Studies on Bovine Factor X. II. Characterization of Purified Factor X. Observations on Some Alterations in Zone Electrophoretic and Chromatographic Behavior Occurring during Purification*

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ABSTRACT: During the development of the large-scale isolation procedure for bovine factor X (Jackson, C. M., Johnson, T. F., and Hanahan, D. J. (1968), *Biochemistry* 7, 4492 (this issue; preceding paper), it was observed that alterations in the disc electrophoretic and chromatographic behavior of the factor X protein were occurring during the purification process. Inclusion of diisopropyl phosphorofluoridate in all protein and buffer solutions completely prevented those changes which were seen by disc electrophoresis. During rechromatography on DEAE-Sephadex A-50, factor X protein, which was homogeneous as indicated by a constant specific activity across chromatographic peaks, by its behavior in the ultracentrifuge, and by disc electrophoresis, gave a complex two-peak elution profile. A number of possible causes for this complex chromatographic behavior were eliminated by consideration of data from a large number of preparations; however, no definitive explanation for this phenomenon has been found. The factor X protein isolated by the procedure cited above has been characterized by its hydrodynamic behavior during analytical ultracentrifugation. Included in the hydrodynamic properties which were determined are the sedimentation and diffusion coefficients, the concentration dependence of the sedimentation coefficient, and the high-speed equilibrium molecular weight averages. The amino acid composition of the purified protein was determined. Preliminary data on the enzymatic properties of factor X after activation with the coagulant protein of Russell's viper venom are also reported. A number of problems associated with the preparation of the enzymatically active form of factor X from the precursor protein are described.

Factor X activity, which has as its basis for definition clinical observations of a blood clotting activity deficiency state, appears to be very similar to autoprothrombin C activity and thrombokinase activity, two blood clotting activities which possess quite different bases for their definition. Autoprothrombin C is a species isolable from prothrombin preparations which have undergone specific treatment to develop coagulant activity and which is defined by the assay procedure developed by Seegers (1964). Thrombokinase is the name given to the species postulated by Morawitz (1905) to be responsible for the conversion of prothrombin into thrombin and has been investigated and described by Milstone (1964). The ability of autoprothrombin C (Seegers, 1964) and thrombokinase (Milstone *et al.*, 1963) to convert prothrombin into thrombin in the absence of any other added components seems well established. The observation that activated factor X could also carry out this reaction (Jackson, 1967; Barton *et al.*, 1967) has made the three differently named activities appear to be very similar, if not equivalent. The similarity of autoprothrombin C activity and activated factor X activity and the chromatographic properties of these two species had been demonstrated by Lechner and Deutsch (1965). In addition to similar blood clotting activity, autoprothrombin C, thrombokinase, and activated factor X all possess the ability to hydrolyze *p*-toluenesulfonyl-L-arginine methyl ester (Esnouf and Williams, 1962; Seegers, 1964; Milstone, 1964).

Although thrombokinase, autoprothrombin C, and activated factor X activities may appear to be equivalent, the molecules possessing these common biological activities as one of their properties may not be identical. For example, the different modes of activation used to generate the enzymatically active forms of these species may well result in distinguishably different molecular species, even if a common precursor exists. These complications justify the limitation discussed previously (Jackson, 1967; Jackson *et al.*, 1968) when comparing activities and the molecules possessing these activities which are involved in the clotting process

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and is the reason for presenting some of the experimental observations reported in this paper.

A recent publication by Seegers *et al.* (1967) presents a number of properties of autoprothrombin III, the species that apparently has the same relationship to autoprothrombin C as factor X does to the activated form of factor X. Autoprothrombin III, a protein derived from prothrombin preparations under carefully defined conditions, possesses no ability to convert prothrombin into thrombin without first being activated by trypsin, Russell's viper venom, or several other agents. These properties may be compared with the very similar results of Papahadjopoulos *et al.* (1964), who used some of the same activating agents with factor X preparations. Data obtained from this study, when compared with those of Seegers *et al.* (1967), indicate a number of additional similarities in the two protein species and will be discussed.

In addition to the possible differences arising from the different activation methods used to obtain the enzymatically active forms of these clotting components, the alterations in electrophoretic and chromatographic behavior of the precursor form of factor X which are reported below add another variable and a source of difference to be considered when comparing macromolecules which possess apparently equivalent clotting activity. On the basis of the confusion existing in the biochemistry of blood coagulation, these alterations, coupled with an investigation of their possible causes, are discussed in some detail. Comparable complications in preparations of human prothrombin have been recently reported by Aronson (1966) and Lanchantin *et al.* (1968).

The factor X protein isolated by the procedure of Jackson *et al.* (1968) has been characterized by its hydrodynamic behavior during analytical ultracentrifugation. Included in the hydrodynamic properties which were determined are the sedimentation and diffusion coefficients, the concentration dependence of the sedimentation coefficient, and the high-speed equilibrium molecular weight averages. The amino acid composition of the purified protein was determined. Preliminary data on the enzymatic properties of factor X after activation with the coagulant protein of Russell's viper venom are also reported. A number of problems associated with the preparation of the enzymatically active form of factor X from the precursor protein are described. In addition to the characterizing of the homogeneous form of purified factor X protein, some characteristics of the altered species are presented and discussed because of the likely occurrence of such alterations in preparations of factor X reported on by other researchers.[1]

Materials and Methods

Bovine factor X was prepared as described previously (Jackson *et al.*, 1968).

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. The purified coagulant protein of Russell's viper venom was prepared (Jackson, 1967) from venom obtained from the Miami Serpentarium, Miami, Fla., by a modification of the procedure of Williams and Esnouf (1962).

Diisopropyl fluorophosphate was purchased from Merck and Co., Rahway, N. J., and was used as a 1 M solution in anhydrous 2-propanol.

Phenylmethanesulfonyl fluoride and soybean trypsin inhibitor were the products of Pierce Chemical Co., Rockford, Ill.

p-Toluenesulfonyl-L-arginine methyl ester and bovine serum albumin (A grade, from Calbiochem, Los Angles, Calif.) were used as provided.

Factor X was assayed by the technique of Bachmann *et al.* (1958) as described previously (Jackson *et al.*, 1968). One unit of factor X activity is defined as that amount present in 1 ml of normal human plasma. Activated factor X, the species resulting from incubation of factor X with the coagulant protein of Russell's viper venom (Williams and Esnouf, 1962), was assayed in the same way as factor X, except that the viper venom was omitted from the cephalin–venom mixture of the factor X assay.

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).

Solutions to be assayed for clotting activities were diluted into 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.

p-Toluenesulfonyl-L-arginine methyl ester esterase activity was determined at 37° by continuous titration using a Radiometer TTT1c, SBR-2, TTA-31 pH-Stat assembly. For routine determinations of activated factor X esterase activity, *p*-toluenesulfonyl-L-arginine methyl ester 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.

