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Bovine Factors X_1 and X_2 (Stuart Factor). Isolation and Characterization†

Kazuo Fujikawa, Mark E. Legaz, and Earl W. Davie*

ABSTRACT: Bovine factors X_1 and X_2 (Stuart factor) were purified by $BaSO_4$ adsorption and elution, DEAE-Sephadex batch adsorption and elution, and DEAE-Sephadex column chromatography. Each protein was homogeneous by immunoelectrophoresis, gel electrophoresis, and zone electrophoresis. Factors X_1 and X_2 were heterogeneous by sedimentation equilibrium centrifugation, but this was attributed to reversible association of the protein. The monomer molecular weight calculated for each protein was about 55,000. The amino acid and carbohydrate compositions of the two proteins are essentially identical and neither protein contains free sulfhydryl groups. The two proteins also appear to have the same immunological properties when tested against antibodies to either factor X_1 or X_2. Each protein contains 10% carbohydrate which includes 3.8% neuraminic acid (*N*-acetylneuraminic acid and *N*-glycolylneuraminic acid), 2.9% hexose (galactose and mannose), and 3.6% hexosamine (galactosamine and glucosamine). Following reduction with 2-mercaptoethanol, a heavy chain with a molecular weight of about 38,000 and a light chain with a molecular weight of about 17,000 were isolated from each protein. The heavy chain from either factors X_1 or X_2 has an amino-terminal sequence of Trp-Ala-Ile-His- and contains nearly all of the carbohydrate. The amino acid composition and carbohydrate content appear to be the same for both heavy chains. The light chain from either factor X_1 or X_2 has an amino-terminal sequence of Ala-Asn-Ser-Phe-, and the total amino acid composition also appears to be essentially the same for these two chains. It was concluded that factors X_1 and X_2 are made up of one heavy and one light chain held together by one or more disulfide bonds. Furthermore, the difference between the two proteins appears to be very minor.

Factor X (Stuart factor)[1] is a plasma protein which participates in intrinsic and extrinsic blood coagulation. It plays a central role in blood coagulation since it can be activated independently by either the intrinsic or extrinsic coagulation system. Once factor X is converted to an activated form (factor X_a), it forms a macromolecular complex in the presence of factor V (proaccelerin), phospholipid, and calcium ions, and this complex is capable of converting prothrombin to thrombin (Papahadjopoulos and Hanahan, 1964; Milstone, 1964; Denson, 1967; Barton *et al.*, 1967; Esnouf and Jobin, 1967; Jobin and Esnouf, 1967; Ferguson *et al.*, 1967).

In order to study in detail the mechanism by which factor X participates in blood coagulation, highly purified preparations with well defined physical–chemical characteristics are required. Accordingly, a number of methods have been developed in recent years for the isolation of factor X from both bovine and human plasma (Hougie and Bunting, 1960; Duckert *et al.*, 1960; Esnouf and Williams, 1962; Papahadjopoulos *et al.*, 1964; Jackson *et al.*, 1968; Aronson *et al.*, 1969). Bovine factor X can also be partially separated into two protein peaks by chromatography on DEAE-Sephadex A-50, and both of these fractions can be converted to factor X_a in the presence of Russell's viper venom (Jackson and Hanahan, 1968). Factor X is presumably identical with prothrombokinase, the precursor of thrombokinase (Milstone, 1964), and autoprothrombin III, the precursor of autoprothrombin C (Seegers *et al.*, 1967; Seegers, 1969).

In the present experiments, bovine factor X was isolated in the presence of several protease inhibitors and separated into factors X_1 and X_2 by chromatography on DEAE-Sephadex A-50. The two proteins were then studied in detail in regard to their size, electrophoretic and immunological properties, amino acid, carbohydrate and subunit compositions, and amino-terminal sequences. The following papers describe some of the molecular events which occur when this protein is activated to factor X_a by a protein present in Russell's viper venom (Fujikawa *et al.*, 1972) and the amino acid sequence of the cyanogen bromide peptide which contains the serine present in the active site (Titani *et al.*, 1972).

Materials

Benzamidine hydrochloride and cyclohexanone were purchased from Aldrich Chemical Co., Milwaukee, Wis. Heparin sodium salt (Grade I), soybean trypsin inhibitor (types I and II), cephalin (rabbit brain extract), neuraminidase (*Clostridium perfringens*), bovine albumin, mannose, galactose,

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[1] The nomenclature for various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).

glucosamine, galactosamine, *p*-dimethylaminobenzaldehyde, acetylacetone, *N*-acetylneuraminic acid, and thiobarbituric acid were purchased from Sigma Chemical Co., St. Louis, Mo. Barium sulfate (X-ray grade) was obtained from Merck & Co., Rahway, N. J. DEAE-Sephadex A-50 and Sephadex G-100 were products from Pharmacia Fine Chemicals, Piscataway, N. J., and Agarose was obtained from Marine Colloids, Inc., Springfield, N. J. Monoiodoacetic acid, 2-mercaptoethanol, *N,N'*-methylenebisacylamide, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Kodak Co., Rochester, N. Y. Acrylamide was obtained from Matheson Coleman & Bell, Norwood, Ohio. Guanidine hydrochloride was purchased from Baker Chemical Co., Phillipsburg, N. J. Lyophilized *Vipera russelli* was obtained from Ross Allen Reptile Institute, Inc., Silver Springs, Fla. Diisopropyl phosphofluoridate (DFP)[2] was purchased from Pierce Chemical Co., Rockford, Ill. Bovine factor X deficient plasma was made by the method of Bachmann *et al.* (1958). Human plasma α_1-glycoprotein was kindly provided by Dr. S. Hakomori, University of Washington, Seattle. All other chemicals were commercial preparations of the highest quality available.

Methods

Factor X concentration was measured by absorption at 280 nm assuming an $E_{280}^{1\%} = 12.4$ (Jackson *et al.*, 1968). Protein concentration was also determined by the biuret method (Gornall *et al.*, 1949) employing crystalline bovine serum albumin as a standard. For carbohydrate analysis, protein was determined by dry weight analysis corrected for 6% moisture content.

Samples were prepared for amino acid analyses by the method of Moore and Stein (1963) and analyzed for amino acids according to the method of Spackman *et al.* (1958) employing a Spinco Model 120 amino acid analyzer. Samples were hydrolyzed in 6 N HCl at 110° for 24, 48, 72, and 96 hr in evaculated tubes. The values for serine and threonine were determined by extrapolation to zero-time hydrolysis. Isoleucine and valine values were calculated from the 96-hr hydrolysis. Tryptophan was estimated by the method of Bencze and Schmid (1957), and half-cystine was determined as cysteic acid by the method of Hirs (1967) or as *S*-carboxymethylcysteine by the method of Crestfield *et al.* (1963). Free sulfhydryl groups were determined by the method of Ellman (1959).

