Isolation and Characterization of Bovine Factor IX (Christmas Factor)†

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ABSTRACT: Factor IX (Christmas factor) has been purified approximately 22,000-fold from bovine plasma with an overall yield of 75%. The isolation procedure involves barium sulfate adsorption and elution, batchwise DEAE-Sephadex adsorption and elution, DEAE-Sephadex chromatography, and heparin–Agarose column chromatography. The final product is homogeneous when examined by zone electrophoresis, gel electrophoresis, and immunoelectrophoresis. The protein was heterogeneous by sedimentation equilibrium experiments; this heterogeneity, however, was attributed to aggregation of the protein. A minimal molecular weight of 55,400 ± 1300 was determined by sedimentation equilibrium. Factor IX is composed of a single polypeptide chain having an amino terminal sequence of Tyr-Asn-Ser-Gly. The protein contains approximately 26% carbohydrate which includes 10.6% hexose, 6.5% *N*-acetylhexosamine, and 8.7% *N*-acetylneuraminic acid. Other properties of this protein including its amino acid composition, isoelectric point, and inhibition by antibodies prepared in rabbits are also reported.

Factor IX (Christmas factor)[1] is a plasma protein which is inactive or absent in individuals with a congenital bleeding disorder known as Christmas disease or hemophilia B. The clinical manifestations of this abnormality are indistinguishable from factor VIII deficiency (classic hemophilia or hemophilia A). Factor IX deficiency, however, can readily be demonstrated in the laboratory since the clotting time of factor IX deficient plasma is corrected *in vitro* by the addition of factor VIII deficient plasma. Factor IX is one of the four coagulation factors (prothrombin, factor VII, factor IX, and factor X) which requires vitamin K for biosynthesis. These four coagulation factors also display the common property of adsorption to and elution from barium sulfate.

Factor IX participates in the middle phase of intrinsic blood coagulation (Davie *et al.*, 1969). In the currently accepted pathway, factor IX is converted to factor IX_a in the presence of factor XI_a (activated plasma thromboplastin antecedent) and calcium ions (Ratnoff and Davie, 1962; Schiffman *et al.*, 1963). Recent evidence indicates that factor IX_a subsequently interacts with thrombin-modified factor VIII to form a complex in the presence of calcium ions and phospholipid, and it is this complex which activates factor X (Stuart factor) (Hougie *et al.*, 1967; Osterud and Rapaport, 1970). The precise role of factor IX_a in these reactions, however, has not been clarified. This is primarily due to the fact that factor IX_a preparations with well-defined physical–chemical properties and free of other clotting factors have not been previously available. Partial purification of factor IX was initially reported by Aggeler *et al.* (1954) from human serum. Since then, several different preparations have been described from human and bovine sources (Harmison and Seegers, 1962; Somer and Castaldi, 1970). In most studies, DEAE-Sephadex and DEAE-cellulose chromatography have been used for the separation of factors VII, IX, X, and prothrombin. With bovine preparations, however, it has been difficult to separate factor IX from prothrombin and factor VII by DEAE-cellulose column chromatography since these proteins are eluted in similar positions. In 1971, Thompson reported that factor IX could be separated from factor VII by affinity chromatography on a heparin–Agarose column. In the present experiments, a method is described for the isolation of a homogeneous preparation of bovine factor IX using heparin–Agarose column chromatography as the final step. This procedure readily separates factor IX from factor VII as well as prothrombin. Factor IX prepared by this method was then characterized as to its size, structure, chemical composition, and immunological properties.

Experimental Section

Materials. Heparin sodium salt (grade I, 170 USP units/mg), soybean trypsin inhibitor (type II-S), rabbit brain cephalin, bovine albumin, mannose, galactose, galactosamine, *p*-dimethylaminobenzaldehyde, acetylacetone, *N*-acetylneuraminic acid, imidazole (grade I), and thiobarbituric acid were obtained from Sigma Chemical Co., St. Louis, Mo. Benzamidine hydrochloride and cyclohexanone were purchased from Aldrich Chemical Co., Milwaukee, Wis. Barium sulfate (X-ray grade) was purchased from Picker Corp., Cleveland, Ohio. DEAE-Sephadex A-50 was a product of Pharmacia Fine Chemicals, Piscataway, N. J., and Bio-Gel A-15m (Agarose), 100–200 mesh, was obtained from Bio-Rad Laboratories, Richmond, Calif. 2-Mercaptoethanol, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Kodak Co., Rochester, N. Y. Cyanogen bromide and 4-vinylpyridine were products of Baker Chemical Co., Phillipsburg, N. J. Kaolin was obtained from Fisher Scientific Co., Fair Lawn, N. J. 5-Sulfosalicylic acid was purchased from Mallinckrodt Chemical Works, St. Louis, Mo. Acrylamide was obtained from Matheson Coleman and Bell, Norwood, Ohio. Guanidine hydrochloride (extreme purity) was purchased from Heico, Inc.,

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

Delaware Water Gap, Pa. Amphojel without flavor (aluminum hydroxide gel) was a product of Wyeth Lab., Inc., Philadelphia, Pa. Adjuvant (Freund) was obtained from Difco Laboratories, Detroit, Mich. Agarose was purchased from Marine Colloids, Inc., Springfield, N. J.

