Studies on Bovine Factor X. I. Large-Scale Purification of the Bovine Plasma Protein Possessing Factor X Activity*

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ABSTRACT: A procedure has been developed whereby approximately 100 mg of factor X protein can be isolated from 100 l. of bovine plasma in a period of 2–3 days. The final product is greater than 16,000-fold purified relative to the starting plasma. It is homogeneous by both velocity and equilibrium sedimentation in the ultracentrifuge, shows only a single component when examined by acrylamide gel electrophoresis using a discontinuous buffer system, and exhibits constant specific activity across chromatographic effluent peaks under conditions of both ion-exchange chromatography and gel filtration. Further chromatography by both ion exchange and gel filtration does not result in an increase in the specific activity of the factor X when the activity is assayed by both blood clotting techniques and the ability of the active enzyme form of factor X to hydrolyze *p*-toluenesulfonyl-L-arginine methyl ester. Isolation of bovine factor X was accomplished by adsorption of factor X onto barium sulfate, followed by extensive washing of the barium sulfate with a dilute sodium citrate containing saline solution, and finally by desorption of the factor X and other coagulation activities from the barium sulfate using a more concentrated citrate solution. The factor X was separated from the other blood coagulation activities present in the protein solution eluted from $BaSO_4$ by selective adsorption onto diethylaminoethylcellulose. The factor X was eluted from the diethylaminoethylcellulose by gradient elution. Purification was completed by gel filtration on Sephadex G-100. During development of the purification procedure, it was found to be necessary to include diisopropylfluorophosphate in the buffer solutions used in chromatography. Failure to include this inhibitor in solutions of factor X during purification resulted in the generation of electrophoretically distinguishable artifact degradation products of the factor X molecule.

In spite of the large volume of research and number of publications pertaining to the components of the process of blood coagulation, especially those concerned with the conversion of prothrombin into thrombin, there is little agreement with respect to the nature or origin of these species. Discussion of the components of blood coagulation is further complicated by the existence of three conceptually different bases for definition and investigation of the process. In one, experimental design and effort have been directed toward isolation of a substance termed "thrombokinase" (Milstone, 1964), the agent postulated by Morawitz (1905) to be responsible for the conversion of prothrombin into thrombin. The second context has its foundation in the observations of Seegers (1964) that activities capable of converting prothrombin into thrombin could arise from apparently homogeneous preparations of prothrombin itself; this school has devised an independent system of nomenclature. The third conceptual model arose from the observation by clinical hematologists of operationally distinguishable hemorrhagic states. Experiments based upon activity assays defined in this last system have implicated some of these factors in the process of prothrombin conversion (Biggs and Macfarlane, 1962).

Although it seems to be generally accepted that some of the differently labeled components of the prothrombin–thrombin conversion process are equivalent, determination of these equivalences is complicated by the necessity for distinguishing between identity of activities and identity of the biological molecules assumed to be responsible for the observed activities. This distinction is necessitated by the observations presented below and those cited in recent publications on the isolation of prothrombin (Aronson, 1966), namely that degradation can occur without detectable change in the activity of the species under the usual conditions of assay. Specific consideration of this situation will be presented in the discussion of the results of this investigation.

This study is based on the model derived from observations of hemorrhagic defects. From the observation of a clinical bleeding tendency, it is inferred that an activity present in the plasma of normal individuals is lacking (missing) or quantitatively reduced in the defective plasma. A particular defect is differentiated from other known hemorrhagic states by the observation that mixtures of plasmas with different defects will exhibit a normal pattern of clotting behavior, whereas a mixture of

* From the Department of Biochemistry, University of Washington, Seattle, Washington 98105. *Received March 1, 1968.* This investigation was supported by grants from the American Cancer Society (P-291) and the Life Insurance Medical Research Fund.

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two samples of plasma possessing the same activity deficiency will not show such correction. It is thus possible to define distinguishable activities necessary for normal clotting behavior as a result of the absence of the activity in the abnormal plasma. Such a definition provides a relatively simple basis for study of the activity deficiency state and provides an assay useable in isolation of the corrective factor from normal plasma.

Factor X was the label given by the International Committee on the Nomenclature of Blood Clotting Factors to the activity missing or reduced in three patients independently reported by Graham and Hougie (1956), Telfer *et al.* (1956), and Bachmann *et al.* (1957). The suggestion by Hougie *et al.* (1957) that Russell's viper venom be used in conjunction with asbestos-filtered plasma to assay factor X activity led to a detailed examination of this possibility by Bachmann *et al.* (1958) and resulted in the assay procedure which actually defined the activity of interest in this study. Using immunochemical techniques, Macfarlane (1961) established quite conclusively that the clotting element acted upon by a component of the viper venom was that possessing factor X and not one of the other known clotting factors.

Although procedures for the isolation of bovine factor X had been published when this study was initiated (Hougie and Bunting, 1960; Duckert *et al.*, 1960; Esnouf and Williams, 1962; Papahadjopoulos *et al.*, 1964), it was found that extensive modifications were necessary in order to obtain reproducibly in sufficient quantity for chemical and physiochemical characterization a protein preparation which would be homogeneous by the currently available criteria.

Materials and Methods

Common inorganic chemicals were reagent grade and were purchased from major domestic suppliers.

Barium sulfate, suitable for X-ray diagnosis (Merck & Co., Rahway, N. J., lot no. E 39785), was used exclusively.

DEAE-cellulose (Selectacel, type 20, 1.0-mequiv/g capacity, lot 1544, Schleicher & Schuell, N. Y.) was washed prior to use successively with 1 N NaOH, distilled H_2O, 1 N HCl, distilled H_2O until neutral, then with 95% ethanol, and finally with absolute ethanol. The ethanol was removed *in vacuo* using a water aspirator for 24–48 hr.

Sephadex G-100 and A-50 (Pharmacia Fine Chemicals, Piscataway, N. J.) were of the beaded form and were equilibrated according to the instructions supplied by the manufacturer.

Russell's viper venom (Stypven, Burroughs Wellcome Co., Tuckahoe, N. Y.) was used in factor X clotting assays.

DFP (Merck & Co., Rahway, N. J.) was used as a 1 M solution in anhydrous 2-propanol.

TAMe[1] (Calbiochem, Los Angeles, Calif., A grade) was used as provided.

BSA (A grade) was obtained from Calbiochem, Los Angeles, Calif., and was the product of Pentex, Inc., Kankakee, Ill.

Bovine blood was obtained locally and was handled in plastic containers. Immediately after being stunned, the animals were hung by the hind legs and the great vessels were cut directly above the heart. A polyethylene bucket was held into the incision and 8–16 l. of blood was collected from each animal within a period of 1–3 min. The blood was transferred to a 25-gal plastic barrel, and approximately one volume of 0.1 M sodium oxalate was added for each nine volumes of blood. The blood and anticoagulant solution were mixed by vigorous hand stirring using a large spatula as a paddle. Very rarely blood collected in this way clotted extensively; in such cases the entire contents of the barrel were discarded. Small clots, however, were frequently detected during transfer of the fresh blood from the collecting bucket to the large barrel. In these cases, the small clots were skimmed from the pooled blood and the bulk of the blood was retained. Approximately 250 gal of blood was collected at each visit to the abattoir and transported at ambient temperature to the laboratory.

The plasma was separated from the cellular components of the blood by centrifugation using a continuous-flow, disk-type separator (DeLaval BRPX 207) with a bowl speed of 6000 rpm. Separation and recovery were optimum when an 86-mm specific gravity disk was used at an input flow rate of 2–3 gal/min. The blood was pumped to the centrifuge by a variable speed peristaltic pump (Vanton "Flex-i-liner," Model XB-T60A) constructed of Teflon and Viton. Foaming of the plasma was minimized by maintaining a back pressure of 60 psi with the separator exit port valve. No control of the temperature was attempted during centrifugation; it therefore ranged from 30 to 40°. The plasma was collected in 1-gal polyethylene jars and was immediately frozen at −28°. The frozen plasma was stored at this temperature.

Approximately 24 hr prior to commencement of the next step of the process, the plasma was removed from the freezer and permitted to thaw in the containers at 28°. As some of the plasma protein failed to redissolve at this temperature and a balloon-like mass of protein was found in each of the containers, the plasma was filtered by gravity force through a Büchner funnel fitted with a pad of glass wool.

Fibrinogen was prepared from bovine plasma by the procedure of Blombäck and Blombäck (1956). Determinations of the amount of protein in the fibrin clot formed after clotting with commercial thrombin (Parke Davis Co.) and of the amount of protein in the solution after clotting, corrected for the total thrombin added, indicated that the fibrinogen was 94–97% clottable, which is in agreement with the findings of Blombäck and Blombäck (1956).