Amino acid analyses were performed by the technique of Spackman *et al.* (1958) using a Beckman 120A amino acid analyzer. Peak areas were determined by automatic integration using Infotronics, Series CRS integrator (Infotronics, Inc., Houston, Texas), for all amino acids except proline. Integrator results were spot checked by manual integration in all cases in which the replicate samples had a variation from the mean which was larger than expected. The protein solutions, freed of NaCl by desalting into phosphate buffer using Sephadex G-25, were hydrolyzed according to the procedure described by Moore and Stein (1963) at 110° for 24, 48, 72, and 96 hr. Simple linear extrapolations with time were made to "correct" for losses of serine and threonine; the average of the values obtained

[1] Similar results with factor X have been observed by Drs. M. P. Esnouf and J. Levison (personal communication).

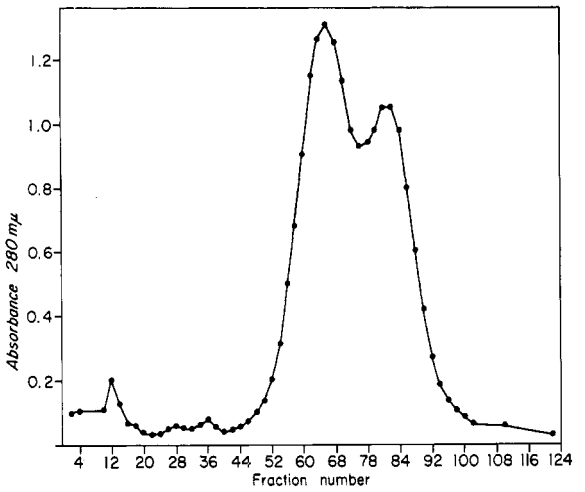

FIGURE 1: Multiple peak formation during chromatography on DEAE-Sephadex A-50 of "undegraded" factor X of maximum specific activity. Column, DEAE-Sephadex A-50, 2.5 × 40 cm. Buffer, 0.05 M Tris-HCl–0.10 M sodium citrate–0.10 M NaCl (pH 8.0); linear gradient in NaCl (0.10–0.50 M) beginning at fraction 8, 500 ml/gradient chamber. Sample, factor X from Sephadex G-100, 40 ml, 10 mg/ml. Flow rate, 120 ml/hr; fraction volume, 10 ml; temperature, 20–25°.

after "plateau" levels were reached was used for leucine, isoleucine, and valine. Tyrosine and tryptophan were determined spectrophotometrically by the procedure of Goodwin and Morton (1946), and the values for tyrosine were included with those determined by amino acid analysis.

Protein concentration was routinely determined by measurement of the absorbance at 280 mμ in either a Beckman DU or DB spectrophotometer. The extinction coefficient for factor X, actually expressed as $E^{1\%}_{1\,cm}$ at 280 mμ, was determined from measurements of the absorbance using a 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} (cm ml)/g was assumed (Armstrong *et al.*, 1947). $E^{1\%}_{1\,cm}$ at 280 mμ was then determined by dividing the absorbance at 280 mμ by the protein concentration. Examination of the change in apparent absorbance between 350 and 320 mμ indicated that light scattering by the solution was negligible; hence no correction was necessary.

Qualitative determination of carbohydrate was done using the anthrone reaction for hexoses (Ashwell, 1957) and the resorcinol reaction for sialic acid (Svennerholm, 1963).

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. Sedimentation velocity determinations were made at 59,780 rpm in aluminum filled epoxy, 2° double-sector centerpieces and with sapphire windows. Sedimentation coefficients were determined from measurements of the rate of movement of the maximum ordinate of the schlieren peak without compensation for radial dilution.

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 an IBM 7094–7040 computer and the program of Teller (1965).

The protein specific volume was calculated from the amino acid composition by the procedure of McMeekin *et al.* (1949).

The diffusion coefficient for the protein was determined using the ultracentrifuge schlieren optical system. Solvent, *i.e.*, dialysate, was layered onto the protein solution at 3000 rpm using a modified capillary-type double-sector synthetic boundary cell, and the diffusion coefficient was determined from boundary spreading. Photographs were taken at 4-min intervals over a period of 1 hr, and the patterns were analyzed by measurement of the peak width at the inflection point of the ordinate. This width was then used to calculate the diffusion coefficient by the procedure outlined by Svedberg and Pedersen (1940). Viscosities and densities of the solvents used in the sedimentation velocity experiments were obtained from Svedberg and Pedersen (1940) or by measurement in a Cannon-Ubbelohde capillary viscometer (Cannon Instruments, State College, Pa.) and a tared class A volumetric flask, respectively.

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. Carbohydrate was stained by the Schiff base–periodate technique.[2]

Preparative disc electrophoresis was done using the apparatus sold by Buchler Instruments Inc., Fort Lee, N. J., as described in the instruction manual provided with the apparatus.

Observations and Results

During development of the large-scale purification procedure for factor X (Jackson *et al.*, 1968), it was observed that the factor X protein which was eluted in a single peak from DEAE-cellulose gave a complex two-peak profile upon chromatography on DEAE-Sephadex A-50. This multiple peak formation was independent of the alterations in the protein seen by disc electrophoresis (*vide infra*) which occurred when DFP was not included in buffer solutions and in the stored samples. This phenomenon of multiple peak formation has occurred in essentially every preparation of bovine factor X processed during a 2-year period. In all the preparations described below, DFP (10^{-3} M) was included during isolation and purification unless stated otherwise, except in gel filtration during which it

[2] The details of this procedure are described in the "Special Procedures for Disc Electrophoresis" sheet of the Canal Industrial Corp., Bethesda, Md. (Feb 12, 1965, 1K).

would have been separated from the protein sample. In no case was activated factor X detectable in the factor X preparations.

An example of multiple peak formation from one batch of factor X protein is shown in Figure 1. The protein was prepared by the aforementioned procedure in which it had first been chromatographed on DEAE-cellulose, then on Sephadex G-100, and finally on Sephadex A-50. The protein applied to this particular column had a clotting specific activity of greater than 96% of the maximum specific activity of the homogeneous protein preparations characterized by assay of all fractions across the chromatographic peaks and described in detail in paper I of this series (Jackson *et al.*, 1968). Examination of the effluent fractions from the column of Figure 1 by disc electrophoresis under the conditions used throughout this study (see Materials and Methods) showed only a single band in all fractions from the entire two-peak complex. A disc gel containing aliquots from both a fraction from the leading edge of the first peak and a fraction from the trailing edge of the second peak also showed only a single band. The factor X clotting specific activity of the material from the leading and the trailing edges was identical with and equal to the maximum obtainable for homogeneous preparation described by Jackson *et al.* (1968). The *p*-toluenesulfonyl-L-arginine methyl ester esterase activity of the material placed onto this Sephadex A-50 column, after activation, was 53 μ-equiv/(ml min mg protein) and that of the pooled factor X from both peaks after chromatography, after activation, was 56 μequiv/(ml min mg). No significant purification was considered to have been achieved by this step. In the particular sample of Figure 1, individual fractions were not examined for differences in *p*-toluenesulfonyl-L-arginine methyl ester esterase activity. The recovery of 280-mμ-absorbing material from the column of Figure 1 was essentially quantitative, *i.e.*, 95–100%.

