonsterile conditions in the supplier's abattoir. It was found that Seitz filtration of the plasma did not have any effect on the activity of the inhibitor.

**Gel Filtration on Sephadex G-200**—As shown in Fig. 1, the inhibitor activity emerged in the albumin region on Sephadex
G-200. No activity was found in the fractions eluted in the first two protein peaks. Concentration of the inhibitor by lyophilization resulted in less than a 10% loss of the activity.

**DEAE-Sephadex Chromatography**—Fig. 2 depicts a typical chromatogram of the inhibitor obtained from Step II on DEAE-Sephadex A-50. Two peaks of protein preceded the elution of the inhibitor. The first protein peak was found to be a γ-globulin and the second protein peak was mainly β-globulin as examined by microzone electrophoresis. No inhibitor activity was detected in either protein peak. A fourth protein peak (not shown in this figure), representing greater than 75% of the total protein chromatographed, could be eluted after the inhibitor peak with 0.2 M NaCl in 0.1 M Tris-HCl, pH 8.32. This fourth peak contained predominantly albumin without inhibitor activity (10). When the inhibitor fractions were pooled and examined by microzone electrophoresis, only a single protein band migrating as an α-globulin was observed. When analyzed at a protein concentration of 200 μg per load on polyacrylamide gel disc electrophoresis, the inhibitor fraction showed a major component which migrated close to transferrin. Three to four additional slow migrating minor components could also be detected on the same gel (Fig. 7B). These bands, however, were diffuse and stained poorly. After lyophilization, another 10 to 15% loss of inhibitor activity was encountered.

**DEAE-cellulose Chromatography**—As shown in Fig. 3A, when 35 mg of the inhibitor from Step III were chromatographed, the activity peaked at Fraction 32. When 35 mg of another preparation from Step III were similarly chromatographed on the same column, the activity peaked at Fraction 26, Fig. 3B, where a definite shouldering of the descending slope could be seen. If 70 mg of the same preparation were then chromatographed on the same column, Fig. 3C, three distinct activity peaks were obtained. The activity peaked at Fractions 18, 24, and 30. That the difference in the eluting positions of the inhibitor depicted in Fig. 3 was not an artifact was supported by the observation that in all three chromatograms, the first protein peak, without inhibitor activity, eluted at Fraction 10. The chromatographic patterns of the inhibitor varied from batch to batch and greatly depended on the duration of storage of the inhibitor initially isolated on the Sephadex gel column before separation on the DEAE-cellulose column. The electrophoretic pattern of the individual inhibitor fraction in all chromatographs was examined by both microzone and analytical polyacrylamide gel disc electrophoresis. In each instance the individual fraction with inhibitor activity showed only a single protein band migrating as an α-globulin of the normal plasma control and the succeeding fractions migrated progressively faster until
Fractions 32 and 34 were in a position between \( \alpha_1 \) and \( \alpha_2 \)-globulins. Control experiments with crystalline bovine serum albumin alone, and bovine serum albumin mixed with the individual fractions, were also performed to rule out possible artifacts. When the inhibitor fractions were pooled and then subjected to electrophoresis, the resulting single band migrated as an \( \alpha_2 \)-globulin. On analytical disc electrophoresis these fractions with activity displayed a single protein band when examined at 100 \( \mu \)g of protein per load. When tested at a protein concentration of 200 \( \mu \)g, one to two additional minor slow migrating components were detected which were diffuse and stained poorly, Fig. 7C. On a few occasions, the fractions displayed a single component even at protein concentration of 300 \( \mu \)g when tested immediately upon elution from the column. This protein band stained positive for glycoprotein with the periodic acid-Schiff reagent. On storage at -20° for only 24 hours these electrophoretically homogeneous fractions now showed one to two additional minor components. This was usually accompanied by some loss of the original activity. Attempts to rechromatograph these fractions on a DEAE-cellulose column resulted in much loss of the activity with fractions exhibiting an increasing number of multiple minor components that also stained positive for glycoprotein. This was usually accompanied by some loss of the original activity. Attempts to rechromatograph these fractions on a DEAE-cellulose column resulted in much loss of the activity with fractions exhibiting an increasing number of multiple minor components that also stained positive for glycoprotein. When the inhibitor fractions were pooled and then subjected to ultrafiltration on the inhibitor is shown below.