The Cephalin used in routine assay of factor X was a chloroform extract of human brain acetone powder prepared according to the procedure of Bell and Alton (1954).

Factor V activity has been assayed by the procedure

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: TAMe, *p*-toluenesulfonyl-L-arginine methyl ester; BSA, bovine serum albumin.

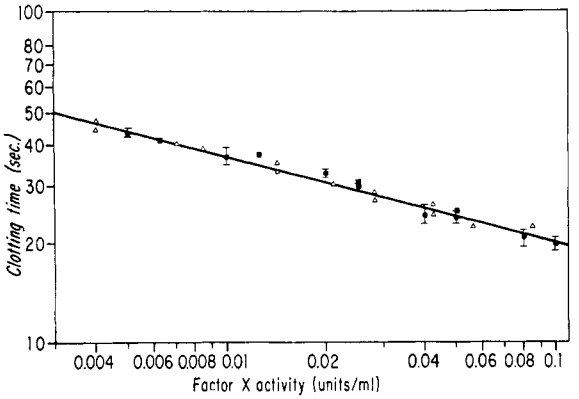

FIGURE 1: Factor X assay dilution curve prepared using reference human plasma and purified factor X. The logarithm of the clotting time was plotted *vs.* the logarithm of the protein concentration for the purified factor X. By subsequent translation along the abcissa the two curves were made superimposable. (△) Points obtained by dilution of purified factor X. (■) Points obtained by serial dilution of reference human plasma. (●) Points obtained by individual dilution of reference human plasma.

of Kappeler (1955) which uses aged human plasma as the factor V deficient substrate plasma.

Prothrombin activity has been determined by the one-stage method of Koller *et al.* (1951).

Thrombin activity has been determined by its ability to clot fibrinogen. Fibrinogen (0.2 ml; 2 mg/ml), $CaCl_2$ (0.1 ml of 0.025 M), and the sample of interest (0.1 ml) were mixed in a 10 × 100 mm culture tube at 37° and the clotting time was determined. As clotting times were usually not less than 5 min and usually more than 1 hr, the mixture was examined initially at 1-min intervals for 10 min and thereafter at intervals of at least 20 min. In a single instance, the unit of thrombin activity was that amount of thrombin which catalyzed formation of a solid clot in 15 sec.

Solutions to be assayed for clotting activities have been diluted with Michaelis buffer (3.6×10^{-2} M sodium acetate, 3.6×10^{-2} M sodium barbital, and 1.45×10^{-1} M NaCl adjusted with HCl to pH 7.35) containing 0.1 mg of bovine serum albumin/ml.

Factor X was assayed by the technique of Bachmann *et al.* (1957). No modifications of this procedure have been made in the course of this study; hence, in an even more limited sense, the protein isolated is the species possessing the activity which is defined by this assay procedure.

A calibration or dilution curve is constructed using normal human plasma as the source of factor X. One unit of factor X activity is defined as that amount present in 1 ml of normal human plasma. In this study, the normal or reference plasma has been that of the first author. (Comparison of the factor X activity in this reference plasma with a preparation of pooled human plasma (Coagulation Control, Ortho Pharmaceuticals) indicated that the level was indistinguishable from that generally considered to represent a normal value.) A linear dilution curve is obtained by plotting the logarithm of the number of units of factor X present in dilutions of the reference plasma *vs.* the logarithm of the clotting time. Such a curve is shown in Figure 1.

In order to ensure the minimum requirement that the calibration curves constructed from the reference plasma and by dilution of preparations of purified factor X possess the same slope, the following was done. (1) Separate dilution curves were constructed using reference human plasma and a sample of purified factor X, and the experimental points were plotted on the separate graphs. For the purified factor X, micrograms of protein replaced units as the independent variable. (2) The two graphs were next superimposed and translated along the abcissa as necessary until the experimental points were lying in the same zone along the curves. As required, it was found that the two curves were coincident (see Figure 1).

An additional check was made by mixing equal volumes of a dilution of the reference plasma and of the purified factor X and then assaying this mixture. The apparent activity determined with the reference plasma–factor X mixture, and a dilution of this mixture, was exactly that expected from the addition of the two activities.

In order to maximize the precision when column fractions were being assayed, a single micropipet was used and the total volume of Veronal–acetate–BSA buffer was varied to obtain clotting times within the usable range.

It has been reported (Esnouf and Williams, 1962) that the activated form of factor X possesses the ability to hydrolyze TAMe. Although TAMe is not a unique substrate for activated factor X, this test was used in conjunction with the Bachmann assay because of its utility in quantitative determination of a factor X related activity.

TAMe esterase activity was determined at 37° by continuous titration using a Radiometer TTT lc, SBR-2, TTA-31 pH-Stat assembly. TAMe was used at a concentration of 0.10 M in 0.10 M NaCl and was titrated with 0.018 N NaOH in 0.10 M NaCl. As a result of the relatively large apparent K_m, saturation was not achieved at this concentration. Rates were therefore always measured from the slope of the pH-Stat curve between 30 sec and less than 2 min after addition of the enzyme to the substrate in the titration cell. No deviation from linearity could be detected in this time period. Less than 10% of the substrate was hydrolyzed under these conditions, thus ensuring reproducibility in the determination. Prior to activation of the precursor factor X with the coagulant protein of Russell's viper venom, column fractions were diluted to a protein concentration of approximately 0.2 mg/ml to permit the use of a single aliquot size in the assays of esterase activity.

Protein concentration was routinely determined by measurement of the absorbance at 280 mμ in a Beckman DU or DB spectrophotometer. In the initial stages of the purification procedure, 1 unit of absorbance measured in a 1.0-cm quartz cell was assumed to represent a protein concentration of approximately 1 mg/ml. The extinction coefficient for factor X, expressed as $E^{1\%}_{1\,cm}$ at 280 mμ, was determined from measurements of the absorbance using the Cary 15 spectrophotometer

and the interference optical system of the Spinco Model E ultracentrifuge as a differential refractometer (Schachman, 1959). A refractive index increment of 1.86×10^{-3} (g cm^{-2} $fringe^{-1}$) was assumed (Armstrong *et al.*, 1947). The change in apparent absorbance between 350 and 320 mμ indicated that scattering by the solution was negligible; hence no correction was necessary.

Sedimentation velocity and sedimentation equilibrium examinations of solutions of factor X were made using the schlieren interference optical system of the Spinco Model E ultracentrifuge.

Molecular weight determinations were made using the high-speed technique of Yphantis (1964) and Teller (1965). The fringe pattern was photographed using a Polaroid 312 filter at the light source aligned parallel to the optical axes of the sapphire windows.

Molecular weight averages, M_n, M_w, and M_z, were calculated at each point in the cell and over the entire cell using the IBM 7094–7040 and the program of Teller (1965).

Disc electrophoresis was done by the technique of Ornstein (1964) using the Tris-HCl–glycinate buffer system devised by Davis (1964) for separation of plasma proteins.

Experimental Section

Isolation and Purification of Bovine Factor X. The procedure developed for purification of factor X is described below; experimental data and considerations which form the basis for this method are presented and discussed later on in this paper. A flow diagram outlining the procedure is presented in Figure 2.

Routine isolation of bovine factor X has been carried out on 100–200-l. batches of plasma. For simplicity, the following description is for a preparation with a starting volume of 100 l. All operations were performed at ambient temperature, 20–25°.

BARIUM SULFATE ADSORPTION. $BaSO_4$ (1 kg; 10 g/l. of plasma) was added to the plasma, and the $BaSO_4$ was dispersed by vigorous stirring. This suspension was stirred for at least 30 min to ensure complete adsorption.

The $BaSO_4$ onto which factor X activity was now adsorbed was separated from the plasma by centrifugation using the BRPX-207 separator as described in the Methods section for plasma preparation. Alternatively, the $BaSO_4$ was allowed to settle overnight from the wash solution in the barrel. However, because of the possibility of partial degradation of factor X at this stage (see paper II of this series: Jackson and Hanahan, 1968), the more rapid separation by centrifugation was used routinely.

The prewashed $BaSO_4$–saline slurry was transferred to 1-l. centrifuge cups. After a brief ambient temperature centrifugation at 1300*g* in either an International PR-2 or a Lourdes 30-R centrifuge, the small amount of remaining wash solution was decanted.