Separate rechromatography on DEAE-Sephadex A-50 of samples taken from the leading edge of the first peak and the trailing edge of the second peak of another preparation of factor X protein from DEAE-Sephadex chromatography did not result in further generation of two peaks. Elution profiles from such rechromatography are shown in Figure 2; the elution conditions are given in the legend to the figure. The factor X containing effluent from both columns was recombined and again chromatographed on Sephadex A-50. Two partially resolved peaks with the same pattern as the starting material were obtained. In the particular experiment shown in Figure 2, alteration of the factor X protein as indicated by disc electrophoresis (*vide infra*) had occurred. However, identical behavior on rechromatography has been found with preparations which gave two peaks on DEAE-Sephadex A-50 but showed only a single band on disc electrophoresis. However, these samples showed no evidence for the type of alteration which led to multiple bands of factor X protein on disc electrophoresis.

Sephadex G-100 gel filtration has been done separately on samples taken from the leading edge of

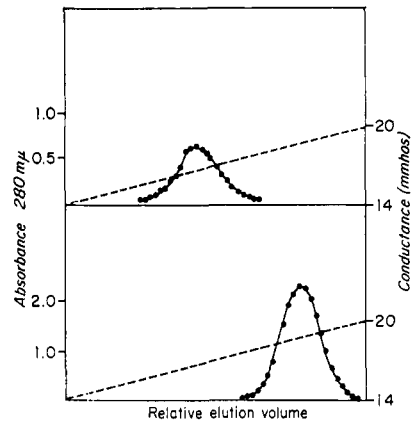

FIGURE 2: Separate rechromatography on DEAE-Sephadex A-50 of samples taken from the leading edge of the first peak (top) and the trailing edge of the second peak (bottom) of the multiple peak complex. Columns, DEAE-Sephadex A-50, 0.95 × 30 cm. Buffer, 0.05 M Tris-HCl–0.10 M sodium citrate (pH 8.0), linear gradient in NaCl (0–0.5 M), 75 ml/gradient chamber. Samples, top: combined fractions of the leading edge of the first peak from Sephadex A-50 (8 mg); bottom: combined fractions from the trailing edge of the second peak from Sephadex A-50 (24 mg); fraction volume, 1 ml.

the first peak and the trailing edge of the second peak of the factor X from DEAE-Sephadex chromatography, but from an early preparation in which the alteration as defined by multiple bands in disc electrophoresis gels had occurred. Although the elution volumes of the two species were significantly different, the molecular weight calculations, using the equation of Determann and Michel (1966) or the relationship of Whitaker (1963), indicated that the apparent molecular weight (size) of the protein from the first peak was 88,000 and of that from the second 80,000. This apparent molecular weight (size) of 80,000–90,000 for factor X from DEAE-cellulose and DEAE-Sephadex chromatography has been found repeatedly by gel filtration. Molecular size differences between the DEAE-Sephadex "differentiable species" have not been checked by gel filtration in any other preparations because of the differences in the apparent molecular weight from gel filtration and the sedimentation equilibrium molecular weight determined by ultracentrifugation (*vide infra*).

Amino acid analyses (of single hydrolysates) of protein taken from each of the two peaks from Sephadex A-50 of factor X protein which was not altered with respect to its disc electrophoretic pattern indicated no significant differences in the amounts of any of the individual amino acids. Nor were there any significant differences in the amounts of any of the amino acids of these distinguishable species when compared with the data from the amino acid composition given in Tables I and II.

As two peaks have been found to occur on DEAE-Sephadex chromatography with both very low and very high ratios of protein to Sephadex A-50 gel (Jackson, 1967), double peak formation is probably not a simple consequence of differences in column loading. Furthermore, as double peaking occurs both in

TABLE I: Amino Acid Composition of Bovine Factor X.

Residue	24 hr[e]	48 hr[e]	72 hr[e]	96 hr[e]	Av
Asp	0.643	0.637	0.634	0.643	0.639
Thr	0.456	0.442	0.419	0.417	0.476[a]
Ser	0.447	0.414	0.376	0.353	0.483[a]
Glu	0.982	0.995	0.987	0.995	0.990
Pro	0.303	0.308	0.299	0.300	0.302
Gly	0.649	0.642	0.645	0.646	0.646
Ala	0.487	0.490	0.494	0.489	0.490
Cys(1/2)	0.340	0.350	0.354	0.358	0.352
Val	0.376	0.402	0.405	0.412	0.406[b]
Met	0.088	0.074	0.083	0.081	0.081
Ile	0.158	0.167	0.187	0.180	0.183[b]
Leu	0.484	0.465	0.463	0.472	0.473
Tyr	(0.117, 0.158)	0.151	0.149	0.151	0.151[c]
Phe	(0.278, 0.348)	0.337	0.335	0.321	0.333[c]
Lys	0.302	0.302	0.306	0.306	0.304
His	0.158	0.157	0.164	0.161	0.159
Arg	0.368	0.368	0.378	0.378	0.374
Trp					0.181[d]

[a] Extrapolated values, determined from a linear extrapolation of micromoles *vs.* time. [b] Determined from values for 72- and 96-hr hydrolyses or from 48-, 72-, and 96-hr values when the variation about an apparent average appeared to be random. [c] Values for the 24-hr hydrolysates of tyrosine and phenylalanine were not included in the average values. [d] Determined spectrophotometrically by the procedure of Goodwin and Morton (1946). [e] Micromoles of amino acid released at various times of hydrolysis at 110°.

the absence and presence of the alterations in the protein which produce three distinguishable bands of factor X protein in disc gels, the type of alteration giving rise to multiple bands by disc electrophoresis is not necessary for double peaking to occur during chromatography on DEAE-Sephadex A-50. An extensive presentation of data and discussion of this complicating feature can be found elsewhere (Jackson, 1967).

An examination by disc electrophoresis of the factor X preparation at each stage of the purification procedure was undertaken because of the double peak formation during Sephadex A-50 chromatography described above. Although no explanation of double peak formation resulted, an additional variable was discovered. Factor X preparations which had been "chromatographed" on DEAE-cellulose by the batch adsorption procedure (Jackson *et al.*, 1968) contained only a single band in the region to which factor X activity was known to migrate; likewise, only one band was present in the protein solution which was eluted from $BaSO_4$ and coelectrophoresed with the factor X protein from DEAE-cellulose (Jackson *et al.*, 1968). After chromatography of factor X from DEAE-cellulose on DEAE-Sephadex A-50 and the separation of the factor X into two peaks, two to three bands were detected in the disc gels. The slowest moving band of the group was found to coelectrophorese with the factor X protein from DEAE-cellulose chromatography.