Barium sulfate–aluminum hydroxide adsorbed plasma was prepared by stirring oxalated normal bovine plasma for 1 hr at 4° with barium sulfate (100 g/l.) followed by centrifugation. This plasma was then treated overnight at 4° with one-fifth volume of aluminum hydroxide gel. Factor X_a was prepared by the method of Fujikawa *et al.* (1972) employing a partially purified protease from Russell's viper venom. Partially purified bovine factor XI_a and antibody prepared in rabbits to purified bovine prothrombin were kindly provided by Drs. H. Kato and M. Coan of our laboratory. The prothrombin antibody was insolubilized by the cyanogen bromide method of Porath *et al.* (1967). All other chemicals were commercial preparations of the highest quality available.

Methods. Protein concentration was estimated by the biuret method (Gornall *et al.*, 1949) with crystalline bovine serum albumin as a standard. Factor IX concentration was measured by absorption at 280 nm employing an $E_{280}^{1\%} = 12.0$. For carbohydrate analysis, protein was determined by dry weight analysis corrected for 6% moisture content.

Amino acid analyses and preparation of samples were carried out by the methods of Moore and Stein (1963) and 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 evacuated tubes. The values for threonine and serine were determined by extrapolation to zero-time hydrolysis. Tryptophan and tyrosine were estimated by the method of Bencze and Schmid (1957), and half-cystine was determined as *S*-pyridylethylcysteine by the method of Friedman *et al.* (1970). Isoleucine and valine values were calculated from the 96-hr hydrolysis. Free sulfhydryl groups were determined by the method of Ellman (1959) in the presence of 6 M urea.

Neuraminic acid was determined by the thiobarbituric acid method of Warren (1959) using *N*-acetylneuraminic acid as a standard. 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.

Zone electrophoresis was carried out in 0.05 M sodium barbital buffer (pH 8.6) on microscope slides (25 × 75 mm) layered with 1.0% Agarose as described by Williams and Chase (1971). Samples (10 μl containing 10 μg of protein) were placed in a small well and electrophoresis was carried out at room temperature for 45 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 1.0% Agarose on microscope slides was performed by the method of Scheidegger (1955). Electrophoresis was carried out in 0.07 M sodium barbital buffer (pH 8.6) for 30 min at 150 V and 5 mA/slide. Samples were diluted in 0.07 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. The slides were then allowed to soak in 0.15 M NaCl for 48 hr to remove any nonprecipitated protein and were then stained with Coomassie Brilliant Blue.

The method of Williams and Reisfeld (1964) was used for polyacrylamide disc gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed by the method of Weber and Osborn (1969). Samples were run for 6 hr with 8 mA/tube. Gels were stained for protein with Coomassie Brilliant Blue and for carbohydrate by the method of Zacharius *et al.* (1969). For the latter, sodium dodecyl sulfate was first removed from the gels by stirring the gels overnight in 5% Cl_3CCOOH and 5% 5-sulfosalicylic acid solution before oxidation with periodic acid.

Ultracentrifugation was carried out in a Beckman Model E 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 automated as described by DeRosier *et al.* (1972). With this procedure, the entire fringe envelope is read at 50-μ intervals and the calculated Fourier transform parameters employed to compute the actual fringe displacement using the program developed by DeRosier *et al.* (1972). These data were then used to calculate the various point-by-point molecular weight averages employing a computer program developed by Teller *et al.* (1969).

Sedimentation equilibrium measurements with *S*-pyridylethyl factor IX were performed at three different concentrations (2.0, 1.0, and 0.75 mg/ml) in 6 M guanidine hydrochloride ($\rho = 1.1415$). Salt-free lyophilized factor IX was dissolved in 6 M guanidine hydrochloride and dialyzed against the same solvent for 48 hr prior to ultracentrifugation, and the appropriate sample concentration was obtained by dilution with dialysate. A method developed by Teller (1973) 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 24,000 rpm. A partial specific volume of $\bar{v} = 0.707$ ml/g was determined by amino acid analysis and corrected for 26% carbohydrate content (Cohn and Edsall, 1943; Longsworth, 1953). The $\bar{v}$ used in determining *S*-pyridylethyl factor IX was assumed to be identical with that of the native protein. Pyridylethylation of factor IX was performed by a minor modification of the method of Friedman *et al.* (1970). This derivative was employed in the sedimentation equilibrium studies in an attempt to reduce protein–protein interaction.

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, three different 10-mg samples of *S*-pyridylethyl factor IX were employed. In the quantitation of the terminal residues, protein concentration was determined in an amino acid analyzer after hydrolysis of the sample. Norleucine was employed as an internal standard to calculate protein recovery.

Rabbits were immunized against factor IX by multisite subcutaneous injection of the $BaSO_4$ eluate or the purified protein (each 1.5 mg) emulsified in Freunds complete adjuvant. Three weeks after the first injections, an anamnestic response was elicited in the rabbits by a second and third injection on consecutive weeks employing 1.5 mg of protein in incomplete

Freunds adjuvant. The rabbits were exsanguinated 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 M 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 at −20° until use.