The dry $BaSO_4$ cake thus obtained was removed from the centrifuge cups and was finely divided using a high-speed homogenizer (Waring Products Co., Model CB-5). After the $BaSO_4$ cake had been dispersed at high speed,

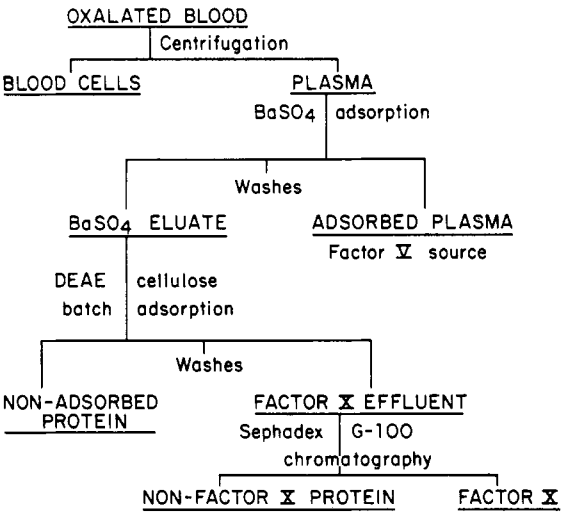

FIGURE 2: Flow diagram of the factor X purification scheme.

the blendor speed was reduced and 2 l. of a wash solution, 0.45% (w/v) in NaCl and 0.001 M in trisodium citrate (pH 7.5), was added. The finely divided $BaSO_4$ was stirred rapidly for 2–3 min. *n*-Octyl alcohol was used to control the foam generated during this procedure. This saline–citrate washing procedure, with separation of the $BaSO_4$ from the wash solution by a brief centrifugation as above, was repeated five to seven times. Washing steps were terminated after the absorbance at 280 mμ of the (0.45 μ Millipore filter clarified) wash solution decreased to less than 0.08 absorbance unit.

Factor X activity was eluted from the washed $BaSO_4$ with 0.06 M trisodium citrate adjusted to pH 5.8 with concentrated HCl. In order to elute the activity efficiently, the "dry" $BaSO_4$ cake from the final wash-centrifugation cycle was first finely divided, with the homogenizer used as described above. Two separate elution steps were performed, each using 5 l. of the citrate elution buffer, or $^1/_{20}$ the initial plasma volume, per elution. In each elution step, the $BaSO_4$ was kept suspended in the eluting solution for at least 30 min. The eluate was separated from the $BaSO_4$ by centrifugation as in the washing process. Occasionally, the $BaSO_4$ was allowed to settle from the eluate overnight; however rapid separation was routine in order to minimize the chance for partial degradation at this stage.

In order to further reduce the chance of degradative changes occurring in the factor X as a result of the action of thrombin, plasmin, or activated factor X possibly present in the $BaSO_4$ eluate, DFP was added to the $BaSO_4$ eluate. A final concentration of 5×10^{-4} M DFP was obtained by the addition of 1 M DFP in anhydrous isopropyl alcohol. All of the steps were carried out at room temperature. Data upon which the final conditions of the elution procedure are based and a discussion of the variability encountered at this stage are given below.

DEAE-CELLULOSE ADSORPTION AND ELUTION. After the factor X activity had been eluted from the $BaSO_4$, further purification could be efficiently achieved by selective adsorption onto DEAE-cellulose. The large volume of solution present at this stage necessitated the use of a batch technique. In order to ensure a reasonably

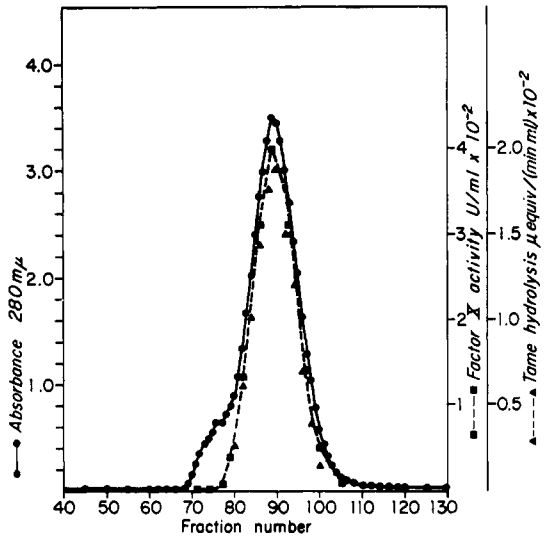

FIGURE 3: Gel filtration of factor X from DEAE-cellulose chromatography on Sephadex G-100. Column, Sephadex G-100, 2.5 × 95 cm; buffer, 0.01 M Tris-HCl–0.50 M NaCl (pH 7.5); sample, factor X from DEAE-cellulose, 21 ml, 7.8 mg/ml; flow rate, 32 ml/hr (upward flow); fraction volume, 2.2 ml; void volume, 154 ml; elution volume, 212 ml; temperature, 20–25°.

uniform product, dry DEAE-cellulose was stirred into the $BaSO_4$ eluate, the factor X adsorbing onto it. The anion exchanger, previously washed and dried, was added in the chloride form (see Materials and Methods). Dry DEAE-cellulose was added to the eluate, 2 mg for each unit of apparent factor X activity present as determined by the Bachmann assay. The pH of the suspension was adjusted to pH 7.1 with 1 M NaOH, and stirring was continued for 15 min. The DEAE-cellulose was rapidly separated from the remaining solution by filtration on a Büchner funnel fitted with a piece of 400-mesh nylon net. The filter cake with its adsorbed protein was next transferred to 600-ml centrifuge cups for batchwise washing. A large portion of the loosely bound protein could be removed by stirring the cellulose first with 0.06 M sodium citrate in 0.05 M Tris-HCl adjusted to pH 6.7 with concentrated HCl, then with 0.06 M sodium citrate, in 0.05 M Tris-HCl (pH 8.0), the volume of each being one-tenth the initial $BaSO_4$ volume. The cellulose was separated from the individual wash solutions by a brief centrifugation at 1370*g* at 20°. The supernatant solution from each wash was discarded, and the sedimented DEAE-cellulose was resuspended in the next wash solution. Upon completion of the final wash, the DEAE-cellulose was transferred to a 2-l. filter flask with 1 l. of 0.08 M sodium citrate, in 0.05 M Tris-HCl (pH 8.0). This slurry was then deaerated using a water aspirator.

The DEAE-cellulose with its adsorbed protein was immediately packed into a Lucite chromatographic column (4.5 × 40 cm) into which a 3–5-cm high column of clean DEAE-cellulose had previously been packed. The packed column was then washed with 0.08 M sodium citrate in 0.05 M Tris-HCl (pH 8.0) until the absorbance at 280 mμ of the effluent solution decreased to less than 0.1 absorbance unit. To achieve this result, 500–1000 ml of buffer was usually required for a column that contained 500 ml of wet cellulose. Subsequently, approximately 100 ml of 0.08 M sodium citrate in 0.05 M Tris-HCl (pH 8.0) containing 0.01 M DFP, as described above, was passed through the column.

Factor X activity was then eluted using a linear gradient in sodium chloride, 0–0.5 M in a buffer mixture containing 0.1 M sodium citrate and 0.05 M Tris adjusted to pH 8.0 with 12 N HCl. Each of the gradient reservoirs contained a volume of buffer two to three times the volume of packed DEAE-cellulose. For the average preparation, the total buffer volume for both gradient chambers was from 1 to 2 l. A flow rate of 30–50 ml/(cm² hr) was maintained during elution.

Fractions of approximately 20 ml each were collected, with the factor X activity eluted in a single peak skewed toward the trailing edge. In routine preparations, the factor X containing fractions were combined immediately after elution and DFP was added to a final concentration of 10^{-3} M. Again all operations were performed at room temperature. The pooled factor X containing solution, approximately 400 ml, was concentrated to a final volume of 10–20 ml using a "Diaflo" ultrafiltration cell (Amicon Corp., Model 400). It is desirable to rinse the concentrator and ultrafiltration membrane with 100 ml of distilled water containing 0.001 M DFP prior to concentration of the sample in order to ensure against possible degradation.

SEPHADEX GEL FILTRATION. Concentrated factor X from the DEAE-cellulose column was warmed and then applied immediately to a column of Sephadex G-200 (2.5 × 95 cm) packed in 0.01 M Tris-HCl (pH 7.5) and 0.5 M NaCl. Gel filtration was carried out in the upward direction (using upward flow adaptors) at a flow rate of 7–10 ml/(cm² hr). Concomitant with this process, the change in buffer permitted the effluent fractions to be assayed easily for both precursor factor X activity by the Bachmann assay and for TAMe esterase activity after total activation of the precursor form of the enzyme with the coagulant protein of Russell's viper venom (Esnouf and Williams, 1962; Jackson and Hanahan, (1968)). An elution profile from such a Sephadex G-100 column is presented in Figure 3.

The specific activity of both activities was essentially constant across the peak, namely, 140 ± 10 units of factor X/mg of protein by the Bachmann assay and 64 ± 5 μequiv of NaOH/(min mg of protein) when assayed with 0.1 M TAMe at pH 7.7 and 37°. Data obtained from a large number of preparations of factor X, each derived from 100 l. of plasma, are given in Table I.