It was further noted that after storage of these factor X preparations, the relative amounts of protein increased in the faster bands and decreased in the slowest band, suggesting that a time-dependent transformation was occurring. Photographs of disc gels showing these multiple bands of factor X can be seen in Figure 3.

Although the expected relationship between the pattern of bands seen by disc electrophoresis and the two Sephadex A-50 peaks is seen in Figure 3, *i.e.*, larger amounts of less anionic species are associated with the first peak off a DEAE-Sephadex column and the more anionic species are associated with the second peak, the data presented previously rule out a necessary relationship between the alterations in the protein responsible for the three bands seen in disc gels and the double peak of Sephadex A-50 chromatography.

The additional observation that after total activation of the factor X protein with the coagulant protein of Russell's viper venom only a single species of activated X could be seen by disc electrophoresis also suggested that all the disc bands were factor X. In view of the previously documented problem of thrombin contamination (Jackson *et al.*, 1968), possible proteolytic degradation resulting from the presence of thrombin at levels too low to be detected unequivocally with fibrinogen was considered a likely cause for these three resolvable protein species seen by disc electrophoresis.

In order to eliminate thrombin activity, DFP (10^{-3}–10^{-2} M) was added to the protein solution eluted from $BaSO_4$ ($BaSO_4$ eluate), the DEAE-cellulose column

TABLE II: Amino Acid Composition of Bovine Factor X.[a]

Amino Acid	g of Amino Acid/ 100 g of Protein	Moles of Amino Acid/10^5 g of Protein	Moles of Amino Acid for a Protein of Mol Wt 55,000	Nearest Integral No. of Residues for Mol Wt 55,000
Asp	11.0	82.4	44.2	44
Thr	7.26	61.0	33.0	33
Ser	6.50	61.9	33.4	33
Glu	18.6	126.8	68.5	68
Pro	4.45	38.7	20.9	21
Gly	6.21	82.7	44.7	45
Ala	5.59	62.8	33.9	34
Cys(1/2)	5.46	45.1	24.4	24
Val	6.09	52.0	28.1	28
Met	1.55	10.4	5.6	6
Ile	3.07	23.4	12.7	13
Leu	7.95	60.6	32.8	33
Tyr	3.50	19.3	10.4	10
Phe	7.05	42.6	23.0	23
Lys	5.69	38.9	21.0	21
His	3.16	20.4	11.0	11
Arg	8.34	47.9	25.9	26
Trp	4.73	23.2	12.6	13

[a] Calculated values for comparison with other proteins and calculated numbers of residues for a given molecular weight.

buffers, and the factor X containing fractions of the column effluent. These preparations of factor X which were isolated in the presence of DFP were then further chromatographed on Sephadex A-50, again using buffer solutions containing DFP (10^{-3} M). Disc electrophoretic examination of the protein eluted from such Sephadex A-50 columns showed only a single band; hence it is fairly definite that the three disc bands of factor X from DEAE-Sephadex were artifacts which could be eliminated by the inclusion of DFP in buffer solutions and factor X samples. One possible explanation for the absence of alteration prior to chromatography on DEAE-cellulose can be found by considering the change in pH which occurs at this stage, namely, the $BaSO_4$ eluate was prepared at pH 5.8 for the reason presented in Jackson *et al.* (1968), but the effluent from the DEAE-cellulose column was at pH 8.0. Thus a change was made from a pH region at which thrombin is relatively inactive to one at which it is enzymatically quite active.

Additional support for the hypothesis that thrombin might be responsible for the partial degradation comes from observations relative to the high mobility band seen previously in disc gels of factor X protein (Jackson *et al.*, 1968). Activated factor X prepared as described in a following section was incubated in Michaelis buffer at 37° with prothrombin obtained as a by-product of factor X preparation. The disc electrophoresis patterns seen after incubation of prothrombin with activated factor X are shown in Figure 4. It can be seen that the band of the most anionic protein noted in the sample of factor X from DEAE-cellulose corresponds to the band which represents the major product from the incubation of activated factor X with prothrombin. For reference, the activated factor X band in the two reaction product gels of Figure 4 is the band which is slightly more anionic than the factor X (major band) of the first gel of Figure 4.

Although it has been asserted in the sections above that all three disc gel bands represent protein with factor X activity, this has been demonstrated by isolation of the material using preparative disc electrophoresis. Complete resolution of the individual factor X disc bands was not achieved; however, fractions containing only a single band by analytical disc electrophoresis were obtained. Assays of fractions containing material associated with each of the three single bands indicated that the factor X specific activity was the same for all three series. However, this particular set of data is not sufficiently precise, when compared with the precision of assay from unaltered factor X protein, to allow any further conclusion. Single amino acid analyses of the individual altered protein species indicated that extensive losses of various amino acids had occurred in these species when they were compared with the unaltered factor X protein amino acid composition.

Ambiguity exists with respect to the evaluation of disc gel patterns for factor X protein. For example, the disc gels shown in Figure 5 are nearly the same with respect to the relative amounts of factor X protein, the major band, and detectable contaminants; however, by clotting assay the specific activities differ by more than a

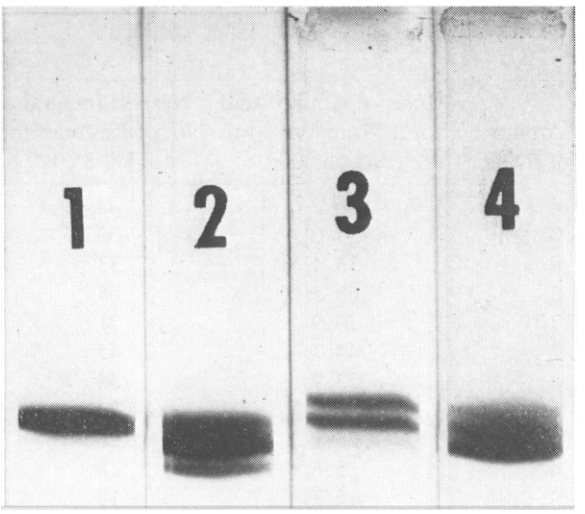

FIGURE 3: Examination of factor X by disc electrophoresis. The demonstration of three distinguishable bands of factor X. Migration is from top (cathode) to bottom (anode). (1) Factor X protein from DEAE-cellulose chromatography; (2) factor X protein after chromatography on DEAE-Sephadex A-50; sample contained an aliquot from each peak; (3) factor X protein from the leading edge of the first peak of the two peak complex of DEAE-Sephadex; and (4) factor X protein from the trailing edge of the second peak of the two-peak complex of DEAE-Sephadex. All samples contained 30–50 μg of protein.