For factor IX assay, two different methods were used. The one-stage assay for factor IX was employed for most of the experiments reported in this paper. In this method, factor IX activity was determined by the kaolin–partial thromboplastin time according to the method of Proctor and Rapaport (1961) with the following modifications. One-tenth milliliter of test sample (diluted in 0.15 M NaCl) was incubated for 10 min at 37° with 0.1 ml of kaolin suspension (20 mg of kaolin/ml of 0.1 M Tris-HCl (pH 7.5)), 0.1 ml of phospholipid solution (1 vial of rabbit brain cephalin, Sigma, suspended in 100 ml of 0.15 M NaCl), and 0.1 ml of human factor IX deficient plasma. A 0.1-ml aliquot of 0.025 M $CaCl_2$ was then added and the clotting times were determined while tipping the tubes gently at 37°. In the assay of factor IX_a, 0.1 ml of 0.1 M Tris-HCl (pH 7.5) was added instead of the kaolin suspension.

A two-stage assay for factor IX was employed for the data shown in Table I. In this procedure, the factor IX sample (10–300 μg of protein) was activated with partially purified bovine factor XI_a (30 μg of protein) in the presence of 5 mM $CaCl_2$–0.05 M Tris-HCl buffer (pH 8.0) in a final volume of 1.0 ml for 30 min at 37°. The reaction was terminated by the addition of 0.1 ml of 0.1 M EDTA. Factor IX_a was then diluted with 0.15 M NaCl and assayed as follows: 0.1 ml of diluted sample, 0.1 ml of phospholipid solution, and 0.1 ml of normal bovine plasma were incubated at 37° for 30 sec. Subsequently, 0.1 ml of 0.025 M $CaCl_2$ was added and the clotting time determined in duplicate. The activity was calculated from a calibration curve where the log of factor IX concentration is plotted against the log of the clotting time. This plot was linear from 70 to 180 sec. Purified bovine factor IX was used as a standard. One unit of activity was defined as that amount of factor IX activity present in 1.0 ml of normal bovine plasma.

Factor X activity was assayed by the method of Bachmann *et al.* (1958) according to Fujikawa *et al.* (1972). Prothrombin activity was assayed by the following procedure: 0.1 ml of barium sulfate–aluminum hydroxide adsorbed plasma, 0.1 ml of diluted sample in saline, and 0.1 ml of factor X_a (16 μg/ml activated with partially purified protease from Russell's viper venom) were incubated for 1 min followed by the addition of 0.1 ml of 0.025 M $CaCl_2$. In this system, 2.5 μg of purified prothrombin gave an 18-sec clotting time, while the blank had a clotting time longer than 500 sec.

Factor VII activity was assayed by the method of Gaston and Spivack (1968).

Heparin–Agarose was prepared according to Cuatrecasas (1970). Fifty milliliters of Agarose A-15m, 100–200 mesh (settled volume), was activated with 10 g of finely divided cyanogen bromide for 13–15 min. The suspension was kept at pH 11.0 by the continuous addition of 6 N NaOH at a temperature between 18 and 20°. One gram of heparin in 50 ml of 0.1 M $NaHCO_3$ (pH 8.3) was added to the activated washed Agarose and stirred overnight at 4°. The heparin–Agarose column was washed extensively with 0.1 M Tris-HCl buffer (pH 9.0) containing 2 M NaCl before use.