The level of prothrombin activity was not determined routinely on the $BaSO_4$ eluates. However, in preparations in which prothrombin was assayed, the plasma contained activity, about 0.6 unit/ml, with 0.6 unit/ml in the $BaSO_4$ eluate (one-tenth starting volume); yield is about 10% of the original activity. No factor V activity could be detected in the $BaSO_4$ eluates, even at eluate protein concentrations 10–25 times greater than normally used for assaying factor X activity.

Each stage of the preparation was examined by the disc electrophoretic technique of Ornstein (1964). Conditions for electrophoresis have been identical with those

TABLE I: Purification of Bovine Factor X.

Stage	Vol (ml)	Act. (units of X/ml)	Total Act. (units)	Protein (mg/ml)	Sp Act. (units of X/mg)	Yield (%)	Purifcn
Plasma (19)[a]	1×10^5	0.71	7.1×10^4	80[b]	8.9×10^{-3}	100	1
$BaSO_4$ eluate (16)	5×10^3	6.5	3.2×10^4	1.08[c]	6.0	54	600
	5×10^3	1.6	0.8×10^4	0.35[c]	4.5		
DEAE-cellulose batch chromatography (8)	300–400	55	$1.6\text{–}2.2 \times 10^4$	0.40[d]	136 ± 10 58 ± 4 μequiv/(min mg)[e]	18	16,000
Sephadex G-100 (4)	60	242	1.5×10^4	1.7[d]	140 ± 10 64 ± 5 μequiv/(min mg)[e]	14	16,000

[a] Number in parentheses is the number of samples included in the calculation. [b] Long (1961). [c] Determined by the absorbance at 280 mμ assuming $E^{1\%}_{1\text{ cm}}$ 10. [d] Determined by the absorbance at 280 mμ using $E^{1\%}_{1\text{ cm}}$ 12.4. [e] TAMe esterase activity, determined after activation with the coagulant protein of Russell's viper venom.

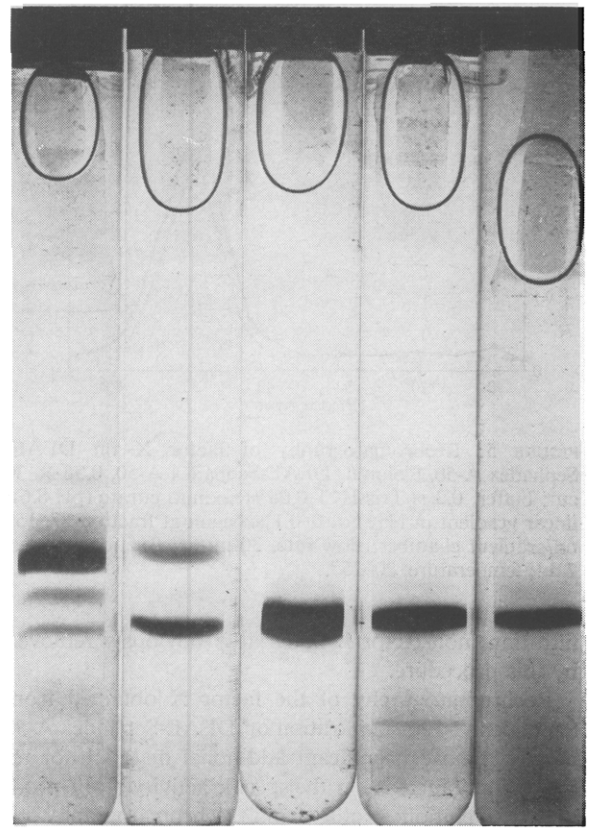

FIGURE 4: Examination by disc electrophoresis of factor X preparations at various stages in the purification scheme. Migration is from top (cathode) to bottom (anode). Samples (from left to right): (1) $BaSO_4$ eluate, 40 μg of protein, factor X specific activity approximately 5 units/mg of protein; (2) $BaSO_4$ eluate, 10 μg of protein plus 5 μg of purified factor X; (3) factor X after Sephadex G-100 gel filtration (fraction 90, Figure 3), 30 μg of protein; (4) factor X from DEAE-cellulose chromatography, the starting material of Figure 3, 20 μg of protein; and (5) factor X after Sephadex G-100 gel filtration (combined fractions, Figure 3), 10 μg of protein.

developed by Davis (1964). No increase in resolution was achieved by increasing the length of the stacking gel, a method which favors dissociation of protein–protein complexes (Ogston, 1946). Photographs of representative gels at each stage are given in Figure 4. Comparison of the gel containing the factor X from the DEAE-cellulose adsorption procedure with those containing the product of Sephadex G-100 gel filtration seems to indicate that a trace component that migrates a relatively short distance into the gel and a species that migrates approximately the same as the bromophenol blue marker are removed by Sephadex G-100 gel filtration. However, disc electrophoretic examination of concentrated material from the first peak seen to elute in the void volume of the G-100 column (Figure 3) indicates that not only is the component seen in the DEAE-cellulose factor X gel removed, but so are three other components with acrylamide gel electrophoretic mobilities between those of the large component(s) and the factor X. Although, as can be seen from the data of Table I, no significant increase in the specific activity of the factor X occurs as the result of Sephadex G-100 gel

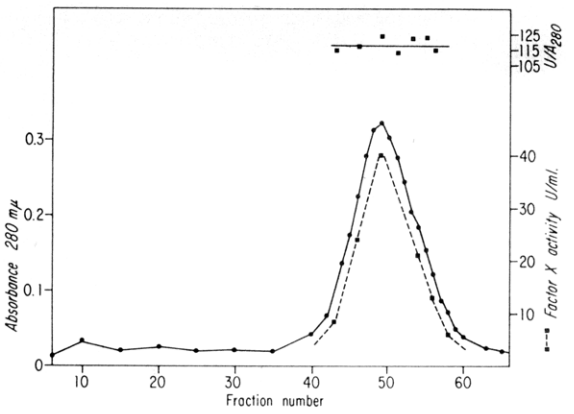

FIGURE 5: Rechromatography of factor X on DEAE-Sephadex A-50. Column, DEAE-Sephadex A-50, 0.95 × 30 cm; buffer, 0.3 M Tris-HCl–0.08 M sodium citrate (pH 8.6); linear gradient in NaCl of 0–0.1 M begun at fraction 27, 150 ml/gradient chamber; flow rate, 30 ml/hr; fraction volume, 2 ml; temperature, 20–25°.

filtration, non-factor X material is obviously removed by this procedure.

Rechromatography of the factor X obtained from Sephadex G-100 gel filtration on DEAE-Sephadex A-50 neither removes significant additional material nor results in any increase in the specific activity of the product. An elution profile from rechromatography on DEAE-Sephadex A-50 can be seen in Figure 5. Similar profiles have been obtained with Sephadex A-50 using a phosphate buffer system.

Complicated elution profiles showing the partially resolvable peaks were frequently obtained after repeated rechromatography of factor X on DEAE-Sephadex A-50. Samples taken from the leading edge of the first peak and the trailing edge of the second peak and gels containing aliquots from both the leading and the trailing edges show only a single band when analyzed by disc electrophoresis. Hence, a phenomenon more complex than inhomogeneity is apparently responsible for double-peak formation. The two factor X species from this apparent partial resolution are also indistinguishable in the Bachmann clotting assay and in their TAMe esterase specific activity. This anomaly and related chromatographic characteristics of factor X are considered in the second paper in this series (Jackson and Hanahan, 1968).

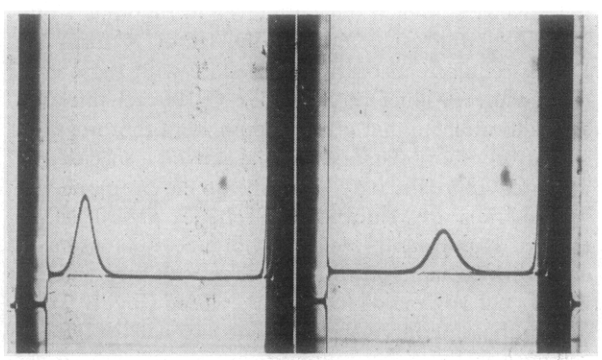

FIGURE 6: Examination of factor X by velocity sedimentation (1) after 64 min and (2) after 160 min (sedimentation is from left to right). Speed, 59,780 rpm; phase-plate angle, 70°; cell, 2°, double sector, sapphire windows; concentration, 6.3 mg/ml; buffer, 0.001 M Tris-HCl–0.10 M NaCl (pH 7.40); temperature, 20.0°.