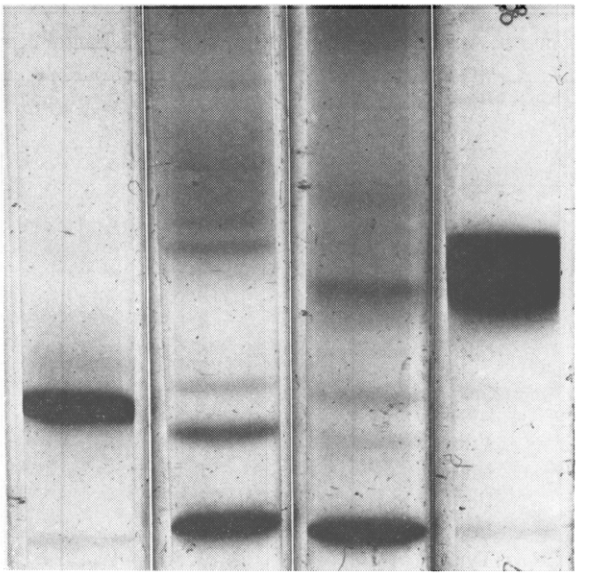

FIGURE 4: Examination by disc electrophoresis of the reaction products from incubation of prothrombin with activated factor X. Possible identification of the most rapidly migrating contaminant of purified factor X preparations. Migration is from top (cathode) to bottom (anode). Samples (from left to right): (1) factor X from DEAE-cellulose (30 μg); (2) reaction mixture containing activated factor X (2.5 μg) and prothrombin (20 μg) after incubation at 37° for 3 hr; (3) reaction mixture as in 2, but containing only 0.25 μg of activated factor X; and (4) starting prothrombin preparation (50 μg).

factor of 2. One possible explanation is that the factor X sample of lower specific activity contains inactive factor X which is electrophoretically indistinguishable from the active species. If this is correct, disc electrophoretic behavior can be seen to be of limited utility in determining the actual cause of the anomalous chromatographic behavior of factor X on DEAE-Sephadex A-50 described previously, and other properties and techniques will have to be used to attack this problem.

TABLE III: Data from Ultracentrifugation of Bovine Factor X.

Sedimentation and Diffusion Data[a]	
$s^0_{20,w}$ (S)	3.56
$D^0_{20,w}$ (cm²/sec)	5.6×10^{-7}
Molecular Weight Averages ($\bar{v}$ = 0.717 cc/g)[a]	
M_{sD}	56,360
M_w at 1.6 mg/ml	54,486
M_w at 1.2 mg/ml	55,945
M_n at 0.8 mg/ml	52,969
M_w at 0.8 mg/ml	54,846
M_z at 0.8 mg/ml	54,921

Frictional Coefficient Ratio and Axial Ratios for Equivalent Ellipsoids of Revolution

	f/f_0	Prolate Ellipsoid	Oblate Ellipsoid
No hydration	1.54	12	10
0.2 g of H_2O/g of protein	1.42	9	8

[a] In 0.001 M Tris-HCl–0.10 M NaCl (pH 7.40) at 20.0°.

Amino Acid Composition. For determination of the amino acid composition of factor X, the protein from both peaks from DEAE-Sephadex A-50 was combined and this mixed sample which had been desalted on Sephadex G-25 was used for all hydrolyses. After analysis of the hydrolysates, the results from the individual samples were normalized to an arbitrary 48-hr hydrolysate by multiplying the number of μmoles of each amino acid by a factor determined by averaging the ratios of glutamic acid, aspartic acid, glycine, alanine, and the total number of μmoles in the given hydrolysate and in the reference 48-hr hydrolysate. The values for tyrosine determined spectrophotometrically and by amino acid analysis agreed to within 1%. The results of the amino acid analyses are presented in Tables I and II. A molecular weight of 55,000 (*vide infra*) was used to calculate the total number of residues in the protein. The calculated values for the number of residues in Table II were determined using methionine and histidine as reference amino acids.

Ultracentrifugation of Factor X. Sedimentation velocity determinations were made at 59,780 rpm and at a temperature of 20.0 ± 0.1°. Sedimentation velocity experiments were performed as a function of concentration in 0.10 M NaCl buffered at pH 7.4 with 0.001 M Tris-HCl. A graph showing the concentration dependence of the sedimentation coefficient is presented in Figure 6. Determination of the coefficient of the

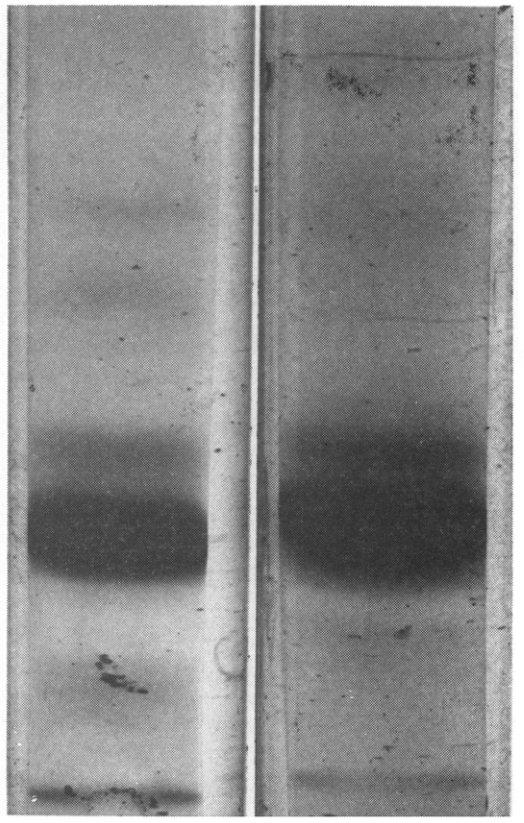

FIGURE 5: Disc electrophoresis of two preparations of factor X which differ in specific activity by a factor of two. Migration is from top (cathode) to bottom (anode). Samples: left: factor X with a specific activity of 60 units/mg of protein; right: factor X with a specific activity of 140 units/mg of protein.

concentration dependence of the sedimentation coefficient from the plot of S *vs.* C gave a value somewhat larger than expected if the molecules were spherical and relatively "normally" hydrated. Sedimentation coefficients and the coefficient of the concentration dependence are given in Table III. Photographs of the schlieren peak taken during velocity sedimentation have been published (Jackson *et al.*, 1968).

Molecular weight averages obtained from equilibrium ultracentrifugation of factor X protein using the high-speed technique of Yphantis (1964) were calculated using the computer program of Teller (1965). These data are also summarized in Table III. The partial specific volume for factor X was calculated from the amino acid composition using the data of McMeekin *et al.* (1949). A plot of the natural logarithm of the concentration *vs.* the square of the radial distance has been published (Jackson *et al.*, 1968).