Chromatography of the inhibitor on DEAE-cellulose columns two to three times the size used in the experiments in Fig. 3 did not improve the elution profile. In fact, recovery of the inhibitor on these large columns was very poor as compared with recovery on a smaller column. The disc electrophoresis pattern of the fractions from the larger columns exhibited multiple minor components, at times more than the starting material that was chromatographed.

The purified inhibitor withstood further concentration by dialysis against polyvinylpyrrolidone at pH 7 better than by either lyophilization or ultrafiltration. The deleterious effect of ultrafiltration on the inhibitor is shown below.

**Fig. 4.** Sephadex gel filtration at 4° of the inhibitor peak shown in Fig. 3B. The light absorbance of the various fractions was read in a Beckman DB-G spectrophotometer at the following wave lengths: inhibitor, 280 nm; bovine serum albumin, 280 nm; cytochrome c, 410 nm; and Blue Dextran 2000 (Pharmacia), 630 nm. \( \bullet - \bullet \), inhibitor activity; \( \bigcirc - \bigcirc \), light absorbance.

Sephadex Gel Filtrations—Filtrations of the inhibitor fraction after DEAE-cellulose chromatography on columns of Sephadex G-75, G-100, and G-200 gave elution profiles similar to that depicted in Fig. 4. The distribution coefficient of the inhibitor was the same as that of crystalline bovine serum albumin in all instances. Passage of the purified inhibitor through Sephadex G-100 or G-200 usually resulted in eliminating the other minor slow migrating components, Fig. 7D, that were previously present in Fig. 7C. In general, a lowering of the specific activity of the inhibitor was observed.

Comparisons between Effect of Ultrafiltration and Polyvinylpyrrolidone and Dialysis on Purified Inhibitor—Although on analytical disc electrophoresis the inhibitor obtained in Step V frequently showed a single protein band at a protein concentration of less than 200 \( \mu \)g in 100 \( \mu \)l per load, it was decided to examine the inhibitor fraction at a much higher protein concentration, without increasing the volume, to detect trace contaminants. To do this, the inhibitor fractions had to be concentrated. Concentration by ultrafiltration through a Dia-Flo membrane was first selected because it is a simple, fast, and widely accepted method for concentrating dilute protein solutions. Approximately 40 to 50 ml of a 0.1% solution of the inhibitor from Step V in 0.10 M NaCl-0.05 M Tris-HCl, pH 8.52, was clarified by centrifugation at 30,000 \( \times \) \( g \) for 60 min at 4° and then concentrated in an Amicon ultrafiltration cell, either model 52 or 202, at 2°. The appropriate size Dia-Flo ultrafiltration membrane PM-10 was used per the accompanying instructions. Mechanical stirring of the solution filtration under \( N_2 \) (less than 10 p.s.i.) was maintained at the fastest revolution rate that did not create a vortex and thus cause the protein solution to foam. The fraction was usually reduced to 10% of its original volume. Approximately half way through the filtration, the previously clear solution in the cell became faintly cloudy and progressively more dense as the volume was reduced. When the desired concentration was achieved, the inhibitor solution inside the cell was observed to contain fine precipitates that could be removed by centrifugation at 3000 \( \times \) \( g \) for 15 min. The clear supernatant was tested for inhibitor activity and found to contain less than 60% of the total activity initially placed in the cell. Examination of the filtrate indicated total absence of inhibitor activity. Therefore, the decrease of activity in the cell retentate was not caused by loss of the inhibitor in its passage through the membrane. To eliminate the possibility that the loss of activity and the appearance of fine precipitates in the purified inhibitor fraction during ultrafiltration were not caused by excessive stirring during the operation, a different batch of inhibitor was subjected to ultrafiltration without stirring. The flow rate progressively slowed and a higher pressure was required to maintain the previous flow rate. At the end of the filtration the retentate appeared relatively clear until the stirrer was turned on slightly for 1 min, after which the fraction appeared cloudy. Following centrifugation of the concentrate the clear supernatant was tested for inhibitor activity and was found to contain less than 70% of the original activity.