Analytical ultracentrifugation of factor X yields a single boundary in velocity sedimentation analysis, which, however, appears to be undergoing self-sharpening at the trailing edge. Photographs of the schlieren patterns of the sedimenting boundary are given in Figures 6 and 7. Examination of the diffusion coefficient measured in the ultracentrifuge at 3000 rpm and the concentration dependence of the sedimentation coefficient indicate that the asymmetry of the sedimenting boundary may be the result of the factor X molecule. Sedimentation equilibrium examination of factor X using the high-speed technique and six-place cell of Yphantis (1964) and analysis of the data using the digital computer program of Teller (1965) further support the hypothesis that factor X purified by the technique described is apparently homogeneous. The plot of the logarithm of the concentration *vs.* the square of the radial distance (Figure 8) and the molecular weight averages obtained from sedimentation equilibrium experiments provide no evidence for inhomogeneity, $M_w/M_n = 1.03$, $M_z/M_w = 1.00$, and $M_{sd}/M_w = 1.03$.

A more exhaustive consideration of the hydrodynamic properties of the factor X molecule is given in paper II of this series (Jackson and Hanahan, 1968).

Development of the Purification Procedure. A number of practical laboratory problems were encountered when large-scale purification of factor X was attempted using published procedures. Variability in the yield and specific activity of the factor X at comparable stages in different preparations, occasional total loss of activity resulting from the precipitation of large masses of protein from the $BaSO_4$ eluates, combined with the necessity of handling large volumes of solution made it appear mandatory that the variables associated with each step in the general procedure be examined in detail. Because of the potential utility of this approach in the development of purification schemes for other clotting activities, a detailed description of the optimization experiments is given in this section.

USE OF FROZEN PLASMA. As was stated in the Methods section of this report, plasma was frozen and stored at −28°. After thawing, however, not all the plasma proteins redissolved, and a stringy, balloon-like mass was present in each of the jars. This mass was extremely fragile and could be easily dispersed by vigorous stirring, although never completely dissolved. Dispersion of $BaSO_4$ in plasma containing the finely dispersed protein appeared to be satisfactory. The $BaSO_4$ settled easily from the plasma in 4–8 hr and could be recovered after removal of the supernatant adsorbed plasma.

When fresh plasma was used and when frozen, thawed plasma was used, significant differences were noted in the physical appearance of the settled $BaSO_4$. In the case of fresh plasma, a fine wet powder sediment of $BaSO_4$ was routinely found on the bottom of the container, frequently topped with a layer of gel-like material. This spongy layer was easily removed and washing yielded a tightly packed $BaSO_4$ cake in the final stages.

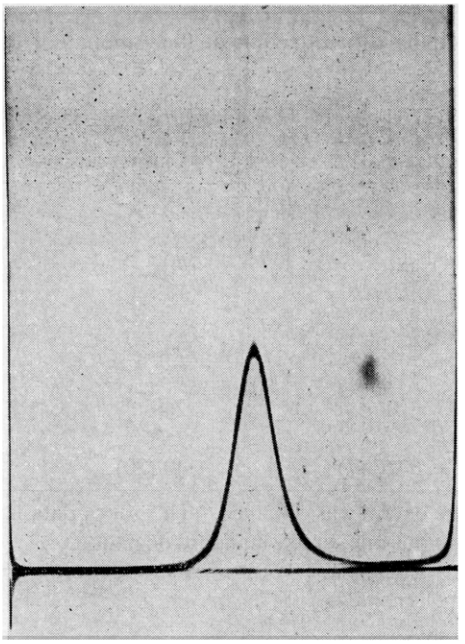

FIGURE 7: Examination of factor X by velocity sedimentation (sedimentation is from left to right). Speed, 59,780 rpm; phase-plate angle, 70°; cell, 2°, double sector, sapphire windows; concentration, 12.6 mg/ml; buffer, 0.001 M Tris-HCl–0.10 M NaCl (pH 7.40); temperature, 19.7°.

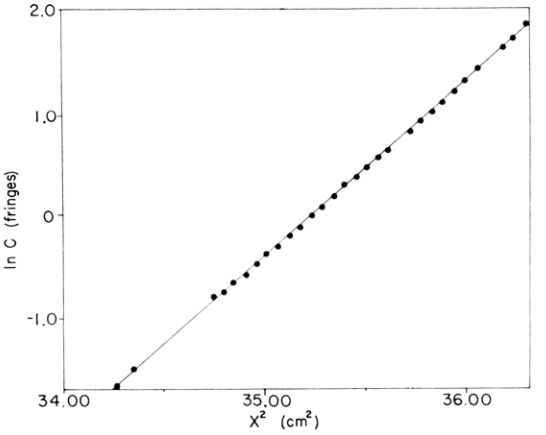

FIGURE 8: Plot of the logarithm of concentration *vs.* the square of the radial distance during high-speed equilibrium ultracentrifugation. Speed, 23,150 rpm; temperature, 20.0°; concentration, 1.6 mg/ml; buffer, 0.001 M Tris-HCl–0.10 M NaCl (pH 7.40); cell Yphantis six-place rectangular channel centerpiece, sapphire windows.

When frozen plasma was used, however, a fibrous, soft, granular sediment of $BaSO_4$ was obtained which could not be efficiently handled in the subsequent washing process and the $BaSO_4$ remained in its original fibrous–granular form. After complete elution of the adsorbed protein from the $BaSO_4$, the absorbance at 280 mμ of the eluate was found to be much higher than when the $BaSO_4$ formed a tightly packed cake. For example, in the poorest preparation, *i.e.*, of very low specific activity, the A_{280} of the eluate was 8.6 units/ml in contrast to an average value of 0.7 unit/ml. The decrease in the specific activity of this preparation was directly proportional to increased absorbance at 280 mμ, and the actual recovery of factor X activity was indistinguishable from that in other preparations.

$BaSO_4$ eluates prepared from moderately granular–fibrous $BaSO_4$ sediments were apparently true solutions at pH 5.8, the pH of the eluting buffer; however, upon addition of DEAE-cellulose and adjustment of the pH to 7.1, fibrous protein was found to precipitate. In the case described in the preceding paragraph, the protein precipitate completely occluded the DEAE-cellulose and terminated the preparation. A satisfactory solution to the problem was obtained by filtering the starting material, thawed plasma, through Büchner funnels fitted with thin pads of glass wool, although centrifugation was even more effective.

CHARACTERIZATION AND OPTIMIZATION OF BARIUM SULFATE ADSORPTION. Experiments described in this section were performed as follows, the only changes being in the variable under investigation. The basic format of these experiments is that described by Papahadjopoulos *et al.* (1964) for preparation of $BaSO_4$ eluates.

Plasma was obtained from bovine blood which had been mixed with 0.1 M sodium oxalate, one volume of oxalate solution to nine volumes of blood. The erythrocytes and buffy coat were separated from the plasma by centrifugation at 1370*g* for 20 min in an International PR-2 centrifuge equipped with a no. 276 swinging-bucket rotor. The plasma was subsequently frozen for storage and thawed prior to use at approximately 30–40°. Any material which did not readily redissolve was removed by centrifugation. Measured volumes of plasma were mixed with $BaSO_4$ and the resulting suspension was stirred for at least 30 min, a period of time shown to be at least three times longer than required for complete adsorption of the factor X activity. The $BaSO_4$ was completely pelleted by a short centrifugation, usually less than 5 min at no more than 1000*g*. If of interest in a particular experiment, the factor X activity remaining in the supernatant plasma was determined. Pelleted $BaSO_4$ was next washed once with a volume of 0.45% (w/v) NaCl solution equal to the volume of plasma used initially. This wash was discarded as occasional assay of it never demonstrated the presence of factor X activity. The factor X activity was eluted using 5% sodium citrate adjusted to pH 5.8 with 12 N HCl. The eluent volume was one-tenth the volume of the starting plasma. Barium sulfate was removed from the eluate by centrifugation as above, and the factor X activity present in the eluate was determined. Extreme care was taken to minimize losses of $BaSO_4$ during the washing procedure.

Although Esnouf and Williams (1962) and Papahadjopoulos *et al.* (1964) used, respectively, 100 and 75 mg of $BaSO_4$ per ml of plasma to adsorb factor X, no reason was given by either group for choosing the particular amount. Because of variable recoveries of factor X in the $BaSO_4$ eluates and the need to optimize each stage of the purification procedure, an investigation was undertaken to determine the minimum amount of barium sulfate required for complete adsorption. In this experiment, the recovery of factor X activity and the specific activity of the factor X in the eluate were investigated

TABLE II: Barium Sulfate Eluate Factor X Activity and Specific Activity as a Function of the Amount of Barium Sulfate.