Neutral sugar was determined by the phenol–sulfuric acid method of Dubois *et al.* (1956) using a 1:1 mixture of mannose and galactose as a standard. For the determination of hexosamine, samples were hydrolyzed in 2 N HCl for 24 hr at 110° and analyzed by the method of Elson and Morgan as described by Gardell (1957) using galactosamine as a standard. Neuraminic acid was determined by the thiobarbituric acid method of Warren (1959) using human α_1-glycoprotein as a standard. This protein contains approximately 11% neuraminic acid (Jeanloz, 1966). Each component of neutral and amino sugar was analyzed by gas chromatography according to the method of Yang and Hakomori (1971). Analyses of *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid were performed by silica gel thin-layer chromatography using the solvent system of Granzer (1962) [propanol–1 N NH_4OH–H_2O, 6:2:1, v/v]. Neuraminic acid was also hydrolyzed from factor X_1 or X_2 essentially by the method of Morell *et al.* (1971) using neuraminidase (*Cl. perfringens*). Following digestion, the reaction mixture was dialyzed against distilled water overnight at 4° and the dialysate was lyophilized and analyzed by thin-layer chromatography. The neuraminic acid was identified by employing the resorcinol–Cu^{2+} reagent (Svennerholm, 1957).

Zone electrophoresis was carried out in 0.05 M sodium barbital (pH 8.6) on microscope slides (25 × 75 mm) layered with 2.0% Agarose and 3% nonpolymerized acrylamide as described by Williams and Chase (1971). Samples (5 μl containing 5–10 μg of protein) were placed in a small well and electrophoresis was carried out at room temperature for 30 min with 150 V and 5 mA/slide. The slides were stained for protein with 0.1% Aniline Blue Black in 7.5% acetic acid.

Immunoelectrophoresis with 2.0% Agarose and 3% nonpolymerized acrylamide on microscope slides was carried out according to the method of Scheidegger (1955). Essentially the same conditions were employed as those for zone electrophoresis. Samples were diluted in 0.05 M sodium barbital buffer (pH 8.6) and run for 30 min. Antibody was added to the center trough and allowed to diffuse for 24 hr.

Polyacrylamide disc gel electrophoresis was performed at pH 9.4 according to the method of Davis (1964). The gels were stained for protein with Aniline Blue Black or for carbohydrate by the method of Zacharius *et al.* (1969). The same procedure was used for urea gel electrophoresis except that the gels were prepared in 6 M urea and the samples were dissolved in the buffer containing 6 M urea.

Ultracentrifugation was carried out in a Beckman Model E-990 analytical ultracentrifuge which was equipped with an electronic speed control. The optics were focused at the two-thirds plane of the cell. The lenses and other optical components were centered on the optic axis. The sedimentation equilibrium data were derived using the short-column, high-speed technique of Yphantis (1964). A six-channel Kel-F centerpiece was employed, and Rayleigh patterns were recorded on Kodak II-G photographic plates. The plates were read on a modified Nikon microcomparator, and computations were preformed using computer programs developed by Harris *et al.* (1969).

Sedimentation equilibrium measurements with native factors X_1 and X_2 were performed at three different concentrations (0.75, 0.5, and 0.25 mg per ml) in 0.05 M Tris-HCl–0.1 M KCl (pH 8.0, $\rho = 1.0028$), while the respective subunit molecular weights were determined in 6 M guanidine-HCl ($\rho = 1.1415$). All samples were dialyzed for 4 days prior to ultracentrifugation, and the appropriate sample concentration was obtained by dilution with dialysate. A method developed by Teller *et al.* (1969) was employed for the determination of optimal rotor speed and the approximate time to attain equilibrium. The attainment of equilibrium was determined by reading the fringe displacement at a given radial position on photographs taken 24 hr after attainment of the desired rotor speed and at 4-hr increments thereafter. A base-line run to correct for window distortion followed each experiment. All determinations were carried out at 20° at a rotor speed of 23,000 rpm for the native factors X_1 and X_2. The rotor speed was 17,000 and 36,000 rpm, respectively, for the heavy and light chains. A partial specific volume of $\bar{v} = 0.717$ ml/g was determined by amino acid analysis and corrected for 10% carbohydrate content (Cohn and Edsall, 1943; Longsworth, 1953). This partial specific volume is derived from the properties of the native protein in dilute solutions. Hade and Tanford (1967) have shown from isopiestic studies that in concentrated guanidine-HCl solutions $\bar{v}$ may be decreased by as much as 0.01 ml/g as a result of preferential guanidination of

[2] Abbreviation used is: DFP, diisopropyl phosphofluoridate.

certain proteins. However, according to Teller[3] this type of preferential interaction is not a ubiquitous property of proteins. Therefore, the $\bar{v}$ used in determining subunit molecular weights was identical with that of the native protein.

Preparation of Heavy and Light Chains of Factors X_1 and X_2. Reduction and alkylation of factor X was performed according to the method of Crestfield *et al.* (1963). The solution containing the carboxymethylated protein was desalted by gel filtration on a Sephadex G-25 column (2 × 40 cm) eluted with 0.2 M ammonium bicarbonate. To ensure the complete inactivation of any DFP-sensitive proteinase(s) that may be present in the factor X preparation, the eluate was treated for 3 hr with 10^{-2} M DFP and lyophilized.

Two polypeptide chains were separated by gel filtration of a solution of the reduced and alkylated factors X_1 and X_2. In these experiments, 100 mg of protein was dissolved in 6–8 ml of 0.2 M ammonium bicarbonate and passed through a Sephadex G-100 column (2.5 × 100 cm) with 0.2 M ammonium bicarbonate as the eluting solution.

Amino-Terminal Sequence. Automated Edman degradations were performed with a Beckman Sequencer Model 890A. The mode of operation of the instrument and the methods of sequenator analysis are adaptions (Hermodson *et al.*, 1972) of the technique of Edman and Begg (1967). For the amino-terminal analysis of the whole protein, 10-mg samples of factor X_1 or X_2 were employed. In experiments dealing with the amino-terminal analyses of the heavy or light chains, sample sizes ranged from 5 to 11 mg of carboxymethylated protein.