PURIFICATION OF BOVINE FACTOR IX. Barium sulfate eluate was prepared as described previously (Fujikawa *et al.*, 1972). After dialysis of the eluate, 500 ml (settled volume) of DEAE-Sephadex (equilibrated with 0.05 M sodium citrate (pH 7.0) containing 1 mM benzamidine) was added to the dialysate and stirred for 20 min at 4°. Subsequent buffers employing benzamidine contained this inhibitor at 1 mM concentration. The suspension was allowed to settle and the supernatant was decanted from the DEAE-Sephadex. The DEAE-Sephadex was then transferred to an empty column (inner diameter 7.5 cm) and the column washed with 1 l. of 0.1 M sodium citrate buffer–benzamidine and then eluted with 0.2 M sodium citrate buffer–benzamidine. The combined washing and first 600 ml of the eluate were then combined with the initial DEAE-Sephadex supernatant. In this step, most of the factor X was separated from factor IX. Another 1000 ml of DEAE-Sephadex (settled volume) was then added to the fraction containing factor IX, and the suspension was stirred for 20 min. DEAE-Sephadex was collected and poured into an empty column as described above. The column was washed with 2 l. of 0.08 M sodium citrate buffer–benzamidine, and factor IX was eluted with 0.2 M sodium citrate buffer–benzamidine. The first 450 ml of the eluate was discarded and the following 700 ml of eluate, referred to as batchwise DEAE-Sephadex fraction, was collected. Soybean trypsin inhibitor (100 mg) and 2.8 ml of 0.5 M benzamidine were added to this fraction and the solution was diluted with two volumes of distilled water. The diluted sample was applied to a DEAE-Sephadex column (5.0 × 20 cm) which was equilibrated with 0.05 M sodium citrate buffer–benzamidine. After adsorption of the protein to the column, it was washed with 500 ml of 0.075 M sodium citrate buffer–benzamidine. Factor IX was then eluted with a linear gradient containing 2 l. of 0.075 and 0.12 M sodium citrate buffer–benzamidine. Factor IX activity eluted at the trailing edge of the major peak which was primarily prothrombin (Figure 1A). The fractions containing factor IX activity (shown with the solid bar) were combined (DEAE-Sephadex column fraction), and soybean trypsin inhibitor (100 mg) and benzamidine were then added. The factor IX solution was dialyzed overnight against 45 l. of 0.05 M imidazole–HCl buffer (pH 6.0). Calcium chloride (final concentration of 2.5 mM) was added to the dialyzed factor IX solution and the mixture was applied to a heparin–Agarose column (2 × 10 cm) which was prewashed with 500 ml of 0.05 M imidazole–HCl buffer containing 2.5 mM $CaCl_2$ and benzamidine. After the sample was applied, the column was washed with 2 l. of 0.05 M imidazole–HCl buffer (pH 6.0) containing 0.25 M sodium chloride, 2.5 mM $CaCl_2$, and benzamidine. Factor IX was then eluted from the column with a linear gradient formed with 500 ml each of 0.25 and 0.7 M sodium chloride in 0.05 M imidazole–HCl buffer (pH 6.0) with 2.5 mM $CaCl_2$ (Figure 1B). The fractions containing factor IX activity were collected (shown by the solid bar) and combined with 0.1 M EDTA at a final concentration of 5 mM. The heparin–Agarose column was reused after washing with 100 ml of 0.05 M Tris-HCl buffer (pH 9.0), containing 2.0 M NaCl.

Concentration of the factor IX to about 50 ml was accomplished by ultrafiltration with an Amicon ultrafiltration apparatus equipped with a PM 10 membrane. The concentrated sample was applied to a Sephadex G-25 column (2.0 × 20 cm) which had been preequilibrated with 0.025 M Tris-HCl buffer (pH 7.2) containing 0.2 M NaCl. The factor IX fraction was then frozen at −20° until further use. For chemical

TABLE I: Purification of Bovine Factor IX.

Purification Step	Vol (ml)	Total Protein (mg)	Total Act.[a] (Units)	Sp Act. (Units/mg)	Recovery (%)	Purification
Plasma	45×10^3	3.5×10^6 [b]	4.5×10^4	0.013	100	1
$BaSO_4$ eluate	4.9×10^3	11.2×10^3 [b]	3.8×10^4	3.39	84.4	261
Batchwise DEAE-Sephadex	700	2.45×10^3 [b]	4.6×10^4	18.8	102.2	1,450
DEAE-Sephadex column	770	308[b]	4.2×10^4	136	93.3	10,500
Heparin–Agarose column	375	117[c]	3.4×10^4	291	75.5	22,300

[a] Activity of factor IX was assayed by the two-stage method described under Methods. Before assay, each sample (1.0 ml) was diluted tenfold with 0.05 M imidazole–HCl buffer (pH 6.0) containing 2.5 mM $CaCl_2$ and passed through a heparin–Agarose column (0.5 × 3 cm). The column was washed with 20 ml of 0.25 M NaCl in the same buffer, and factor IX was eluted with the same buffer containing 0.6 M NaCl. [b] Protein concentration was determined by the biuret method. [c] Protein concentration was determined by absorbance at 280 nm using $E^{1\%}_{280} = 12.0$.

analysis, salt-free protein was obtained by gel filtration through a Sephadex G-25 column (2.5 × 20 cm) with 0.1 M NH_4HCO_3 followed by lyophilization.

Results

Preparation of Bovine Factor IX. The various steps in the purification of a typical preparation of bovine factor IX are shown in Table I. The procedure involves a barium sulfate adsorption and elution, batchwise DEAE-Sephadex adsorption and elution, DEAE-Sephadex column chromatography, and heparin–Agarose column chromatography. The purification was ~22,000-fold with an overall yield of 76%.

In the purification procedure, factor X activity was partially separated from factor IX in the batchwise DEAE-Sephadex step and completely separated from factor IX in the DEAE-Sephadex column chromatography step. Assay of factor IX activity across the protein peak indicated that factor IX was eluted from the DEAE-Sephadex column at the trailing edge of the main peak at a salt concentration between 0.1 and 0.11 M of sodium citrate (Figure 1A). Factor X activity was eluted at a concentration of sodium citrate higher than 0.12 M (Fujikawa *et al.*, 1972). The assay of factor IX by the one-stage assay method was not always reproducible on partially purified preparations, apparently due to the presence of inhibitor activity. The assay of heparin–Agarose fractions, however, gave reproducible results (Figure 1B). The protein from the pooled factor IX fraction from the DEAE-Sephadex column contained 70% prothrombin, approximately 30% factor IX, and several other minor contaminants.