$BaSO_4$ (mg/ml of plasma)	Eluate Factor X Act. (units/ml)	Factor X Sp Act. (units/A_{280})	Recovery (% of plasma act.)
100	1.8 (1)[a]	0.07	35
80	2.1 (1)		
75	2.1 (1)	0.11	40
70	1.8 (1)	0.10	18
60	2.0 (1)		
50	2.0 (1)	0.24	24
40	2.1 (1)		
30	2.3 (2)	0.36	26
10	2.2 (2)	0.93	25
10	2.2 ± 0.4 (20)[b]	0.84 ± 0.16 (7)[b]	40 (20)

[a] The figure in parentheses is the number of samples from which the datum was obtained. [b] This set of data is from large-scale preparations, *i.e.*, 30–40 l. of plasma, and is given plus and minus one sample standard deviation.

as functions of the quantity of adsorbent. The data are summarized in Table II.

It was found that the recovery of factor X activity was not dependent upon the quantity of $BaSO_4$ used. On the other hand, prothrombin activity adsorption is dependent. Thus, the use of 10 mg of $BaSO_4$/ml, which is optimum for high factor X specific activity, achieves partial separation of factor X and prothrombin.

No significant differences were found between the yields obtained at room temperature and those obtained in experiments performed at 4–5°.

A series of experiments, designed to determine the stability of factor X as a function of the time that the plasma and $BaSO_4$ were in contact, were conducted using 10 mg of $BaSO_4$/ml of plasma. The results are shown in Table III.

WASHING OF THE BARIUM SULFATE–PROTEIN PRECIPITATE. Prior to elution with a citrate buffer, the $BaSO_4$ adsorbent–protein precipitate had traditionally been washed with a solution of NaCl to remove occluded and relatively loosely adsorbed, noncoagulant protein (Papahadjopoulos *et al.*, 1964; Esnouf and Williams, 1962). In the procedure of Papahadjopoulos *et al.*, 0.45% NaCl was used as the washing solution. This solution was used initially in these experiments.

The major problem involved in the efficient washing of the $BaSO_4$-adsorbed protein cake was the quick and effective dispersion of the $BaSO_4$ in the 0.45% NaCl washing solution. Because the consistency of the $BaSO_4$ protein cake was not unlike partially set concrete under the best conditions, and was a granular mass of flocculent protein and occluded $BaSO_4$ prior to routine filtration of the thawed plasma, stirring was ineffective as a means of dispersion. Use of the Waring Blendor vastly

TABLE III: Stability of Factor X Adsorbed onto Barium Sulfate.

Time (hr)	Recov (%)
1 (control)	38
16[a]	40 (24–57)[b]
30	26
50	26

[a] This figure is from 20 large-scale preparations. [b] The numbers in parentheses indicate the range encountered.

TABLE IV: Effect on the $BaSO_4$ Eluate of Washing the Barium Sulfate–Adsorbed Protein Cake with Solutions Containing Sodium Citrate.

Citrate Concn[a] (M)	Act. (units of X/ml)	A_{280}	Sp Act. (units of X/A_{280})	Yield (%)
0	2.1	0.85	2.5	42
	3.0	0.98	3.1	60
0.001	2.35	0.54	4.3	47
	2.45	0.48	5.1	49
0.005	2.05	0.32	6.4	41
	2.05	0.32	6.4	41
	2.25	0.47	4.8	45
	2.01	0.48	4.4	42
0.010	0.75	0.21	3.6	15
	1.00	0.46	2.2	20
0.005[b]	1.50	0.44	3.4	30
	1.65	0.46	3.6	33

[a] Wash solutions contained 0.45% (w/v) NaCl plus sodium citrate at the stated concentration. [b] $BaSO_4$ plus 0.5 g of Celite (Johns-Manville Co.)/100 ml of plasma as an aid in dispersing the $BaSO_4$.

improved the washing procedure. The entire washing procedure was therefore carried out using the Waring Blendor. Excessive foaming of the protein $BaSO_4$ suspension was easily controlled using 1-octanol. For comparison, the $BaSO_4$ eluate from $BaSO_4$ protein cakes (10 mg/ml of plasma) washed by a previously described procedure (Papahadjopoulos *et al.*, 1964) possessed a specific activity of 0.84 ± 0.16 unit of factor X activity per A_{280} unit, whereas the factor X specific activity in the eluates in which initial dispersion was achieved using the Waring Blendor was 1.55 ± 0.23 units X per A_{280}. Thus it can be seen that the more effective dispersion obtained with the Waring Blendor resulted in a doubling of the specific activity of factor X in the $BaSO_4$ eluate.

In a paper describing the purification of bovine thrombokinase, Milstone (1955) stated that prothrombin could be partially eluted from $BaSO_4$ using a phosphate buffer of concentration less than that required to elute the thrombokinase activity. This suggestion led to an investigation of the ability of dilute citrate solutions to elute prothrombin and other "loosely" bound proteins selectively from the barium sulfate.

Factor X and the accompanying proteins were adsorbed using 10 mg of $BaSO_4$/ml of plasma. The Waring Blendor was used to disperse the $BaSO_4$ in a trisodium citrate–0.45% sodium chloride wash solution. Each sample was washed twice with a volume of citrate–NaCl solution twice the starting plasma volume. The results are shown in Table IV. Use of HyFlo Supercel (Johns-Manville Co.) as an aid in dispersing the $BaSO_4$ was also investigated. As can be seen from Table IV, the amount of protein in the eluate was not significantly decreased, and, in fact, some factor X activity was lost, probably by adsorption onto the diatomaceous earth. For comparison with the small-scale pilot determinations, Table V contains a compilation of data from large-scale preparations. In contrast to the results of Milstone, no selective elution of prothrombin could be demonstrated.

ELUTION OF FACTOR X FROM BARIUM SULFATE. Discovery that the amount of barium sulfate required to adsorb factor X completely could be reduced to 10 mg/ml of plasma suggested that the concentration of the citrate solution used to elute the factor X might also be decreased. If that were possible, direct adsorption onto DEAE-cellulose might then become feasible without an intermediate concentration step, the single most troublesome step in published schemes. Variation of the concentration of citrate between 0.03 (pH 5.8) and 0.19 M (pH 5.8) was found to yield no significant difference in the factor X recovery (38–46%) in the eluate.

In a subsequent investigation to determine the effect of varying the pH of the eluting citrate solution from pH 5.0 to 8.0, there was no detectable difference in the recovery of the factor X at pH 5.8 and above. The recovery at pH 5.0, however, was only one-half that at the higher pH values.

The use of 0.06 M sodium citrate (pH 5.8) as the eluting solution in the final scheme was decided from two other considerations. (1) Factor X could be adsorbed directly onto DEAE-cellulose without dilution of the

TABLE V: Barium Sulfate Eluate Factor X Activity and Specific Activity Resulting from Various Conditions of Washing and Handling.

Washing and Handling Procedures[a]	Factor X Act. (units of X/ml)	A_{280}	Sp Act. (units of X/A_{280})	Yield (%)
1. Fresh plasma, *no* citrate. The $BaSO_4$ cake was broken with a spatula; the suspension was stirred for 30 min using a magnetic stirring motor.	2.3 (7)[b] (1.8–3.1)[c]	3.5 (7) (1.3–7.0)	0.66 (7) (0.3–1.4)	37 (6) (32–43)
2. Fresh plasma, *no* citrate. The Waring Blendor was used in washing. Each $BaSO_4$–protein precipitate was washed five times.	2.2 (14) (1.0–3.6)	1.3 (14) (0.8–1.4)	1.7 (14) (1.1–3.8)	42 (12) (24–57)
3. Frozen plasma was prepared using the DeLaval separator. Washes *contained* sodium citrate. The Waring Blendor was used in washing. The $BaSO_4$ was permitted to settle from the eluate overnight.	1.6 (5) (1.0–2.9)	0.75 (4)[d] (0.5–0.9)	2.0 (4) (1.0–3.5)	Not available, but not likely different from above
4. Identical with 3 except that the plasma was filtered through glass wool.	1.8 (4) (1.2–2.7)	0.50 (4) (0.40–0.60)	3.2 (4) (2.1–5.6)	See 3
5. Identical with 4 except that the $BaSO_4$ was immediately separated from the eluates by centrifugation.[e]	1.0 (16) (2.7–6.5)	0.71 (16) (0.48–0.92)	5.2 (16) (2.0–10.5)	54 (16) (23–86)

[a] Ten milligrams of $BaSO_4$ was used per milliliter of plasma; all wash solutions contained 0.45% (w/v) NaCl and the concentration of citrate, when present, was 0.001 M. [b] Number in parentheses is the number of samples included in the calculation. [c] The range encountered. [d] Excluding the most aberrant preparation to date, *i.e.*, A_{280} 8.6. [e] Data in this set are actually from two elutions with $^1/_{20}$ the plasma volume each, but they have been converted into a single $^1/_{10}$ volume basis for comparison. See text for a discussion of the apparently high activity levels.