Antibody Preparation. Rabbits were immunized against factor X_1 or X_2 by multisite subcutaneous injection of the purified protein (each 1.5 mg) and Freunds complete adjuvant. Three weeks after the first injections, the rabbits were given a second and third injection in consecutive weeks in the marginal ear vein with 1.0 mg of protein suspended in 0.75% aluminum potassium sulfate and 0.15 M NaCl. The rabbits were then bled 1 week later by heart puncture, and the blood was allowed to clot and retract overnight in the cold room. The sera were treated with $BaSO_4$ (100 mg/ml) for 30 min at room temperature and then centrifuged. Saturated ammonium sulfate was added to 33% saturation. The pellet obtained by centrifugation was dissolved in a half-original volume of 0.15 N NaCl. This procedure was repeated twice and the solutions were dialyzed extensively against 0.001 M phosphate buffer (pH 7.4). The precipitated euglobulin fraction was removed by centrifugation, and NaCl was added to the supernatant until the final concentration was 0.15 M. The supernatant which contained the antibody was stored frozen until use.

Clotting Assays. Factor X activity was assayed by the method of Bachmann *et al.* (1958). The test sample was diluted with Michaelis buffer (3.6×10^{-2} M sodium acetate, 3.6×10^{-2} M sodium barbital, and 1.45×10^{-1} M sodium chloride, pH 7.4) containing 0.1 mg/ml of bovine serum albumin. The diluted sample (0.1 ml) was incubated at 37° for 30 sec with 0.1 ml of phospholipid–Russell's viper venom mixture (one vial of Sigma rabbit brain extract and 0.5 mg of crude Russell's viper venom dissolved in 100 ml of 0.15 M NaCl) and 0.1 ml of factor X deficient plasma. A 0.1-ml solution of 0.025 M $CaCl_2$ was added to the mixture and the clotting time was determined. Normal human plasma was used as a standard. One unit of activity was defined as that amount of factor X activity present in 1.0 ml of normal human plasma. Clotting assays for thrombin, prothrombin, factor VII, and factor IX were carried out as previously described (Schmer *et al.*, 1972).

Purification of Bovine Factor X. Bovine blood was collected in ten 10-l. polyethylene buckets, each containing 1 l. of anticoagulant solution [13.4 g of sodium oxalate, 100 mg of heparin (17,000 units), 10 g of benzamidine-HCl, and 100 mg of crude soybean trypsin inhibitor]. Mixing of the blood with anticoagulant was facilitated by pouring the blood into a second container and then into a 20-l. polyethylene vessel. The blood was centrifuged at 4° for 15 min at 6,000*g* in a Sorvall RC3 centrifuge or for 70 min at 1500*g* in an International Model PR-2 centrifuge. The plasma was mixed with barium sulfate (40 g/l.) for 30 min, and the barium sulfate which adsorbs factor X was collected by centrifugation at 4° for 20 min at 1500*g*. The $BaSO_4$ precipitate was suspended in about 3.5 l. of 0.005 M sodium acetate by mixing at high speed for 1 min in a 4-l. Waring Blendor followed by centrifugation. The washing of the $BaSO_4$ precipitate was repeated four times. Factor X was eluted from the barium sulfate by mixing in the Waring Blendor (1 min at high speed) with 4 l. of 0.2 M sodium citrate (pH 7.0) containing 10^{-2} M benzamidine-HCl and 160 mg of crude soybean trypsin inhibitor followed by slow stirring for 30 min. The eluate was then dialyzed overnight against approximately 40 l. of distilled water. The dialysate was centrifuged at 4° for 15 min at 6000*g*. The supernatant was stirred for 15 min with 500 ml of DEAE-Sephadex A-50 (settled volume) previously equilibrated with 0.05 M sodium citrate (pH 7.0). After the DEAE-Sephadex was allowed to settle for 15 min, the supernatant was decanted and employed for the preparation of bovine factor IX. DEAE-Sephadex was then poured into an empty column (5.0 × 50 cm) and washed with 1 l. of 0.1 M sodium citrate buffer (pH 7.0) containing 10^{-3} M benzamidine-HCl. Factor X was eluted with 0.2 M sodium citrate buffer (pH 7.0) containing 1×10^{-3} M benzamidine-HCl. The first 300 ml of eluate were combined for the preparation of factor IX and the next 1.5 l. of eluate were collected for factor X. Purified soybean trypsin inhibitor (30 mg) was then added to the factor X fraction which was then diluted with an equal volume of ice–water and applied to a DEAE-Sephadex column (4.0 × 20 cm). This column was previously equilibrated with 0.1 M sodium citrate buffer (pH 7.0) containing 10^{-3} M benzamidine-HCl. After washing the column with 0.5–1.0 l. of the same buffer, the protein was eluted with a linear gradient prepared from 3 l. of 0.1 M sodium citrate buffer and 3 l. of 0.2 M sodium citrate buffer, each containing 1×10^{-3} M benzamidine-HCl. The first 2 l. of eluate were discarded and the remaining eluate was collected in 25-ml fractions at a flow rate of approximately 50 ml/hr. Factor X was separated into two biologically active protein peaks by this step. The first peak eluted from the DEAE-Sephadex column was called factor X_1, and the second peak was called factor X_2. Each of the peaks containing factor X_1 or X_2 was combined separately and each was concentrated to about 50 ml by ultrafiltration with an Amicon ultrafiltration apparatus employing a PM-10 Diaflow membrane. The pH of each solution was raised to 8.0 with 1.0 M Tris-HCl buffer (pH 8.4) and then made 1×10^{-2} M with a stock solution of 1.0 M DFP dissolved in 2-propanol. The solutions were allowed to stand overnight at 4°. Each solution was then dialyzed for 2 days against two changes of 4 l. of 0.025 M Tris-HCl (pH 8.0) containing 0.2 M sodium chloride. The solutions of factors X_1 and X_2 were then frozen at −20° until further use. For the chemical analysis, salt-free preparations were obtained by gel filtration through a Sephadex G-25 column (4.0 × 40 cm) with 0.2 M ammonium bicarbonate followed by

[3] D. Teller, personal communication.

TABLE I: Purification of Bovine Factors X_1 and X_2.

Purifcn Step	Vol (ml)	Total Protein (mg)	Total Act. (Units)	Sp Act. (Units/mg)	Recov (%)	Purifcn
Plasma	44,000	3.32×10^6 [a]	32,600[b]	0.00876	100	1
$BaSO_4$ eluate	5,000	1.69×10^4 [a]	27,400[c]	1.62	83.9	185
First DEAE-Sephadex	1,680	722	23,400	32.3	71.2	3,680
Second DEAE-Sephadex						
X_1	520	102[d]	9,410	92.3		
X_2	480	61[d]	6,530	107		
$X_1 + X_2$		163	15,900	~97.5	48.9	10,100

[a] Protein concentration was determined by the biuret method. [b] The original factor X activity was determined on a small sample collected in the absence of heparin, benzamidine, and soybean trypsin inhibitor. [c] This sample was passed through a DEAE-Sephadex column (1.0 × 1.0 cm) which was equilibrated with 0.2 M sodium citrate. [d] Protein concentration was determined by absorbance at 280 nm.

lyophilization. The lyophilized salt-free proteins had a moisture content of 6%.