On the other hand, when the purified inhibitor was concentrated by dialysis against 40% polyvinylpyrrolidone at 4°, a process requiring 8 to 12 hours, the inhibitor retained greater than 90% of its original activity without any visible precipitation. When both inhibitor concentrates prepared by ultrafiltration and by polyvinylpyrrolidone dialysis were initially examined at 500 \( \mu \)g of protein per load on analytical disc electrophoresis, multiple protein bands were found in fractions concentrated by both
methods; and, in addition, a striking difference in the electrophoretic pattern between these two fractions was evident. These two samples were then diluted to their preconcentration protein levels and re-examined on disc electrophoresis. The fraction concentrated by ultrafiltration (Fig. 7E) demonstrated much smearing with multiple, heavily stained protein bands. On microzone electrophoresis two protein bands corresponding to α1- and α2-globulin were seen. On the other hand, the concentrate prepared by polystyrene dialysis (Fig. 7E) revealed one major component with two to three additional fine bands close to it. No extremely slow migrating components similar to those in Fig. 7E were seen. On microzone electrophoresis the polystyrene-concentrated inhibitor revealed only one protein band. The preconcentration electrophoretic patterns of these two fractions were identical with that of Fig. 7D.

Preparative Disc Electrophoresis—The purified inhibitor obtained in Step V was concentrated by dialysis against polystyrene for preparative disc electrophoresis. Because the inhibitor was most stable at pH 6 and 8 (see below), it was necessary to perform the preparative electrophoresis as close to these values as possible without affecting either the activity of the inhibitor or the resolving power of the technique. Since the inhibitor was relatively heat stable even at 50° (see below), electrophoresis at 2° was believed to pose no hazard to the protein. The imidazole-HCl system with a running pH of 8.1 was the most reasonable one encountered. Figs. 5 and 6 show the behavior of two different preparations of concentrated purified inhibitor on polyacrylamide gel preparative disc electrophoresis at 2°. In Fig. 5, the distribution of the multiple activity peaks was narrower than that shown in Fig. 6. It is, however, significant to note that in the latter figure, three out of the four minor protein peaks still had some detectable inhibitory activity against activated Factor X. The inhibitor activity eluted from the main peak was found to be very unstable. The specific activity across the peak was fairly constant. At 4° it retained less than 50% of its original activity after 12 hours. It lost greater than 60% of its activity after 24 hours especially at 25° and 60°. Fig. 7 shows the various disc electrophoretic patterns of the inhibitor fraction from the main peak of Fig. 6 before and after storage at -60°. The sample, prior to freezing, appeared as a single, broad and diffuse band, Fig. 7H. Forty-eight hours after storage at -60°, the fraction now showed multiple, heavily stained components, Fig. 7I. At this stage, it had completely lost its biological activity to inhibit activated Factor X. Inhibitor fractions similarly obtained were also labile on storage at -20°, but appeared slightly more stable at 4°. Some of the more concentrated minor components in the disc gel stained lightly with periodic acid-Schiff. Control studies of the stability of the inhibitor from Step V incubated in the same imidazole buffer at 4° and -60° ruled out the loss of activity of the preparative disc electrophoresis fraction as caused by the particular buffer employed. The addition of 0.14 M β-mercaptoethanol to the preparative disc electrophoresis system did not improve the stability of the inhibitor, nor did the prior removal of the ammonium persulfate from the separating gel by electrophoresis (19). The underlying causes leading to the extensive loss of the inhibitor activity after electrophoresis on polyacrylamide gel remain to be determined.