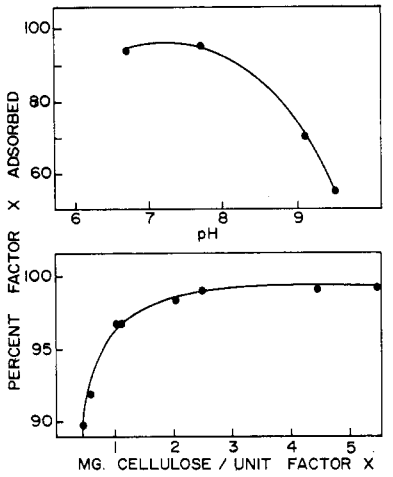

FIGURE 9: Adsorption of factor X onto DEAE-cellulose as a function of (1) pH, and (2) the amount of DEAE-cellulose. Top: DEAE-cellulose, 1 mg/unit of factor X activity, pH measured after equilibration. Bottom: DEAE-cellulose, specified amount added, pH adjusted to 7.1.

$BaSO_4$ eluate, and (2) precipitation of the stringy mass of protein mentioned above did not occur in pH 5.8, 0.06 M citrate solution. Although adjustment to pH 7 did result in precipitation of protein in preparations in which the thawed plasma had not been glass wool filtered, the concomitant loss of a large amount of factor X activity prevented its use as a purification step.

ADSORPTION OF FACTOR X ONTO DEAE-CELLULOSE BY BATCH TECHNIQUE. Chromatography of barium sulfate eluates on DEAE-cellulose constitutes a major step in the factor X purification schemes of both Esnouf and Williams (1962) and Papahadjopoulos *et al.* (1964). Although it was possible to obtain a reasonable purification of factor X by the DEAE-cellulose procedure of Papahadjopoulos *et al.* (1964), the large volume of barium sulfate eluate and the frequent clogging of the column during sample application made this type of procedure impractical for large-scale preparations.

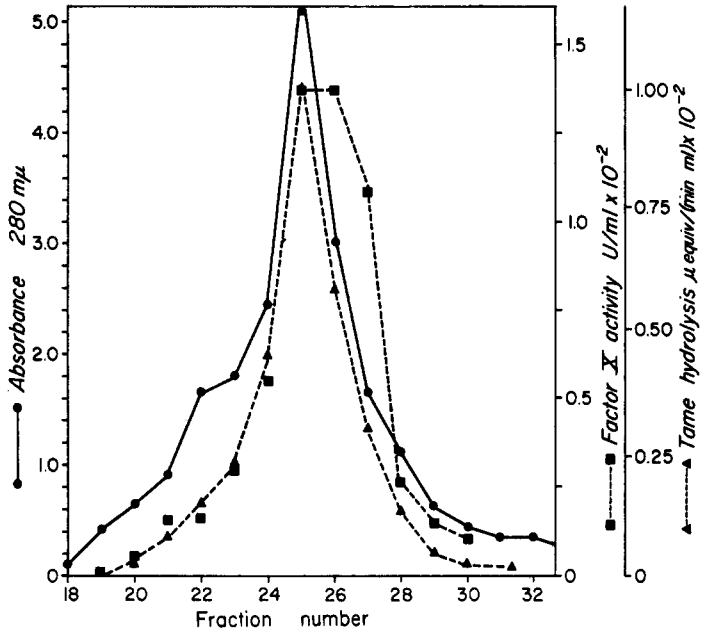

FIGURE 10: Elution profile of factor X after batch adsorption onto DEAE-cellulose, packing into a chromatographic column, and gradient elution. Column, DEAE-cellulose, 4.5 × 43 cm; buffer, 0.1 M sodium citrate–0.05 M Tris-HCl (pH 8.0); linear gradient in NaCl of 0–0.5 M, 500 ml/chamber; sample, $BaSO_4$ eluate, 25,000 units of factor X; flow rate, 300 ml/hr; temperature, 20–25°.

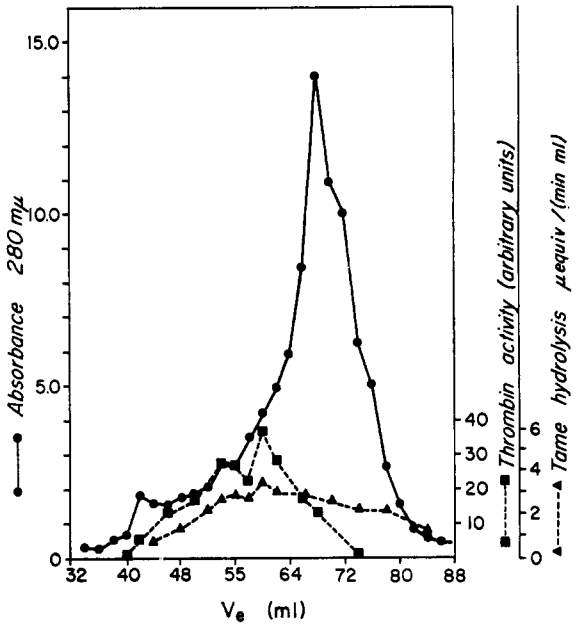

FIGURE 11: Gel filtration on Sephadex G-100 of factor X from DEAE-cellulose chromatography. An example of an elution profile for a low specific activity preparation. Column, Sephadex G-100, 1.9 × 50 cm; buffer, 0.05 M Tris-HCl–0.10 M NaCl (pH 7.4); sample, factor X from DEAE-cellulose (fractions 25–27 of Figure 10), 8 ml, 17 mg/ml; flow rate, 5 ml/hr; fraction volume, 2 ml; temperature, 20–25°.

Batch adsorption onto DEAE-cellulose was attempted by using washed, wet DEAE-cellulose; however, because of the difficulty of adding reproducible amounts of wet DEAE-cellulose, the use of dry DEAE-cellulose which had been washed and subsequently dried was adopted (see Materials and Methods section).

In order to exploit the selectivity possible with DEAE-cellulose adsorption, experiments were first performed to determine the pH at which maximum adsorption of the factor X activity would occur. It was known that at pH 7 adsorption was nearly complete when 1 mg of DEAE-cellulose was used per unit of factor X activity; hence this quantity of cellulose was used in the pH optimization experiments. Adsorption was apparently maximum at pH 6.8–7.8. The adsorption procedure was routinely done at pH 7.1 in order to minimize the pH effect on precipitation of the fibrous protein mentioned above.

The minimum amount of dry DEAE-cellulose required to adsorb factor X completely from a 0.06 M sodium citrate solution (pH 5.8) was determined using weighed amounts of dry DEAE-cellulose. The pH of the $BaSO_4$ eluate–cellulose suspension was adjusted to the desired value after the cellulose had been added. The results are shown in Figure 9. In order to provide suffi-

TABLE VI: A Comparison of Factor X Specific Activity and TAMe Esterase Specific Activity at Comparable Stages in Two Preparations during Refinement of the Purification Procedure.

Preparation Description	$BaSO_4$ Eluate	DEAE-cellulose Effluent	Factor X: TAMe ratio
Preparation of Figures 10 and 11[a]	2.6 units of X/ml 1.2 A_{280} units/ml 2.1 units of X/A_{280}	27 units of X/A_{280} 20 μequiv/(min A_{280})	1.35
Preparation of Figures 3–5	5.2 units of X/ml 0.6 A_{280} unit/ml 8.8 units of X/A_{280}	120 units X/A_{280} 45–54 μequiv/(min A_{280})	2.4

[a] Fractions 25–27, the constant specific activity fractions, of Figures 10 and 11.

cient latitude for variation in the determination of the eluate factor X activity, 2 mg of DEAE-cellulose/unit of factor X was chosen as the quantity to be used routinely in the preparations.

In preparations of $BaSO_4$ eluate possessing specific activities greater than 1 unit of factor X activity/A_{280} unit, the factor X activity could not be eluted from the DEAE-cellulose with 0.08 M sodium citrate (pH 7–8). Consequently, the DEAE-cellulose was washed extensively in the centrifuge cups which were used for centrifugally separating the cellulose from the wash solution, thus eliminating transfer losses at this stage. Initially, washing was done with 0.06 M sodium citrate (pH 6.7) to guarantee retention of factor X activity. However, because of the greater affinity of the factor X for the DEAE-cellulose in the high specific activity eluates, or, more likely, less competitive binding of other proteins to the cellulose, the DEAE-cellulose could also be washed with 0.08 M sodium citrate (pH 8.0) without loss of factor X activity.

ELUTION OF FACTOR X FROM DEAE-CELLULOSE. Column chromatography of $BaSO_4$ eluates on DEAE-cellulose under the conditions of Papahadjopoulos *et al.* (1964) indicated a nonlinear distribution isotherm, *i.e.*, elution profiles for both factor X activity and absorbance at 280 mμ showed marked skewing toward the trailing edge.