The complete separation of factor IX and prothrombin was achieved on the heparin–Agarose column where factor IX elutes after prothrombin and factor VII (Figure 1B). These proteins are found in the first peak. Factor IX prepared by this method was completely free of factor VII, factor IX_a, factor X, and all other known coagulation factors except prothrombin. This was shown by testing 0.1 ml of a factor IX solution containing 0.1 mg of protein/ml for various coagulation factors, as described under Methods. It still contained traces (less than 0.05%) of prothrombin. The prothrombin was readily removed, however, by adsorption with an insolubilized antibody prepared in rabbits to purified bovine prothrombin.

Polyacrylamide Disc Gel and Sodium Dodecyl Sulfate Gel Electrophoresis of Bovine Factor IX. A single, sharp protein band for factor IX was obtained in polyacrylamide disc gel electrophoresis, pH 8.0 (sample 1, Figure 2). Also, sodium dodecyl sulfate–polyacrylamide gel electrophoresis of factor IX before and after reduction with 2-mercaptoethanol gave a single protein band (samples 2 and 3, respectively). Sample 4 is reduced factor IX stained for carbohydrate with the periodic acid–Schiff reagent. These experiments provide good evidence for the homogeneity of the factor IX preparation and indicate that this plasma protein is a glycoprotein composed of one polypeptide chain. Many of the reduced factor IX preparations contained a minor band which amounted to 2–3% of the total

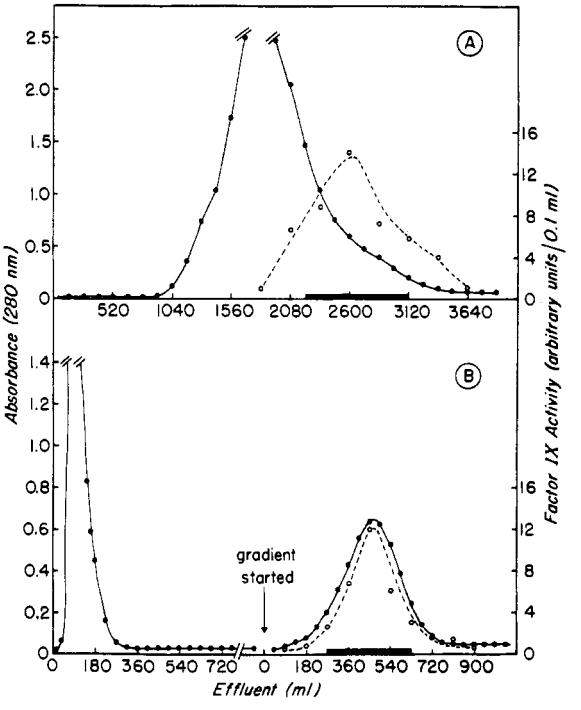

FIGURE 1: The top figure (A) is the elution pattern for bovine factor IX from the DEAE-Sephadex column. Protein was eluted from the column (5 × 20 cm) with a linear gradient formed by 2 l. each of 0.075 and 0.12 M sodium citrate buffer, as described under Methods. Fractions (27 ml) were collected at a flow rate of ~140 ml/hr. Factor IX activity was determined by the one-stage method described under Methods: (●——●) absorbance at 280 nm; (O---O) clotting activity. The bottom figure (B) is the elution pattern for bovine factor IX from a heparin–Agarose column. Protein was eluted from the column (2 × 12 cm) with a linear gradient consisting of 500 ml each of 0.25 and 0.7 M NaCl in 0.05 M imidazole (pH 6.0) as described under Methods. Fractions (9 ml) were collected at a flow rate of 300 ml/hr: (●——●) absorbance at 280 nm; (O---O) clotting activity.

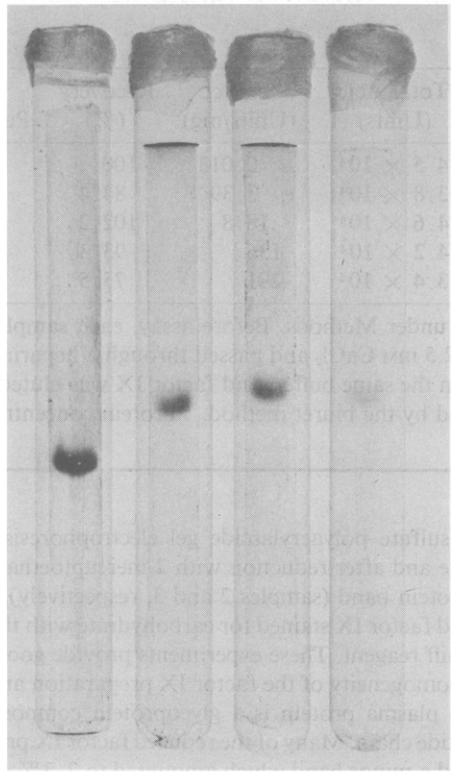

FIGURE 2: Polyacrylamide disc gel and sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified bovine factor IX. All samples contained 5 μg of protein. Gels, 1, 2, and 3 were stained for protein with Coomassie Brilliant Blue, and gel 4 was stained for carbohydrate with the periodic acid–Schiff base reagent. Gel 1 was a polyacrylamide disc gel, and gels 2, 3, and 4 were sodium dodecyl sulfate–polyacrylamide gels. The anode was at the bottom of the gel.

protein as estimated by densitometer tracings of the gels. The amount of this component dramatically increased when factor IX was prepared in the absence of proteolytic enzyme inhibitors. It was concluded from these observations that factor IX readily undergoes partial degradation during its isolation in the absence of protease inhibitors without a major loss of activity.