Results

Preparation of Bovine Factors X_1 and X_2. The steps and the purification achieved for a typical preparation of bovine factors X_1 and X_2 are shown in Table I. The procedure involves a barium sulfate adsorption and elution, a DEAE-Sephadex batch adsorption and elution, and DEAE-Sephadex column chromatography. The separation of factors X_1 and X_2 by DEAE-Sephadex column chromatography is shown in Figure 1. In this step, factor X_1 (the first peak containing factor X activity) is readily separated from factor X_2. Upon rechromatography, factor X_1 or X_2 is eluted from the DEAE-Sephadex column in the same position as that found for the mixture.

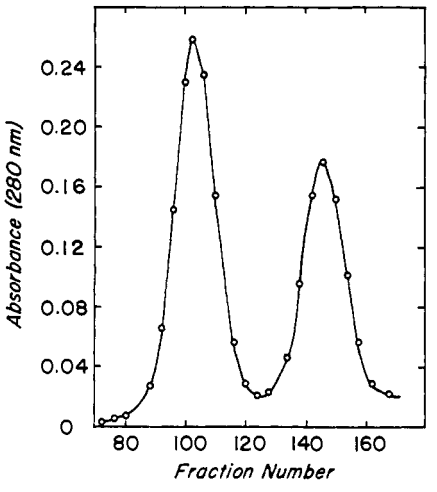

FIGURE 1: Separation of factors X_1 and X_2 by DEAE-Sephadex A-50 column chromatography. The eluate from the first DEAE-Sephadex batch adsorption and elution was applied to a column (4.0 × 20 cm) of DEAE-Sephadex which was equilibrated with 0.1 M sodium citrate (pH 7.0) containing 1×10^{-3} M benzamidine. The protein was eluted by a linear gradient of sodium citrate buffer containing 1×10^{-3} M benzamidine, as described in Methods. The first 2.0 l. of the eluate were discarded, and the eluate was then collected in 25-ml samples at a flow rate of 50 ml/hr. The absorbance due to protein was calculated by subtracting the basal absorbance of 1×10^{-3} M benzamidine.

Similar results for rechromatography of these two proteins have been published by Jackson and Hanahan (1968) who first described the two forms of factor X. The overall recovery from plasma for factor X activity ranged from 40 to 50% with a purification of 10,000- to 12,000 fold. The final specific activity ranged from 90 to 130 for various preparations. This deviation, however, was due to variations from 1 month to another in the substrate plasma employed in the assay. The weight ratio of factor X_1 to factor X_2 was consistently found to be about 1.6–1.7:1.0 in all preparations which were routinely made from the blood of 10 animals. The high yield of factor X was dependent upon the collection of blood in oxalate, heparin, benzamidine, and soybean trypsin inhibitor and fractionation of factor X in the presence of benzamidine and soybean trypsin inhibitor to limit proteolysis. Factors X_1 and X_2 prepared in the absence of these inhibitors often had decreased activity, were isolated in low yield, and underwent degradation and slow activation to factor X_a during preparation and storage. Factors X_1 and X_2 prepared by this method were also free of the other vitamin K dependent coagulation factors. This was shown by testing 0.1 ml of an undiluted factor X_1 or factor X_2 solution (1 mg/ml) for factors VII, IX, thrombin, and prothrombin, as described in Methods.

Polyacrylamide Gel Electrophoresis of Bovine Factors X_1 and X_2. Single sharp protein bands were obtained by polyacrylamide gel electrophoresis of factors X_1 and X_2 (Figure 2). Gel number 1 is factor X_1, and gel number 2 is an equal mixture of factors X_1 and X_2. Gel number 3 is factor X_2. It is clear from these experiments that factors X_1 and X_2 are homogeneous by gel electrophoresis and are indistinguishable from each other under these conditions. Factor X is a glycoprotein, and this is shown by gel electrophoresis experiments followed by staining with the periodic acid–Schiff base reagent (Figure 3). In these experiments, factor X_1 was stained for protein with Aniline Blue Black (gel on the left), and this protein band corresponds to the carbohydrate-positive band (gel on the right). Similar results were obtained for factor X_2.

Zone Electrophoresis of Bovine Factors X_1 and X_2. For further evidence of purity, factors X_1 and X_2 were subjected to electrophoresis at pH 8.6 employing microscope slides layered with Agarose–nonpolymerized acrylamide (Figure 4). After electrophoresis for 30 min, a single protein spot was observed for factor X_1 (sample 1) and for factor X_2 (sample 2). A mix-

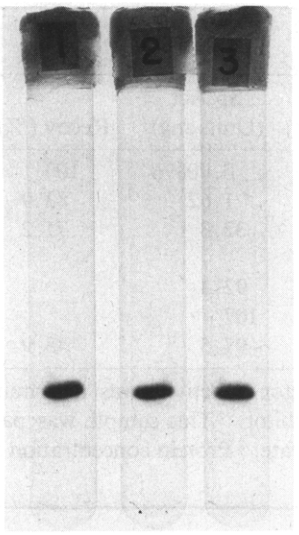

FIGURE 2: Disc gel electrophoresis of bovine factors X_1 and X_2. Gel electrophoresis was carried out in 7.5% polyacrylamide gels at pH 9.4. Factors X_1 and X_2 obtained from DEAE-Sephadex column (Figure 1) were subjected to electrophoresis, as described in Methods, and stained for protein with 1% Aniline Blue Black in 7.5% acetic acid. Gel 1 is 40 μg of factor X_1, gel 2 is 20 μg each of factors X_1 and X_2, and gel 3 is 40 μg of factor X_2.

ture containing equal amounts of factors X_1 and X_2 also yields a single protein spot (sample 3) under these conditions.

Immunoelectrophoresis of Bovine Factors X_1 and X_2. Purified factors X_1 and X_2 were also subjected to electrophoresis on Agarose–nonpolymerized acrylamide slides followed by immunodiffusion against rabbit antibody prepared from either factor X_1 or X_2. Sample 1 in Figure 5 shows factor X_1, and sample 2 shows factor X_2 after electrophoresis and precipitation by rabbit antibody to factor X_1 which was added to the center trough. Samples 3 and 4 show similar experiments for factors X_1 and X_2 employing rabbit antibody to factor X_2. It is clear from these experiments that a single sharp precipitin line is formed for each protein, and these precipitin lines are formed equally well with antibody prepared against either factor X_1 or factor X_2. The antibodies employed in these experiments also readily neutralized factor X_1 or factor X_2 activity when measured in the regular factor X assay (Figure

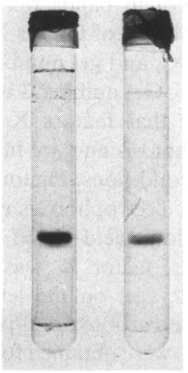

FIGURE 3: Disc gel electrophoresis pattern for bovine factor X_1 stained for protein and carbohydrate. Approximately 50 μg of factor X_1 was added to each 7.5% polyacrylamide gel, and the protein was stained with a solution of 1% Aniline Blue Black (left) and the carbohydrate was stained with the periodic acid–Schiff base reagent (right), as described in Methods.