Elution employing a linear concentration gradient in sodium citrate was investigated, but this method did not reduce significantly the volume of factor X containing effluent. A single attempt to avoid the presence of citrate in the eluting buffer and to elute the factor X activity from DEAE-cellulose with NaCl, 0–1.0 M buffered with Tris-HCl at pH 7.5, failed to elute any activity from the column. A solution of 0.1 M citrate–0.05 M Tris-HCl (pH 8.0) and a linear gradient in NaCl of 0–0.5 M was found to be the most satisfactory combination for eluting factor X in a minimum volume. Elution could be achieved in $^1/_{25}$ the $BaSO_4$ eluate volume, *i.e.*, approximately 400 ml after starting with 10 l. of $BaSO_4$ eluate. Buffer solutions containing 0.1 M sodium phosphate (pH 8.0) in place of sodium citrate did not result in a distinguishably different elution profile.

Attempts to elute factor X from DEAE-cellulose by the batch technique, *i.e.*, stirring with an eluting citrate solution (no NaCl) followed by filtration on a Büchner funnel, were extremely unsatisfactory since this resulted in very large volumes of factor X containing solutions.

The following discussion of a typical preparation from a $BaSO_4$ eluate possessing specific activity of less than 1 unit of factor X activity/A_{280} unit illustrates some of the problems encountered at this stage during development of the final procedure. It should be noted that these problems were not encountered in DEAE-cellulose batch chromatography when high specific activity eluates were used as starting material. Figure 10 shows the apparent correspondence of the activity and the protein eluted from a batch adsorption column. The two "shoulders" preceding the major peak probably represent protein displaced from the DEAE-cellulose as a result of the factor X peak; this has not been detected when using the final scheme. The trailing of the factor X activity behind the protein peak results from trace amounts of activated X in the preparation. Although activated X is independently assayable, no correction factor exists that would permit a quantitative assessment of factor X in the presence of the activated species. Aliquots of the peak fractions were dialyzed to remove the citrate; they were then activated with the coagulant protein of Russell's viper venom (Williams and Esnouf, 1962; Jackson and Hanahan, 1968) and the TAMe esterase activity was determined. This assay, which requires conversion of the factor X into its activated form, demonstrates further the correspondence between the activity and the protein elution pattern. A constant specific activity of 24 μequiv/(min mg) was found in fractions 24–27, or approximately 65% of the material of this peak was constant specific activity factor X. Fractions 25–27 were combined, concentrated, and examined by gel filtration on Sephadex G-100 (Figure 11). It is now clear that the apparent correspondence of activity and protein from DEAE-cellulose columns was probably coincidental.

The data from the columns of Figures 10 and 11 and a comparison of the ratios of TAMe esterase activity to clotting activity given in Table VI demonstrate the inadequacy of simple protein activity correspondence in a purification step as evidence for homogeneity. Ta-

ble VI further illustrates the problems which could arise if a single activity, one specific but difficult to quantitate accurately or one quantitative but relatively nonspecific, were used alone to follow the purification procedure.

Discussion

A protein preparation possessing factor X activity as defined by the Bachmann assay procedure (Bachmann *et al.*, 1958) has been isolated from bovine plasma and purified 16,000-fold, based upon a comparison of the specific activity of factor X in the starting plasma and the specific activity of the finally purified product. Variation in the degree of purification does occur and the range encountered is from 10,000- to 30,000-fold. This has been, however, exclusively the result of variability in the factor X activity in various batches of bovine plasma. With this isolation procedure, it has been possible to prepare repeatedly 0.8–1.2 mg of factor X protein from 1 l. of bovine plasma.

Optimization of each step of the original procedure of Papahadjopoulos *et al.* (1964) and Duckert *et al.* (1960) provided a reproducible method for the isolation and purification of factor X in 100-mg quantities that required 3–4 days from plasma to final product. During development of the purification procedure it was found necessary to include DFP in buffer solutions during chromatography on DEAE-cellulose. DFP also had to be included in solutions of factor X which were to be stored frozen. Data relevant to this requirement for DFP as an inhibitor of proteolytic enzymes can be found in Jackson and Hanahan (1968).

Individual preparations of factor X with less than maximum specific activity after Sephadex gel filtration do occur, but can be brought to maximum specific activity by rechromatography on an anion-exchange material. In these cases, chromatography on DEAE-Sephadex A-50 under the same conditions as were used with DEAE-cellulose has been found to be very satisfactory. However, sufficiently reproducible multiple peaking has been found to occur during rechromatography on Sephadex A-50 to merit independent consideration; this complication is discussed in Jackson and Hanahan (1968). Claims for homogeneity of a protein preparation derive from failure to demonstrate inhomogeneity in the preparation. Operationally, therefore, homogeneity or indemonstrable inhomogeneity is dependent entirely upon the technique used for detection of inhomogeneity. Furthermore, given an assay procedure which defines the particular biological activity, the preparation isolated should not be further purifiable by currently available techniques; that is, application of other purification techniques to the preparation should not result in a further increase in the specific activity of the preparation. In addition, the specific activity across the peaks in chromatographic elution profiles should be constant within the limits of error of the assay. It can be seen from Table I that the specific activity of the factor X preparations could not be increased by rechromatography. Constancy of the specific activity across the peaks during gel filtration on Sephadex G-100 (Figure 3) and chromatography on DEAE-Sephadex A-50 (Figure 5) has been obtained. These criteria can be seen to be satisfied after gel filtration by both the clotting activity of precursor factor X and the TAMe esterase activity of the activated form of factor X. Attempts to chromatograph factor X preparations at low pH on cation-exchange materials have been unsuccessful due to the instability of the activity at pH values below pH 5.5.

Data obtained from examination of factor X preparations in the analytical ultracentrifuge by both velocity and equilibrium sedimentation provide no evidence for inhomogeneity. (The experimental data are presented in Jackson and Hanahan 1968.)

Only a single band is seen when factor X protein is examined by the disc electrophoretic technique of Ornstein (1964) and Davis (1964) when single gels are loaded with 10–30 μg of protein. A single gel containing 250–300 μg of factor X protein did, however, exhibit additional bands with gel electrophoretic mobilities comparable with those of the other components of the $BaSO_4$ eluate. Visual estimation of the quantities of these additional components indicated that they probably represent less than 5% and possibly only 1% of the total stained material in the gel. Elimination of these contaminants appears to be possible by preparative disc electrophoresis and will undoubtedly become a step in the purification of factor X for studies of the mechanism of its activation and its biochemical role in blood coagulation. (Initial experiments indicate that preparative disc electrophoresis is capable of the resolution required.)

Apparently homogeneous factor X preparations are obtained relatively easily by this procedure and in sufficient quantity for chemical and physiochemical studies. Nevertheless, in certain types of experiments on the physiological role of factor X in which high concentrations of protein might be required, the data must be analyzed carefully because trace contaminants are known to be present in the factor X preparations. Additional relevant data are presented and discussed in Jackson and Hanahan (1968).

Because of the difficulty in interpreting assay data for contaminant clotting activities in the presence of factor X at 100 times the concentration of the probable contaminants, such assays were not performed. Instead, an estimate was made based upon contaminant bands which could be seen by disc electrophoresis of 200-μg samples of protein (*vide supra*). Final assessment of contaminants probably must await the isolation of relatively pure preparations of the contaminating species, the preparation of antibodies to these proteins and immunoelectrophoretic examinations.

The observation that differential adsorption of prothrombin and factor X activities onto $BaSO_4$ occurs, is not consistent with the prothrombin derivative hypothesis of Seegers (1964). Experiments similar to those performed here using other adsorbents and eluting solutions might well aid in the resolution of some of the controversy associated with research on the components of the process of prothrombin activation. The results of this study which were obtained by varying the amount of $BaSO_4$ are qualitatively in agreement with those of

Voss (1965) but are quantitatively different, possibly owing to qualitative differences in the $BaSO_4$ used.

The protein bands seen on disc electrophoresis were not independently identified with the exception of the factor X band; however, the most darkly staining band of the $BaSO_4$ eluate was found to disappear after conversion of the prothrombin of the $BaSO_4$ eluate to thrombin. A new band with a gel electrophoretic mobility greater than factor X was found to appear, presumably representing the less anionic but less asymmetric, hence encountering less frictional resistance, thrombin protein. If the major band is assumed to represent prothrombin, the observed relative mobilities of the stained bands in the disc gel are comparable with the mobilities found by Pechet *et al.* (1960) for clotting activities from eluted bands from starch gel electrophoresis. Thus, it can be proposed that the band between the factor X and prothrombin bands may represent factor VII. Obviously the proteins must be isolated to confirm such a proposal. A more detailed discussion of disc electrophoretic data relevant to factor X and the associated proteins is found in Jackson and Hanahan (1968), along with data from the partial characterization of the factor X protein.

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