The specific activity and the recovery of an average preparation of activated Factor X inhibitor at various purification steps are listed in Table I. Individual steps in the procedure had been performed 16 to 24 times, and the results as a rule did not deviate markedly from those presented.

Ultraviolet Spectrum—The absorption spectrum of the inhibitor in 0.1 M NaCl, with a path length of 1 cm, measured in a Beckman DB-G spectrophotometer, exhibited an ultraviolet spectrum with a maximum at 278 μm.

Trichloroacetic Acid Solubility—The solubility of the inhibitor in trichloroacetic acid was determined (Table II). It was 100% soluble in 2.5% trichloroacetic acid, but totally insoluble in 5% trichloroacetic acid. Under the same experimental conditions, bovine serum albumin was 13.6% soluble, whereas trypsin was totally insoluble in 2.5% trichloroacetic acid.

Carbohydrate Content—The activated Factor X inhibitor contained 4.1% hexose and 4.6% sialic acid. Other carbohydrates were not determined.

1 Kindly performed by Mr. William A. Frazier and Dr. Craig M. Jackson, Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri.
Fig. 7. Analytical disc electrophoresis on polyacrylamide gel (pH 9.5) of activated Factor X inhibitor pooled fractions at different purification steps. A, Step I (300 μg); B, Step III (150 μg); C, Step IV (200 μg); D, Step V (200 μg); E, fraction from Step V concentrated on Dia-Flo membrane (200 μg); F, fraction from Step V concentrated by polyvinylpyrolidone dialysis (200 μg); G, starting fraction used in the preparative disc electrophoresis (200 μg); H, fraction eluted on preparative disc (200 μg); and I, preparative disc fraction (same as sample H) after 36 hours of storage at -60° (200 μg). Samples A to F were run on 4 cm of 7.5% separating gels, and G to I were on 1 cm spacer gels with 4 cm of 12.5% separating gels. Sample gel was not used in any tests. The sample volume was 100 μl and electrophoresis was conducted from 60 to 90 min at 3 ma per gel. The stained samples were stored in 7% acetic acid for photography.

### TABLE I

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<th>Purification step and fraction</th>
<th>Specific activity range</th>
<th>Recovery range</th>
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<tr>
<td>I. Rabbit plasma</td>
<td>0.042</td>
<td>95-100%</td>
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<tr>
<td>II. Sephadex G-200 pool</td>
<td>0.21-0.27</td>
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<td>III. DEAE-Sephadex pool</td>
<td>4.5-6.0</td>
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<td>IV. DEAE-cellulose pool</td>
<td>7.8-15.6</td>
<td>15-25%</td>
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<td>V. Sephadex G-200 pool</td>
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*One unit of activated Factor X inhibitor activity is tentatively defined as the amount capable of neutralizing 1 unit of activated Factor X in 10 min, at 37° (see "Methods").

### TABLE II

<table>
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<th>Trichloroacetic acid solubility of activated Factor X inhibitor</th>
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<td>The fractions mixed with trichloroacetic acid were left at 20° for 30 min. The 280 nm absorbance was measured before the addition of trichloroacetic acid, and in the supernatant solution after centrifugation at 10,000 X g for 30 min.</td>
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<table>
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<th>ml</th>
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<th>Bovine serum albumin</th>
<th>Assp per ml</th>
<th>Trichloroacetic acid</th>
<th>Assp per ml of supernatant</th>
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*Trichloroacetic acid, 5%.
†Trichloroacetic acid, 10%.