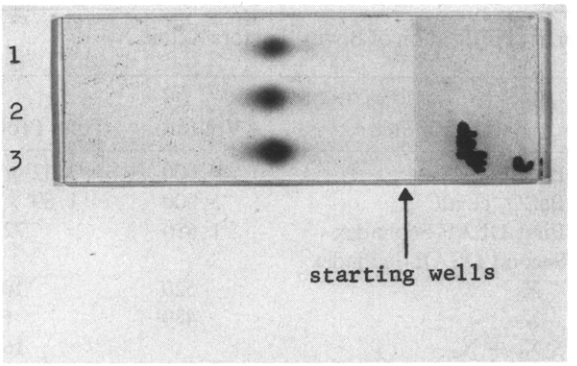

FIGURE 4: Zone electrophoresis of factor X_1, factor X_2, and an equal mixture of both proteins. Factor X_1 (5 μl containing 8 μg of protein), factor X_2 (5 μl containing 9 μg of protein), and a mixture of factor X_1 (5 μl) and factor X_2 (5 μl) were subjected to electrophoresis in 0.05 M sodium barbital buffer (pH 8.6) for 30 min at room temperature, with 150 V and 5 mA/slide. The protein was then stained with Amido Black. Sample 1 contained factor X_1, sample 2 contained factor X_2, and sample 3 contained equal amounts of factor X_1 and factor X_2. The anode is at the left of the photograph.

6). In these experiments, factor X_1 or X_2 was incubated for 5 min with increasing concentrations of antibody to factor X_1 or X_2, and aliquots were then assayed for factor X activity. The inhibition of factor X_1 by increasing concentrations of antibody to factor X_1 or X_2 was identical (solid circles). Similarly, the inhibition of factor X_2 by increasing concentrations of antibody prepared against factor X_1 or X_2 was the same (open circles). These experiments are consistent with the conclusion that factors X_1 and X_2 have essentially the same immunological properties, and the single protein precipitin line observed in the immunoelectrophoresis experiments (Figure 5) was due to the presence of factor X_1 or X_2 and not some contaminating protein.

Ouchterlony Experiments with Bovine Factors X_1 and X_2. Factors X_1 and X_2 yield single and continuous precipitin lines

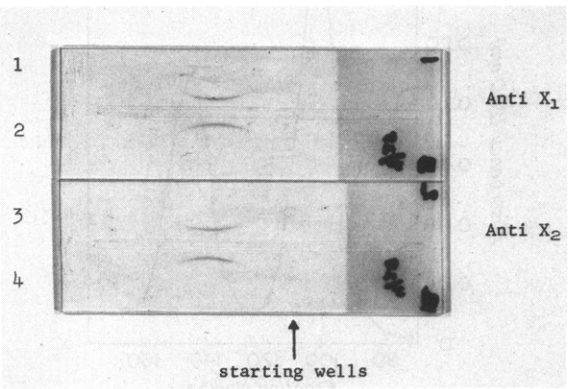

FIGURE 5: Immunoelectrophoresis of factor X_1 and factor X_2. Factor X_1 (5 μl containing 8 μg of protein) was placed in wells 1 and 3, while factor X_2 (5 μl containing 9 μg of protein) was placed in wells 2 and 4. Electrophoresis was carried out in 0.05 M sodium barbital buffer (pH 8.6) for 30 min at room temperature, and at 150 V and 5 mA/slide. Following electrophoresis, 50 μl of rabbit antibody against factor X_1 (1.5 mg/ml) and 50 μl of rabbit antibody against factor X_2 (1.8 mg/ml) were placed in the center trough of the top and bottom slide, respectively. After 24 hr, the precipitin lines were stained as outlined in Methods. The anode is at the left of the photograph.

TABLE II: Molecular Weight of Bovine Factors X_1 and X_2 by Sedimentation Equilibrium.

Sample	M_n	M_w	M_z	M_1[a]
Factor X_1	57,100 ± 1640	61,600 ± 1900	74,000 ± 2700	53,000 ± 2400
Factor X_2	59,000 ± 1460	62,400 ± 1800	77,300 ± 2400	56,000 ± 2800
Factor X_1, heavy chain	38,400 ± 800	41,700 ± 1200	47,800 ± 1400	36,000 ± 1800
Factor X_2, heavy chain	43,600 ± 1000	47,000 ± 1300	52,600 ± 1600	40,700 ± 2100
Factor X_1, light chain	18,900 ± 700	22,300 ± 900	27,000 ± 1200	16,000 ± 1100
Factor X_2, light chain	21,200 ± 800	24,600 ± 1000	30,400 ± 1400	18,000 ± 900

[a] M_1 refers to the smallest molecular weight species calculated by the methods described by Teller *et al.* (1969). The M_n, M_w, and M_z values shown above are the whole cell averages of experiments from three different protein concentrations, as described in Methods.

with no detectable spurs against rabbit antibody to factor X_1 (left side of Figure 7). Similar results were obtained with factors X_1 and X_2 when antibody to factor X_2 was added to the center well (right side of Figure 7). These results provide further evidence for the marked similarity of factors X_1 and X_2.

Sedimentation Equilibrium Studies on Bovine Factors X_1 and X_2. Sedimentation equilibrium studies on native factors X_1 and X_2 demonstrated that these two proteins were heterogeneous at all protein concentrations tested (Table II). This heterogeneity, however, was attributed to limited association of the native protein. Further analysis of the data derived from the computer program of Teller *et al.* (1969) indicated that a weak association of monomeric units was occurring with both proteins. Thus, approximately 10% of the factor X_1 or X_2 molecule was present as dimers and the remaining 90% as monomers. The molecular weight calculated for the smallest species of factor X_1 was 53,000 ± 2400 and 56,000 ± 2800 for factor X_2. In 6 M guanidine-HCl, no association of factor X_1 occurred, and the minimal molecular weight calculated for the denatured protein was 55,000 ± 1800.