Diffusion coefficients for factor X were determined from boundary spreading at low speed in the ultracentrifuge. A capillary-type double-sector synthetic boundary cell was modified to permit layering at 3000 rpm and the diffusion coefficient was determined at this speed. These data are presented in Table III. Values of f/f_0 and axial ratios for equivalent ellipsoids of revolution were calculated using the treatment of Oncley (1941). Two photographs of the schlieren peak seen during one diffusion coefficient determination are given in Figure 7.

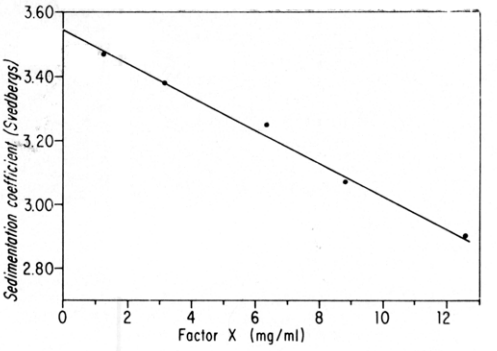

FIGURE 6: Concentration dependence of the sedimentation coefficient of factor X. Speed, 59,780 rpm; phase-plate angle, 70°; cell, 2°, double sector, sapphire windows; buffer, 0.001 M Tris-HCl–0.10 M NaCl (pH 7.40); temperature, 20°. Sedimentation coefficients were determined from the slopes of the plots of the logarithm of the radial distance (measured to the maximum ordinate) *vs.* time.

The sedimentation coefficient of maximally purified factor X at a concentration of 3.5 mg/ml was examined in the presence of: (1) 0.10 M sodium citrate, (2) 0.1 M sodium citrate plus 0.5 M NaCl, and (3) 1.0 M NaCl, all buffered at pH 8.0 with 0.05 M Tris-HCl. The results indicate that at high ionic strengths the sedimentation coefficient decreased (Table IV). Interestingly, the decrease found in the presence of citrate ions was significantly greater than that found with NaCl at the same ionic strength, which may suggest that a structural change occurs in factor X in citrate solutions. This effect of citrate can be considered to be a reversible effect as the conditions of examination by sedimentation velocity are the same as encountered by the protein during chromatography on DEAE-cellulose and DEAE-Sephadex. Whether this is related to the anomalous chromatographic behavior discussed above remains to be determined. However, the fact that such an effect is demonstrable makes quantitative examination of this behavior a logical future project.

TABLE IV: Velocity Sedimentation of Factor X in High Ionic Strength Sodium Citrate and NaCl Solutions.

Conditions	Ionic Strength[a]	$s^0_{20,w}$ (S)
0.10 M NaCl–0.001 M Tris-HCl, pH 7.0, 20°	0.1	3.46
0.10 M Trisodium citrate–0.05 M Tris-HCl, pH 8.0, 20°	0.6	3.09
0.10 M Trisodium citrate–0.5 M NaCl–0.05 M Tris-HCl, pH 8.0, 20°	1.1	2.97
1.0 M NaCl–0.05 M Tris-HCl, pH 8.0, 20°	1.0	3.25

[a] Calculated values.

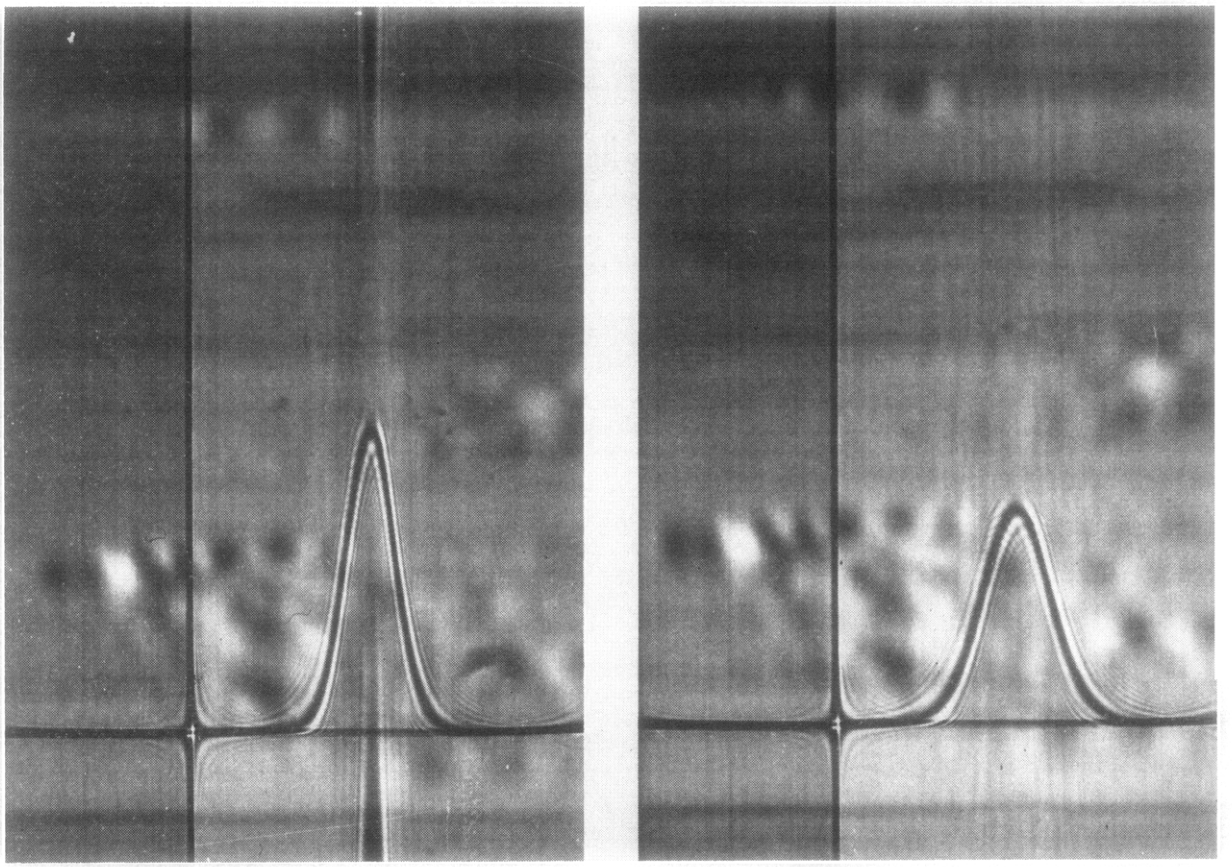

FIGURE 7: Diffusion coefficient determinations. Schlieren patterns seen during boundary spreading at low speed in the Spinco Model E ultracentrifuge. Speed, 3000 rpm; phase-plate angle, 70°; cell, 2°, double sector, synthetic boundary, modified to permit layering at 3000 rpm; buffer, 0.001 M Tris-HCl–0.10 M NaCl (pH 7.40); temperature, 20.0°. Samples, factor X (12.8 mg/ml); left: after 20 min; right: after 72 min.