Zone Electrophoresis and Immunoelectrophoresis of Bovine Factor IX. As further evidence of purity, factor IX was subjected to zone electrophoresis in barbital buffer, pH 8.6, employing microscope slides layered with Agarose (Figure 3). Following electrophoresis for 45 min, a single protein spot was observed for factor IX. Electrophoresis of factor IX at a series of different pH values indicated that its isoelectric point is 3.7. This value was determined in 0.025 M formate buffer in the pH range of 3.5–4.0.

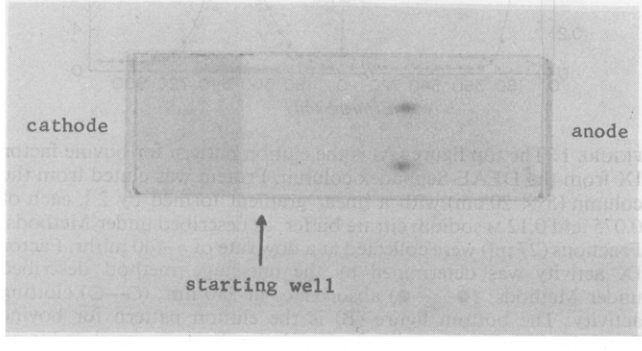

FIGURE 3: Zone electrophoresis of two different samples of bovine factor IX preparations. Factor IX (10 μg) was applied to each of the wells and electrophoresis was performed as described under Methods.

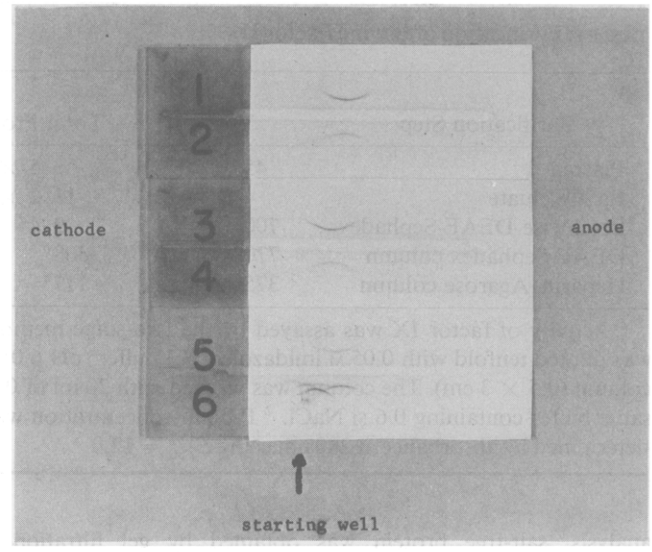

FIGURE 4: Immunoelectrophoresis of purified bovine factor IX and proteins present in the barium sulfate eluate. Wells 1 and 2 contained two different purified factor IX preparations (10 μl containing 5–10 μg of protein) and the center trough contained antibody to the purified factor IX. Well 3 contained 15 μg of purified factor IX and well 4 contained 35 μg of protein from the $BaSO_4$ eluate. The center trough in this experiment contained antibody to the purified factor IX. Well 5 contained purified factor IX and well 6 contained protein from the $BaSO_4$ eluate. The center trough in this experiment contained antibody made in rabbits against the $BaSO_4$ eluate protein. Immunoelectrophoresis and staining were carried out as described under Methods.

Electrophoresis of factor IX followed by immunodiffusion against rabbit antibody prepared from the purified factor IX is shown in Figure 4. A single, sharp precipitin line was obtained with two different preparations of purified factor IX (samples 1 and 2). Similarly, this antibody gives a single precipitin line with pure factor IX (sample 3) and with $BaSO_4$ eluate (sample 4), a protein fraction which contains a substantial amount of factor IX. An antibody prepared against the $BaSO_4$ eluate forms one sharp precipitin line with the pure factor IX (sample 5) but many precipitin lines are obtained with the $BaSO_4$ eluate (sample 6). These results are consistent with the removal of many contaminating proteins during the later stages of the purification.

Factor IX antibodies employed in these experiments readily neutralize factor IX activity, as measured in the one-stage assay using factor IX deficient plasma (Figure 5). In these studies, factor IX was incubated for 1 hr at 37° with increasing concentrations of antibody to purified factor IX, and aliquots were then appropriately diluted and assayed for clotting activity. These experiments demonstrate quite clearly that the rabbit antibody prepared against bovine factor IX readily inactivates factor IX activity. This antibody, however, has no effect on the clotting activity of purified prothrombin or factor X (Fujikawa *et al.*, 1973). These investigations provide good evidence that the single protein precipitin line observed in the immunoelectrophoresis experiments shown in Figure 4 was due to the presence of bovine factor IX and not some contaminating protein.