Amino Acid and Carbohydrate Compositions of Bovine Factors X_1 and X_2. The amino acid and carbohydrate compositions of factors X_1 and X_2 are shown in Table III. No significant difference in the amino acid compositions of the two proteins was observed. These amino acid analyses are very similar to the data published by Jackson and Hanahan (1968) for a mixture of factors X_1 and X_2 (also shown in Table III). No free sulfhydryl groups were found in either factor X_1 or X_2 as determined by the method of Ellman (1959).

The carbohydrate content for both proteins is about 10% and includes 2.8% hexose, 3.6% hexosamine, and 3.8% neuraminic acid. The hexose was identified as galactose and mannose by gas chromatography, and these two sugars are present in the approximate ratio of 1.2:1.0, respectively, in both proteins. The hexosamine was identified as galactosamine and glucosamine (and/or mannosamine), and these sugars were present in the ratio of 2.2:3.0 in both proteins. The neuraminic acid was tentatively identified as *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid. No fucose, xylose, or arabinose was detected. The total neuraminic acid content in factors X_1 and X_2 reported in Table III may be approximately 10% low. This was indicated by experiments in which the neuraminic acid was hydrolyzed from factor X_1 or X_2 by incubation with neuraminidase at pH 5.6 at 37° for 75 min with a substrate-to-enzyme ratio of 50:1 (on a weight basis). In these experiments, the neuraminic acid content in

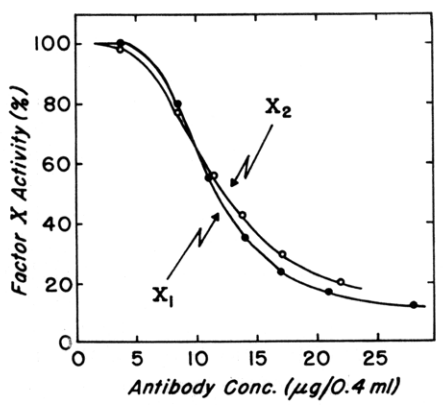

FIGURE 6: Neutralization of factor X_1 and factor X_2 by a rabbit antibody against either factor X_1 or factor X_2. Serial dilutions of an antibody against factor X_1 (8.4 mg/ml) or factor X_2 (8.6 mg/ml) were preincubated for 5 min with a solution containing factor X_1 (0.75 μg/ml) or factor X_2 (0.85 μg/ml). The samples were then assayed in the standard factor X assay, as described in Methods. The solid circles are factor X_1 solutions neutralized with antibody to factors X_1 and X_2, and the open circles are factor X_2 solutions neutralized against either antibody to factors X_1 or X_2.

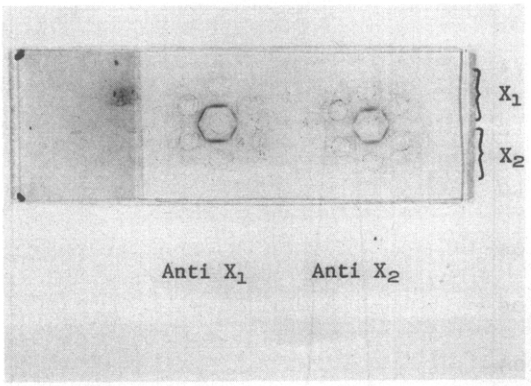

FIGURE 7: Ouchterlony immunodiffusion of factor X_1 and factor X_2 against a rabbit antibody prepared from factor X_1 or factor X_2. Factor X_1 (5 μl of 0.4, 0.6, or 0.9 mg per ml) was placed in the upper three wells, while factor X_2 (5 μl of 0.4, 0.6, or 0.9 mg per ml) was placed in the bottom three wells. A rabbit antibody against factor X_1 (5 μl of 1.5 mg/ml) and factor X_2 (5 μl of 1.8 mg/ml) was placed in the left and right center wells, respectively. After 48 hr, the excess unprecipitated protein and antibody were removed by immersing the slides in saline overnight. The slides were then allowed to dry and precipitin lines were stained with Aniline Blue Black, as outlined in Methods.

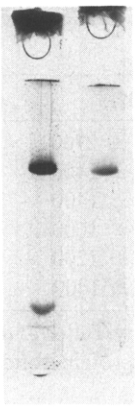

FIGURE 8: Disc gel electrophoresis of reduced factor X_1. Factor X_1 was reduced and carboxymethylated, as described in Methods, and 50 μg of protein was added to 7.5% polyacrylamide gels (pH 9.4) containing 6 M urea. The sample was stained for protein with Aniline Blue Black (left), or stained for carbohydrate with the periodic acid–Schiff base reagent (right).

factors X_1 and X_2 was approximately 10% higher than that obtained by acid hydrolysis of the glycoprotein. These results indicate that some destruction of neuraminic acid has occurred in the nonenzymatic analysis, perhaps during the hydrolysis step by dilute sulfuric acid.

Separation of Bovine Factors X_1 and X_2 into Two Chains. In the presence of 2-mercaptoethanol, factors X_1 and X_2 are broken into two chains, a heavy and a light chain, and these two chains are readily separated by gel electrophoresis (Figure 8). The gel on the left is reduced and carboxymethylated factor X_1 which was stained for protein, and the gel on the right is reduced and carboxymethylated factor X_1 which was stained for carbohydrate. Thus, the heavy or slow-moving chain contains nearly all of the carbohydrate in factor X_1. Similar results were obtained for factor X_2.

Prior reduction of factor X_1 or X_2 with 2-mercaptoethanol is necessary in order to separate the two chains by gel electrophoresis. This was shown in experiments with factor X_1 or X_2 in which the gel electrophoresis was carried out in the presence of 0.1% sodium dodecyl sulfate and in the absence of 2-mercaptoethanol. Under these conditions, the two proteins migrate as a single band.

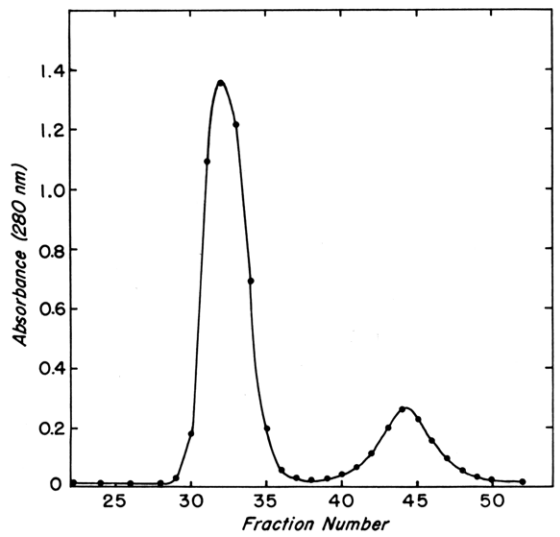

FIGURE 9: Separation of the heavy and light chains of factor X_1 by gel filtration on a Sephadex G-100 column. Reduced and carboxymethylated factor X_1 (100 mg) was subjected to gel filtration on a Sephadex G-100 column (2.5 × 90 cm). Elution was carried out with 0.2 M ammonium bicarbonate, as described in Methods.