During the course of isolation and characterization of the factor X protein, a variety of properties have been observed. It has been found that factor X cannot be dialyzed against distilled water or freeze dried without nearly complete loss of activity. Furthermore, the purified factor X protein precipitates in low ionic strength solutions. Factor X activity appears to be stable in solutions at pH above 5.5; at pH values below this, precipitation and concomitant total loss of activity occur. The observation that the band of factor X protein revealed by Amido Black staining of disc electrophoresis gels can also be detected by the Schiff base–periodate staining technique suggested that factor X is a glycoprotein. Qualitative determination of carbohydrate by the anthrone reaction (Ashwell, 1957) and the resorcinol reaction for sialic acid (Svennerholm, 1963) confirmed this proposal.

Activated Factor X. The *p*-toluenesulfonyl-L-arginine methyl ester esterase activity of activated factor X which was reported by Esnouf and Williams (1962) was confirmed in this study. The process of activation of factor X by the coagulant protein of Russell's viper venom was investigated to permit use of the *p*-toluenesulfonyl-L-arginine methyl ester esterase activity of activated factor X as a supplementary assay. In agreement with Williams and Esnouf (1962), it was found that Ca^{2+} was required for activation of factor X by the venom protein. In contrast to their findings, however, the coagulant protein isolated for use in these studies did not itself possess *p*-toluenesulfonyl-L-arginine methyl ester esterase activity (Jackson, 1967).

Activation was achieved by incubating at 37° a factor X protein preparation (0.2–1.6 mg/ml) possessing the maximum attainable specific activity with the coagulant protein (0.05–37 μg/ml) and $CaCl_2$ (0.005–0.020 M) in 0.05 M Tris-HCl (pH 7.4–7.9). Maximum activation was determined by measuring either the clotting activity or the *p*-toluenesulfonyl-L-arginine methyl ester esterase activity of the activated factor X. Maximum activation was obtained in 10–15 min using 0.1 μg/ml of the coagulant protein. The time course of activation over a broad range of coagulant protein concentrations provided no evidence for an autocatalytic process. In all cases, the rate curves for development of clotting activity and of *p*-toluenesulfonyl-L-arginine methyl ester esterase activity were coincident. Attempts to activate factor X at protein concentrations of 10–20 mg/ml always led to incomplete activation and precipitation of protein.

The dependence of activated factor X *p*-toluenesulfonyl-L-arginine methyl ester esterase activity on pH and *p*-toluenesulfonyl-L-arginine methyl ester concentration were investigated titrimetrically. At 37° the pH optimum was found to lie between pH 7.4 and 8.0 (Figure 8), and the apparent Michaelis constant deter-

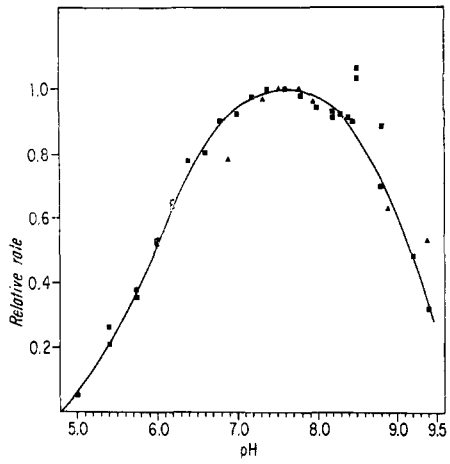

FIGURE 8: pH dependence of *p*-toluenesulfonyl-L-arginine methyl ester hydrolysis by activated factor X. Reaction mixture: 1.0 ml of 0.1 M *p*-toluenesulfonyl-L-arginine methyl ester in 0.10 M NaCl, 40 μl of activated factor X (0.4 mg/ml) (0.016 mg/ml in reaction mixture); pH maintained constant by addition of 0.018 N NaOH in 0.10 M NaCl; temperature, 37°. ▲, ■ represent different batches of activated factor X.

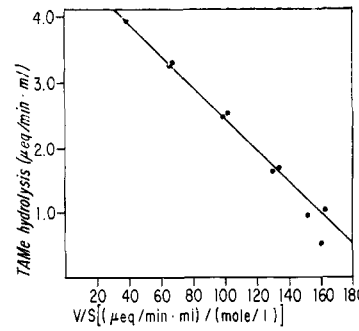

FIGURE 9: Rate of *p*-toluenesulfonyl-L-arginine methyl ester hydrolysis by activated factor X as a function of *p*-toluenesulfonyl-L-arginine methyl ester concentration. Reaction mixture: 1.0 ml of *p*-toluenesulfonyl-L-arginine methyl ester of specified concentration in 0.10 M NaCl. 40 μl of activated factor X (1.5 mg/ml) (0.07 mg/ml in reaction mixture); pH maintained constant by addition of 0.018 N NaOH in 0.10 M NaCl; temperature, 37°.

mined from an Eadie plot (Eadie, 1952; Hofstee, 1952) was 2.5 × 10^{-2} M (Figure 9). Both values agreed with those previously determined by Esnouf and Williams (1962). The extrapolated value of the turnover number was 72 μequiv/(min mg ml).

A preliminary investigation of common inhibitors of proteolytic enzymes, which also hydrolyze *p*-toluenesulfonyl-L-arginine methyl ester, was conducted to learn which of these might also inhibit activated factor X. Soybean trypsin inhibitor had been shown to inhibit the clotting activity of activated factor X (Breckenridge and Ratnoff, 1965; Lundblad and Davie, 1965); this was confirmed in this study. However, inhibition of *p*-toluenesulfonyl-L-arginine methyl ester esterase activity which was assayed at much greater protein concentrations than clotting activity appeared to be a reversible reaction and dependent upon the soybean trypsin inhibitor concentration. Both the clotting and the *p*-toluenesulfonyl-L-arginine methyl ester esterase activities were found to be inhibited by DFP and phenylmethanesulfonyl fluoride, and at the same rate. However, the concentration of DFP and of phenylmethanesulfonyl fluoride required for essentially complete inhibition in 1–2 hr was greater than 10^{-2} M. No investigation was made of the stoichiometry of binding for either inhibitor, and no other data are available which bear on inhibition at such high inhibitor concentrations.

The activation of factor X by the coagulant protein of Russell's viper venom has been followed by disc electrophoresis. It was found that under conditions in which activation was complete in less than 15 min, a single electrophoretic band of activated factor X was observed. If, however, the incubation mixture was examined 12–18 hr later, a band of activated factor X protein with a gel electrophoretic mobility greater than that of the precursor factor X was observed. No detectable change had occurred in either the activated factor X clotting activity or the *p*-toluenesulfonyl-L-arginine methyl ester esterase specific activity during this time. Variation of the concentration of coagulant protein used in the experiment did not appear to change the rate of conversion of the first form of activated factor X into the second, thus making it unlikely that the coagulant protein or a contaminant in it was responsible for this change. The observation of a quantitatively very minor, rapidly migrating band in the activated factor X disc gels suggests that what has been tentatively identified as a form of thrombin might be responsible for this second change.