TABLE II: Molecular Weight of Bovine Factor IX by Sedimentation Equilibrium.[a]

Sample	M_1	M_n	M_w	M_z
S-Pyridylethyl factor IX	56,500 ± 1300	60,500 ± 1300	65,300 ± 1800	69,300 ± 2200
Factor IX[b]	55,400 ± 1300			

[a] M_1 refers to the smallest molecular weight species calculated by the methods described by Teller *et al.* (1969). M_n, M_w, and M_z refer to the number average molecular weight, the weight average molecular weight, and the Z-average molecular weight, respectively, as defined by Kraemer (1940). [b] Corrected for 17.2 *S*-pyridylethyl residues.

Sedimentation Equilibrium Studies on Bovine Factor IX. Sedimentation equilibrium studies on *S*-pyridylethyl bovine factor IX demonstrated that the glycoprotein was heterogeneous at all concentrations tested (Table II). The heterogeneity, however, was attributed to limited association of the protein, and calculations by the method of Teller *et al.* (1969) indicated that approximately 10% of factor IX was present as dimers and the remaining 90% was present as monomers. The apparent heterogeneity was attributed to monomer aggregation rather than a contaminating protein species, as indicated by the fact that the M_n, M_w, and M_z *vs.* concentration plots are superimposable at all protein concentrations tested (see Table II). A complete discussion of this method has been summarized by Harris *et al.* (1969). The minimal molecular weight calculated for the *S*-pyridylethyl protein was 56,000 ± 1300. When corrected for 17.2 *S*-pyridylethyl residues, the molecular weight was found to be 55,400 for factor IX.

Amino Acid and Carbohydrate Compositions of Bovine Factor IX. The amino acid and carbohydrate compositions of factor IX are shown in Table III. Factor IX is low in methionine, but contains a substantial amount of cystine. No free sulfhydryl groups were detected by the method of Ellman (1959). These experiments also show that factor IX is a glycoprotein containing 26% carbohydrate. The carbohydrate includes hexose, hexosamine, and neuraminic acid. This is equivalent to 41,000 g of protein and 14,000 g of carbohydrate per 55,000 g of glycoprotein.

Amino-Terminal Sequence of Bovine Factor IX. The amino-terminal analysis of the *S*-pyridylethyl derivative of bovine factor IX was carried out on a Beckman sequencer. Factor IX was found to contain the amino-terminal sequence Tyr-Asn-Ser-Gly-. The yield of tyrosine was 0.77 equiv/mol of protein on the first cycle in the sequenator. The protein also contains approximately 0.2 equiv of amino-terminal asparagine which occurs in position 2 of the major sequence. On the second cycle, asparagine was the primary amino acid identified along with small amounts of serine which was found in position 3 of the major sequence. On the third cycle, serine was the primary amino acid identified along with small amounts of glycine. The ratio of glycine in cycles three and four was 1:4. This is the same ratio as that found for aspara-

TABLE III: Amino Acid and Carbohydrate Compositions of Bovine Factor IX.

Components	Factor IX (Residues/55,400)
Amino acid	
Lysine	27.6
Histidine	8.1
Arginine	17.0
Aspartic acid	36.3
Threonine	20.1
Serine	29.0
Glutamic acid	46.6
Proline	13.1
Glycine	29.7
Alanine	18.8
Half-cystine[a]	17.2
Valine	24.9
Methionine	2.6
Isoleucine	19.3
Leucine	18.7
Tyrosine[b]	9.2
Phenylalanine	15.2
Tryptophan[b]	11.4
Carbohydrate	
Hexose	32.6
N-Acetylhexosamine	16.4
N-Acetylneuraminic acid	15.6
Protein (%)	74.2
Carbohydrate (%)	25.8

[a] Determined as *S*-pyridylethylcysteine by the method of Friedman *et al.* (1970). [b] Determined by the spectrophotometric assay of Bencze and Schmid (1957).

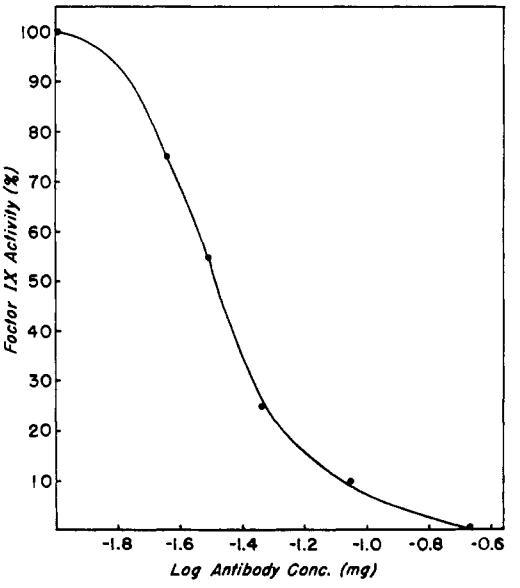

FIGURE 5: Neutralization of bovine factor IX activity with an antibody prepared in rabbits against the purified factor IX. Factor IX (0.5 mg/ml) was incubated at 37° for 1 hr with increasing concentrations of antibody and then diluted 1:100 in 0.01 M Tris-HCl (pH 7.4) containing 0.15 M NaCl. Factor IX was then assayed by the one-stage procedure outlined under Methods.

gine and tyrosine in the first cycle. These experiments support the conclusion that factor IX is a glycoprotein composed of a single polypeptide chain. Furthermore, minor heterogeneity occurs at the amino-terminal end of this protein, apparently due to limited hydrolysis of the terminal tyrosine by aminopeptidase *in vivo* or during the isolation procedure.