TABLE III: Amino Acid and Carbohydrate Compositions of Bovine Factors X_1 and X_2.

Components	Mol/55,000 Factor X_1	Factor X_2	Factor X[a]
Amino acid			
Lysine	22.0	22.3	19.0
Histidine	10.2	11.0	10.0
Arginine	25.4	25.4	23.4
Aspartic acid	39.5	38.8	40.0
Threonine	29.9	28.3	29.9
Serine	28.6	29.7	30.2
Glutamic acid	56.3	57.1	62.0
Proline	18.0	18.2	19.0
Glycine	37.7	37.2	40.4
Alanine	30.0	28.6	30.7
Half-cystine[b]	19.1	19.2	22.1
Valine	26.2	26.5	25.4
Methionine	6.4	6.3	5.0
Isoleucine	11.7	12.3	11.5
Leucine	30.0	31.1	29.7
Tyrosine	8.7	8.9	9.4
Phenylalanine	19.9	20.1	20.8
Tryptophan[c]	9.9	9.3	11.4
Carbohydrate			
Hexose[d]	9.8 (2.9%)	9.1 (2.7%)	
Hexosamine[e]	9.7 (3.6%)	9.7 (3.6%)	
Neuraminic acid[f]	6.8 (3.8%)	6.8 (3.8%)	
Protein (%)	88.7	88.9	
Carbohydrate (%)	10.3	10.1	

[a] Analysis of Jackson *et al.* (1968) corrected for 10% carbohydrate. [b] Determined as cysteic acid. [c] Determined by the spectrophotometric assay. [d] Hexose included both galactose and mannose. [e] Hexosamine which included galactosamine and glucosamine was calculated as residues of *N*-acetylhexosamine. [f] Neuraminic acid which included both the *N*-acetyl and *N*-glycolyl derivatives was calculated as residues of *N*-acetylneuraminic acid.

The heavy and light chains of both proteins are also readily separated by gel filtration on Sephadex G-100 columns (Figure 9). The molecular weight for the monomer of the heavy chains of factors X_1 and X_2 was determined by sedimentation equilibrium studies and was found to be 36,000 ± 1800 for factor X_1 and 40,750 ± 2150 for factor X_2 (Table II). The molecular weight for the monomer of the light chains of factors X_1 and X_2 was found to be 16,000 ± 1100 and 18,000 ± 900, respectively. These values are in good agreement with the total molecular weight of 55,000 assuming one heavy and one light chain per mol of protein.

The amino acid and carbohydrate compositions for the heavy and light chains of factors X_1 and X_2 are shown in Tables IV and V. The amino acid composition for each of the heavy chains was calculated in moles per 38,000 g of glycopro-

TABLE IV: Amino Acid and Carbohydrate Compositions of the Heavy and Light Chains of Factor X_1.

Components	Heavy Chain (Mol/38,000)	Light Chain (Mol/ 17,000)	Heavy and Light Chains	Factor X_1 (Mol/ 55,000)
Amino acid				
Lysine	15.0	6.6	21.6	22.0
Histidine	7.0	2.5	9.5	10.2
Arginine	17.6	8.3	25.9	25.4
Aspartic acid	27.4	15.3	42.7	39.5
Threonine	21.9	7.0	28.9	29.9
Serine	18.6	12.2	30.8	28.6
Glutamic acid	36.3	32.8	69.1	56.3
Proline	17.1	2.7	19.8	18.0
Glycine	27.7	16.7	44.4	37.7
Alanine	22.8	8.5	31.3	30.0
Half-cystine[a]	9.2	16.4	25.6	19.1
Valine	23.3	6.4	29.7	26.2
Methionine	4.6	0	4.6	6.4
Isoleucine	9.9	2.4	12.3	11.7
Leucine	22.8	8.1	30.9	30.0
Tyrosine	6.2	3.2	9.4	8.7
Phenylalanine	10.4	8.1	18.5	19.9
Tryptophan[b]	7.2	1.4	8.6	9.9
Carbohydrate				
Hexose[c]	8.1 (3.5%)	1.3 (1.2%)		
Hexosamine[d]	6.3 (3.4%)	0.3 (0.4%)		
Neuraminic acid[e]	5.3 (4.5%)	0.2 (0.4%)		
Protein (%)	88.4	98.0		
Carbohydrate (%)	11.6	2.0		

[a] Determined as *S*-carboxymethylcysteine. [b] Determined by the spectrophotometric assay. [c] Hexose included both galactose and mannose. [d] Hexosamine which included both galactosamine and glucosamine was calculated as residues of *N*-acetylhexosamine. [e] Neuraminic acid which included both the *N*-acetyl and *N*-glycolyl derivatives was calculated as residues of *N*-acetylneuraminic acid.

TABLE V: Amino Acid and Carbohydrate Compositions of the Heavy and Light Chains of Factor X_2.

Components	Heavy Chain (Mol/38,000)	Light Chain (mol/ 17,000)	Heavy and Light Chains	Factor X_2 (Mol/ 55,000)
Amino acid				
Lysine	15.2	7.3	22.5	22.3
Histidine	7.8	3.1	10.9	11.0
Arginine	17.0	8.0	25.0	25.4
Aspartic acid	27.0	15.1	42.1	38.8
Threonine	23.8	7.2	31.0	28.3
Serine	20.7	10.5	31.2	29.7
Glutamic acid	33.6	29.1	62.7	57.1
Proline	16.3	3.0	19.3	18.2
Glycine	25.9	15.1	41.0	37.2
Alanine	22.7	6.4	29.1	28.6
Half-cystine[a]	9.4	15.4	24.8	19.2
Valine	20.8	5.5	26.3	26.5
Methionine	4.5	0	4.5	6.3
Isoleucine	9.3	2.2	11.5	12.3
Leucine	23.1	7.4	30.5	31.1
Tyrosine	7.1	2.9	10.0	8.9
Phenylalanine	12.5	7.9	20.4	20.1
Tryptophan[b]	7.5	1.5	9.0	9.3
Carbohydrate				
Hexose[c]	7.7 (3.3%)	0.6 (0.6%)		
Hexosamine[d]	6.6 (3.6%)	0.2 (0.2%)		
Neuraminic acid[e]	5.4 (4.4%)	0.2 (0.4%)		
Protein (%)	88.7	98.8		
Carbohydrate (%)	11.3	1.2		