**Effect of pH on Inhibitor Activity**—The pH dependency of the activated Factor X inhibitor activity was performed by incubating at 37°, 0.1 ml of inhibitor (30 μg), 0.4 ml of buffer of indicated pH, and 0.1 ml of activated Factor X (9 units in 10% bovine serum albumin in 0.145 M NaCl) for 20 min, followed by the addition of 0.4 ml of neutralizing buffer (e.g., to neutralize reaction mixture of pH 5, buffer of pH 9 was used, and vice versa). An aliquot of the final mixture was removed and checked for residual activated Factor X activity. Buffer was Tris-maleate, 0.025 M. Control experiments in the absence of the inhibitor indicated complete stability of activated Factor X activity over the pH range studied. The results indicated maximum inhibitor activity at pH 7 to 9. A sharp drop in activity was noted at pH below 7 and above 9.

**Effect of pH on Stability of Inhibitor at 37°**—The pH stability of the activated Factor X inhibitor activity was determined by incubating 0.1 ml of the inhibitor (30 μg) with 0.9 ml of buffer at 37° for 24 hours in a stoppered tube. At the end of the incubation period, the fraction was titrated to near neutrality by adding 1.0 ml of "neutralizing" buffer (e.g., to neutralize an incubation mixture of pH 2, a buffer of pH 12 was added, and vice versa). From this, 0.2 ml was removed and added to a tube containing 0.7 ml of 1% bovine serum albumin in 0.025 M Tris-maleate, pH 7.3, and 0.1 ml of activated Factor X (9 units), then incubated at 37°. At 15 s after the addition of the inhibitor fraction, an initial activated Factor X value was assayed, and another sample tested 30 min later. The percentage of stability was calculated based on a fresh inhibitor control (no prior incubation) taken as 100%. The buffer at pH 2 to 3 was 0.025 M glycine-HCl; at pH 4 to 9, 0.025 M Tris-maleate; and at pH 10 to 12, 0.025 M glycine-NaOH.

Control experiments indicated that the final ionic strength of the buffers did not influence the coagulation assay system, nor
did it influence the stability of activated Factor X in the absence of the inhibitor.

The results indicated that at pH 6 to 8, the inhibitor activity was completely stable under the experimental conditions. At pH 2, it lost 90% of its activity after 24 hours, whereas, at pH 12, only 30% was lost at this time interval. The fraction remained liquid and clear at both pH extremes.

**Influence of Temperature on Inhibitor Activity**—Temperature dependency of the activated Factor X inhibitor activity was performed by incubating at the indicated temperatures, 0.1 ml of inhibitor (30 μg), 0.8 ml of 1% bovine serum albumin in 0.025 M Tris-maleate, pH 7.3, and 0.1 ml of activated Factor X (9 units per ml). At incubation times of 10, 20, and 30 min, an aliquot (0.1 ml) was removed from the incubation mixture and assayed for residual activated Factor X activity as described under “Experimental Procedure.” At 1°, there was virtually no detectable activity during the 30-min period. The inhibitor activity at 37° was the best of the three different temperatures studied at pH 7.30.

**Stability of Inhibitor at 56°**—Three milliliters of inhibitor, 50 μg per ml of 0.01 M Tris-maleate, pH 7.5, were incubated in a stoppered tube in a 56° water bath for 6 hours. At hourly intervals, 0.2 ml of the incubation mixture was removed and tested for inhibitor activity towards activated Factor X, as described under “Experimental Procedure.” The results indicated a greater than 80% stability of the inhibitor at 56° in the 1st hour and at the end of the 6th hour only 20% remained. The fraction remained clear throughout the 6-hour incubation period. At lower inhibitor concentration (15 μg per ml), the stability of the inhibitor at 56° was insignificantly altered.

**DISCUSSION**

Although the inhibitor to activated Factor X is present in both plasma and serum, we have chosen to work with the former for several reasons. By definition, serum is the fluid remaining after whole blood has coagulated. During blood clotting the plasma coagulation factors interact and intermediates and by-products are formed. One of the intermediate products formed is activated Factor X, which is rapidly neutralized by its inhibitor. There is a significant difference between the level of activated Factor X inhibitor in plasma and serum. In addition, it is not known whether the inhibitor in serum, even though active, may not have been modified chemically or physically as a result of interactions between it and other proteins such as plasmin and thrombin. An analogous example is the action of thrombin and plasmin on Factor V (20). Because of these considerations it seemed preferable that activated Factor X inhibitor be isolated from plasma as close as possible to its native form.