Discussion

The most important step in the present purification method for factor IX is the heparin–Agarose column chromatography procedure carried out in the presence of calcium ions. This step provides a clear-cut separation of factor IX from prothrombin and factor VII. In the absence of calcium ions, these proteins also bind to the heparin–Agarose column and are eluted separately. However, under these conditions factor IX is often contaminated with prothrombin. Indeed, washing of the heparin–Agarose column with a large volume of 0.05 M imidazole–HCl buffer (pH 6.0) in the presence of 0.025 M $CaCl_2$ is required for the removal of prothrombin from factor IX.

Hougie *et al.* (1967) observed that factor IX binds to Dextran Blue in the presence of calcium ions. The binding of factor IX to heparin–Agarose was also enhanced by calcium ions (Thompson, 1971). These observations are similar to the precipitation of lipoproteins by heparin and divalent metal ions (Burstein *et al.*, 1970). Purified factor IX, however, does not contain lipid as tested by Sudan Black staining of factor IX on polyacrylamide gels (Thompson, 1971). In view of the high carbohydrate content of factor IX, a carbohydrate–heparin interaction bridged by calcium ions is possible. Considerable specificity in the heparin interaction with factor IX appears to be involved since other sulfonic groups such as sulfethyl Sephadex failed to bind factor IX under similar conditions. This specificity may be related in part to heparin inhibition of the early phases of intrinsic coagulation at the point where heparin blocks the activity of factor IX_a (O'Brien, 1958; Shanberge *et al.*, 1959; Ratnoff and Davie, 1962; Lundblad and Davie, 1964; Pitlick *et al.*, 1969). Insolubilized heparin has also been used by others in the purification of lipoprotein lipase (Egelrud and Olivecrona, 1972) and heparin cofactor (Andersson and Miller-Andersson, 1972). Gentry and Alexander (1973) have recently noted that heparin–Agarose removes significant amounts of factors IX and XI from other clotting factors in human plasma in the presence of calcium ions.

Another important consideration in the isolation of factor IX in high yield (75%) and quality from bovine plasma is the use of heparin, benzamidine, and soybean trypsin inhibitor, in addition to citrate or oxalate during the isolation procedure. These protease and coagulation inhibitors were introduced into freshly obtained whole blood and were present in each subsequent step of the purification procedure. With these inhibitors, factor IX can be isolated as a single polypeptide chain. These inhibitors have also been employed in our laboratory in the preparations of factors VIII and X to restrict protease activity (Schmer *et al.*, 1972; Legaz *et al.*, 1973; Fujikawa *et al.*, 1972). Factor IX purified in the presence of lower concentrations or in the absence of inhibitors usually forms two or three bands on sodium dodecyl sulfate gel electrophoresis in the presence of reducing agents. These preparations appear as a single band, however, on regular disc gel electrophoresis and sodium dodecyl sulfate–polyacrylamide gels in the absence of reducing agents. Thus, factor IX is more susceptible to minor protease degradation by plasma enzymes than factor X and is readily split into several peptides which are held together by disulfide bonds.

Factor IX has a molecular weight of approximately 54,000 and is smaller than prothrombin (70,000). It appears to be essentially the same size, however, as bovine factor X (55,000) (Jackson and Hanahan, 1968; Fujikawa *et al.*, 1972). The amino-terminal sequences of these three proteins are similar in that prothrombin contains Ala-Asn-Lys-Gly and the light chain of factor X contains Ala-Asn-Ser-Phe, while factor IX contains Tyr-Asn-Ser-Gly. Thus, all three proteins contain Asn in the second position. A great deal of homology is apparent in the first 15–20 amino-terminal acids of these three proteins, and the results of these experiments will be published elsewhere. The homology of the amino-terminal sequences and active-site regions of factor X_a and thrombin has been noted previously (Titani *et al.*, 1972).

Factor IX contains 26% carbohydrate, a value which is much larger than that found in bovine prothrombin or factor X which contain 10.2 and 9.5% carbohydrate, respectively (Fujikawa *et al.*, 1972). A high carbohydrate content has also been observed for bovine factor VIII which contains 20% carbohydrate (Schmer *et al.*, 1972). Factor VIII, however, has a low content of neuraminic acid in comparison to bovine factor IX, prothrombin, and factor X. The low content of neuraminic acid in bovine factor VIII presumably enables a favorable interaction between terminal hexose residues with concanavalin A, in contrast to factor IX, prothrombin, and factor X which are not precipitated by concanavalin A.

The mechanism of activation of factor IX by factor XI_a remains to be clarified. Investigations employing the highly purified bovine factor IX are presently in progress in our laboratory to study this question.

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