[a] Determined as *S*-carboxymethylcysteine. [b] Determined by the spectrophotometric assay. [c] Hexose included both galactose and mannose. [d] Hexosamine which included both galactosamine and glucosamine was calculated as residues of *N*-acetylhexosamine. [e] Neuraminic acid which included both the *N*-acetyl and *N*-glycolyl derivatives was calculated as residues of *N*-acetylneuraminic acid.

tein. The amino acid composition for each of the light chains was calculated in moles per 17,000 g of protein. These two molecular weights are the average of the heavy and light chains from factors X_1 and X_2. Averaging these values is justified in that the heavy chains from factors X_1 and X_2 have the same mobility in sodium dodecyl sulfate gel electrophoresis. Likewise, the light chains also have the same mobility. These results indicate that the molecular weights of the two heavy chains are essentially identical. Also, the molecular weights of the two light chains are the same. There is reasonably good agreement for the amino acid composition of the intact protein with that obtained by summation of the two chains. Some amino acids, such as glutamic acid and half-cystine, however, show poor agreement. The poor agreement for half-cystine may be due in part to the fact that it was determined as cysteic acid in the intact protein and *S*-carboxymethylcysteine in the light and heavy chains. The methionine levels for light- and heavy-chain analyses may also be low due to some alkylation during the carboxymethylation reaction.

It is also apparent from these experiments that nearly all of the carbohydrate in factors X_1 and X_2 is located in the heavy chain. This is further indicated by the fact that the hexosamine and neuraminic acid present in the light chain is less than 1 equiv/mol of protein. It is not clear at this time whether the minor difference in the hexose content in the two light chains is significant.

Amino-Terminal Sequences of the Heavy and Light Chains. Amino-terminal analyses of the heavy and light chains of factor X_1 or X_2 were carried out on a Beckman Sequencer. The heavy chain from factor X_1 or X_2 was shown to contain the amino terminal sequence of Trp-Ala-Ile-His-. The light chain from either factor X_1 or X_2 was shown to contain the amino-terminal sequence of Ala-Asn-Ser-Phe-. During each cycle in the sequenator with the heavy or light chains, only one amino acid residue was observed. These results were consistent with the amino-terminal analysis on the intact factor X_1 or X_2 in which tryptophan and alanine were identified as the N-terminal amino acids and were present in approximately equimolar amounts. In the second cycle on the sequenator, alanine and asparagine were identified as the next two residues in each protein. These data indicate that factor X_1 or X_2 is composed of two different polypeptide chains, a heavy and a

light chain, and these two chains are present in equimolar amounts in the intact protein.

Discussion

In the present experiments, bovine factors X_1 and X_2 have been isolated in good yield and high stability in the presence of protease inhibitors. Diisopropyl phosphofluoridate was previously employed by Jackson and Hanahan (1968) for the preparation of factor X to restrict plasma protease activity. Some preparations of factor X undergo spontaneous activation during storage (Papahadjopoulos *et al.*, 1964). This may be due in part to the presence of trace amounts of other proteins, such as factor VII which can slowly activate factor X (Aronson and Mustafa, 1971). Factor X prepared by the present methods, however, is very stable during storage, suggesting that contaminants of this type are not present.

Factors X_1 and X_2 have very similar physical, chemical, and immunological properties. Furthermore, each is homogeneous by immunoelectrophoresis, gel electrophoresis, zone electrophoresis, and amino-terminal analysis. Sedimentation equilibrium studies, however, indicated that each protein in the native form was heterogeneous. This heterogeneity, however, is due to association of monomeric units in the absence of denaturing agents.

The monomer molecular weight calculated for each protein by sedimentation equilibrium was about 55,000. This value is in good agreement with that previously reported by Jackson and Hanahan (1968) for factor X. These investigators, however, did not observe association of their factor X preparation which included both factors X_1 and X_2. A molecular weight of 55,000 is much lower than that reported by Esnouf and Williams (1962) for their bovine factor X preparation. These investigators found a value of 84,800 ± 700 by sedimentation equilibrium and 87,000 by sedimentation velocity and diffusion data. It appears probable that the high values reported by Esnouf and Williams were due to substantial association of their preparation.

The weight ratio of factor X_1 to factor X_2 in bovine plasma was consistently found to be about 1.6–1.7:1.0 in all preparations made during the past several years in our laboratory. Furthermore, no evidence was obtained to suggest that these two molecules were interconvertible during their isolation or storage. Although a small difference in their molecular weight was observed, this difference was not considered significant. The separation of these two proteins by DEAE-Sephadex column chromatography could be due to a single amino acid replacement. Although their amino acid composition is essentially the same, a small difference would not be detected by this analysis. Additional studies will be required to test this possibility.

Factor X has been highly purified from human plasma by Aronson and coworkers (1969). Peptide maps of tryptic digests of human factor X yielded 42 peptides, and this number was increased to about 50 upon reduction and alkylation. This is about the number of peptides one would expect to find with bovine factor X which contains approximately 47 lysine and arginine residues per 55,000 g of protein (Table III). These data suggest that factor X from human and bovine sources may be very similar in composition and structure.

The present data indicate that bovine factor X is a glycoprotein present in plasma at a concentration of about 7 μg/ml. The carbohydrate present in this protein includes hexose, hexosamine, and neuraminic acid. Galactosamine was identified as one of the hexosamines by gas chromatography. A peak in the gas chromatogram was also observed for glucosamine and/or mannosamine. Since mannosamine has not been found in plasma glycoproteins, it appears most likely that the other hexosamine in factor X is galactosamine. The presence of carbohydrate in factor X was also noted earlier by Jackson and Hanahan (1968) who observed that factor X could be detected by the periodic acid–Schiff base reagent in gel electrophoresis experiments. More recently, Jackson (1972) has reported that factor X_1 contains 1–2 fewer neuraminic acid and hexose residues per mol of protein than factor X_2. In the present experiments, no significant difference in carbohydrate content between the two proteins was noted.

Tryptophan was found to be the amino-terminal residue in the heavy chains of factors X_1 and X_2, and alanine the amino-terminal residue in the light chains of factors X_1 and X_2. Our results differ from those previously published by Esnouf and Williams (1962) who found an amino-terminal glycine and alanine in their factor X preparation by the 1-fluoro-2,4-dinitrobenzene procedure. The reasons for the discrepancy between the present work and that of Esnouf and Williams are not known. It may be due in part to the tendency of tryptophanyl residues in proteins to form an *N*-formylkynurenine derivative under acidic conditions, and such a derivative present as an amino-terminal residue would not be readily detected (Spande *et al.*, 1970). Also, some proteolytic degradation of factor X may have occurred during the isolation by Esnouf and Williams yielding a new terminal glycine residue. Minor protease degradation is possible since these investigators did not employ protease inhibitors during their purification procedure.

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