Occasionally, the fibrinogen fraction of the pooled rabbit plasma precipitated out during Sephadex gel filtration and clogged the column. This was especially true when the plasma had been previously frozen. Slow thawing of the frozen material at 4° enhanced the initial precipitation of a large portion of the fibrinogen and facilitated the subsequent gel filtration step. The common practice of defibrinating plasma by heating at 56°, or the prior removal of the fibrinogen from the plasma by salting-out with ammonium sulfate was avoided. In the latter technique there was a substantial loss of the inhibitor activity, unless the salt was immediately removed from the plasma.

Gel filtration of the plasma concentrate was best carried out at room temperature (22-26°). At lower temperatures there was a strong possibility of fibrinogen precipitation. Chromatography of the inhibitor peak isolated from Step II on DEAE-Sephadex at room temperature afforded a faster flow rate than at 4°, without any difference in the elution pattern. The elution flow rate was not a determining factor in the effective separation of the activated Factor X inhibitor from the bulk of other proteins, especially the albumin. However, undue slow flow rates, even at low temperature, often resulted in low recovery of the inhibitor, and, therefore, we employed the highest flow rate possible without causing the gel bed to collapse. Although greater than 90% of the fractions containing inhibitor activity always demonstrated a single protein band on microzone electrophoresis migrating as an α-globulin, they invariably showed other minor components on disc electrophoresis.

In contrast to the activated Factor X inhibitor isolated from human plasma reported in our preliminary communication (10), the rabbit inhibitor had less affinity for DEAE-cellulose. The latter could be eluted between 0.03 and 0.045 M NaCl, whereas the human inhibitor required 0.08 to 0.09 M NaCl (10). Furthermore, the rabbit inhibitor was eluted over a broad peak on the DEAE-cellulose column, whereas the human inhibitor was eluted in a narrow peak. The electrophoretic pattern on disc gels of the DEAE-cellulose chromatographed rabbit inhibitor fractions varied considerably. Homogeneity of the individual fraction containing high activity could be obtained, provided that the starting material for this step was chromatographed with a minimum of delay from the time the pool was obtained from the DEAE-Sephadex run. However, even if minor components were present in the peak fractions, the total contamination was minute. These minor components could be produced even in fractions that were previously homogeneous, but had been stored frozen for a short period. This suggested protein denaturation. Rechromatography of these fractions on DEAE-cellulose invariably created a greater number of minor bands on subsequent disc electrophoresis. That failure to obtain a homogeneous species on rechromatography was not related to oxidation of the protein by the ammonium persulfate present in the polymerized gel was established by removing the catalyst (19) and adding 2-mercaptoethanol to the electrophoresis system. Thus, if the minor components were indeed denatured protein from the inhibitor, denaturation must have occurred during or after ion exchange chromatography. Rechromatography of the DEAE-cellulose column also caused considerable lowering of the specific activity of the inhibitor. Removal of the minor components could be achieved effectively on either a Sephadex G-100 or G-200 column. Again, the specific activity was often lowered, but not to the extent lost by rechromatography on the DEAE-cellulose column. Perhaps the greatest impact on the inhibitor, when it was exposed to purification procedures based on charge effect, was the preparative electrophoresis on polyacrylamide gel. The fresh product traveled as a single but diffuse band on the analytical disc electrophoresis (Fig. 7F). The diffuse pattern suggested that the protein was already undergoing a certain amount of denaturation. Within a short period during storage the fraction underwent extensive denaturation of the protein molecule with a concomitant loss of activity and the appearance of multiple, heavily stained, slowly migrating bands on disc electrophoresis (Fig. 7F).