Although factor X can be reproducibly isolated and purified, after activation of different preparations which are apparently indistinguishable by specific activity and disc electrophoresis, marked variability in activated factor X is found following DEAE-Sephadex A-50 chromatography. Single batches of protein have been obtained which, after activation, yield a single activated factor X peak of constant specific activity and show a single disc electrophoretic band. However, some preparations have been found to give multiple peaks, some of which are inactive, and which, on examination by disc electrophoresis, appear to have been partially degraded, *i.e.*, the disc gels show five to seven protein bands. Also, in some preparations of activated factor X, the *p*-toluenesulfonyl-L-arginine methyl ester esterase specific activity appeared to increase after chromatography on DEAE-Sephadex; however, this was found to be due to a change in $E_{1\,cm}^{1\%}$ at 280 mμ, rather than to further purification. Apparently, during activation or chromatography proteolysis occurred which resulted in the separation of high content tryptophan or tyrosine peptides from the part of the factor X polypeptide chain possessing the active site. Examples of such preparations and an extensive consideration of these problems can be found elsewhere (Jackson, 1967).

Discussion

The observations reported in this paper make it apparent that considerable work remains to be done

before isolation of pure factor X can be achieved without some alteration during purification. Alterations in factor X which were responsible for the three bands of factor X protein seen by disc electrophoresis were shown to be eliminated by inclusion of DFP in protein samples and buffer solutions used for chromatography. Evidence from a comparison of bands from contaminants in factor X preparations (Figure 4) by disc electrophoresis has been presented which suggests that thrombin may be responsible for these alterations. The demonstration of gross changes in amino acid composition in the isolated fractions from preparative disc electrophoresis isolation of altered protein coupled with the prevention of these alterations by DFP and the evidence supporting the presence of thrombin in factor X protein eluted from DEAE-cellulose columns strongly support the hypothesis that the alterations are the result of proteolytic degradations.

On the basis of the observations concerning DFP-preventable alteration in factor X protein, the hypothesis may be entertained that the double peaking observed during chromatography on DEAE-Sephadex A-50 also is the result of a similar, but much less extensive, degradation. If this is so, at least two likely possibilities exist, namely, proteolytic degradation which does not sufficiently alter the gel electrophoretic properties of the protein to permit detection of the change by this technique, or the loss of carbohydrate residues since factor X appears to be a glycoprotein. This first possibility is made quite plausible by the observations on disc gel patterns and specific activity shown in Figure 5. Even though a significant difference in the apparent molecular weight as determined by gel filtration could be demonstrated in factor X altered with respect to the disc electrophoretic criterion, the homogeneity with respect to the various molecular weight averages obtained by equilibrium ultracentrifugation of the mixture of the protein from the two peaks (Table III) indicates that there is no gross difference in the molecular weight of the two chromatographically distinguishable species. Further investigation is being carried out to resolve this problem.

The gross difference in molecular weight determined by sedimentation equilibrium and the apparent molecular weight from gel filtration would appear to be explained by the asymmetry of the factor X protein molecule which is seen from the calculated axial ratios for equivalent ellipsoids of revolution (Table III). One qualification must be made on the molecular weight reported here, however. Inasmuch as factor X appears to be a glycoprotein, the molecular weight calculated using specific volumes of amino acids and the factor X amino acid composition would have to be revised downward after a value of $\bar{v}$ is determined by pycnometric means. A value for $\bar{v}$ of 0.690 ml/g, which is a reasonable estimate for a plasma glycoprotein (Gottschalk, 1966), would result in a decrease in the molecular weight to about 50,000.

The observation that a change in the sedimentation coefficient of factor X occurs in the presence of citrate ions may indicate a specific effect on the factor X molecule; however, further investigation is required before this phenomenon can be accepted as representing more than gross nonideality of the protein–citrate solution.

There is apparently little agreement between the physicochemical data obtained by Esnouf and Williams (1962) on their preparations of bovine factor X and that reported here from examination of the protein prepared according to Jackson *et al.* (1968). Values for the molecular weight, sedimentation coefficients, and the diffusion coefficients for these two groups of factor X protein preparations exhibit significant differences. Inasmuch as the reference points for determination of the degree of purification and the factor X assays used by Esnouf and Williams were different from those used in this study, unambiguous comparison is impossible, however.

On the other hand, comparison with the data reported for autoprothrombin III by Seegers *et al.* (1967) indicates considerable agreement in the physicochemical characteristics of the two protein preparations. Both the values for the sedimentation coefficient extrapolated to zero concentration and the concentration dependence of the sedimentation coefficient appear to agree quite well, namely, $s^0_{20,w}$ values of 3.4 and 3.56 S and, at 12 mg/ml, 3.0 and 2.9 S for autoprothrombin III and factor X, respectively. After conversion of the amino acid data of Seegers *et al.* (1967), which were given as number of residues for a protein of mol wt 15,000, into those for a protein of mol wt 55,000, the similarity in the two species is again striking. No sedimentation equilibrium value or diffusion coefficient for calculation of a sedimentation–diffusion value of molecular weight was given by Seegers *et al.* (1967): however, a protein with a sedimentation coefficient of 3.4 S would be expected to have a minimum molecular weight at least twice that chosen by these workers for calculation of their amino acid data. If further experiments indicate more extensive agreement between these two species, some of the confusion in the models for blood coagulation may be eliminated also. In contrast to the observation reported in this paper that activated factor X activity could be inhibited by DFP and phenylmethanesulfonyl fluoride, autoprothrombin C activity has been reported as not inhibited by these agents (Seegers *et al.*, 1962, 1965). However, observations in this study indicate that no significant inhibition of activated factor X would have been detected at the low level of DFP used by Seegers *et al.* (1962); hence, until autoprothrombin C inhibition is attempted using higher DFP concentrations, this difference will remain uncertain.

The failure to obtain reproducibly preparations of activated factor X of constant clotting specific activity indicates problems that remain in this area. On the basis of the variation in $E^{1\%}_{1\,cm}$ observed in different preparations of activated factor X, alterations obviously occur in factor X preparations during the activation process. One explanation for which some evidence exists is that during the latter process the activated factor X converts contaminant prothrombin to thrombin which in turn may degrade the activated factor X. If this is true, it may be possible to eliminate the problem of degradation by activation of factor X in the presence

of DFP at concentrations sufficient to inhibit thrombin but without significant inhibition of the activated factor X. This is currently under investigation.

Although ambiguity still exists with respect to some of the properties of factor X, sufficient data are now available for guiding additional research on this component of the blood coagulation process.

Acknowledgments

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