The failure to detect any casemolytic or tosylarginine methyl ester esterase activity in these purified fractions4 tends to argue

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4 E. T. Yin and S. Wessler, unpublished data.
against possible proteolysis of the polypeptide chains by a contaminating proteolytic enzyme. Proteolysis, however, could have occurred at an earlier stage of purification. Indeed, it is difficult to determine the significance of the occasional electrophoretic heterogeneity seen at high inhibitor protein concentrations of some of the preparations obtained in Steps IV and V. The main concern is in deciding whether the observed heterogeneity reflects the presence of other trace protein contaminants, the products of denaturation or dissociation of the inhibitor protein itself. Based on the evidence from the preparative disc electrophoresis experiments, and the observations on the subsequent appearance of additional minor components in purified fractions previously exhibiting electrophoretic homogeneity, however, it would appear that altered inhibitor protein must be responsible for the observed apparent heterogeneity. Despite the instability of the inhibitor protein, the present purification technique offers an opportunity to obtain a “homogeneous” activated Factor X inhibitor fraction. In this report, the claim for protein homogeneity is based on disc electrophoretic analyses on polyacrylamide gel. We are fully aware of the fact that no single test by itself is sufficient to prove that a final product consists of only one protein or enzyme. Apparent homogeneity by electrophoresis, however, provides better evidence than that obtained by ultracentrifugation. If the molecular species under study is unstable, then the inability to demonstrate homogeneity by either technique does not necessarily indicate heterogeneity. In such a case, the ability to isolate a homogeneous fraction initially and to produce an experimentally induced or “spontaneous” heterogeneity of the same preparation lends strong support to a claim of homogeneity. These demonstrations are presented in this report.

The coefficient distribution ($K_d$) of the purified inhibitor on the Sephadex gel columns is identical with that of crystalline bovine serum albumin. Assuming that the inhibitor is a globular protein, the data suggest that its molecular weight, as judged by molecular sieving technique, is approximately 67,000. However, since the inhibitor is a glycoprotein with a high carbohydrate content, caution should be used in accepting any molecular weight data obtained by this type of exclusion chromatography until confirmations by other techniques are obtained. The true molecular weight may indeed be less than 40,000, because we are able to obtain inhibitor activity in filtrates of some of the purified fractions subjected to ultrafiltration on the PM-30 Dia-Flo membrane. These results, however, are inconstant reproducibly.

The action of the inhibitor on activated Factor X is enhanced several fold in the presence of a trace amount of heparin (21), a type of catalysis reminiscent of the effect of phospholipid in prothrombin conversion at the level of prothrombin activator formation (22). Heparin and heparin-like substances have been observed to exist in mammalian blood and it has been inferred that they participate in regulating normal hemostatic balance (23). Therefore, the influence of heparin on the inhibitor activity coupled with the findings that in the isolated test systems the action of the inhibitor alone on activated Factor X follows a time-dependent course, renders the accurate establishment of a unit of activity in a purified inhibitor fraction difficult until more is known about its relationship with heparin or heparin-like substances.

The results on the pH requirement, pH stability, ultraviolet absorption spectrum, trichloroacetic acid solubility, heat stability, and the temperature dependency of the inhibitor indicate that it possesses properties characteristic of proteins with biological activities. The solubility of the inhibitor in 2.5% trichloroacetic acid suggests that it may be a protein with substantially high carbohydrate content (24). The purified activated Factor X inhibitor was found to contain 4.1% hexose and 4.6% sialic acid. Presumably other carbohydrates were also present, but these were not analyzed.

The above experiments have been performed with the purified inhibitor fraction obtained in Step V of the purification procedure. These fractions all demonstrated homogeneity (single protein band) on disc electrophoresis tested at a protein concentration of approximately 700 μg per gel.

The biological actions of activated Factor X inhibitor, its identity with what is presently termed antithrombin III (progressive antithrombin), heparin plasma cofactor, and its lack of identity to a crude liver homogenate claimed to be capable of neutralizing activated Factor X, are presented in the succeeding reports (25, 26).

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