Isolation and Characterization of Bovine Factor XI (Plasma Thromboplastin Antecedent)†

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ABSTRACT: Factor XI (plasma thromboplastin antecedent) has been purified approximately 28 000-fold from bovine plasma with an overall yield of about 30%. The isolation procedure involves barium sulfate adsorption of contaminants, ammonium sulfate precipitation, and chromatography on heparin-agarose, CM-Sephadex, and DEAE-Sephadex. The final product was homogeneous when examined by polyacrylamide gel electrophoresis and immunoelectrophoresis. A minimal mol wt of 124 000 was determined by sedimentation equilibrium. Factor XI is composed of two similar or identical polypeptide chains (mol wt of approximately 55 000), and these two chains are held together by a disulfide bond(s). Factor XI is a glycoprotein which contains approximately 11% carbohydrate including 5.4% hexose, 4.7% *N*-acetylhexosamine, and 1.0% *N*-acetylneuraminic acid. Other properties of this coagulation factor including its amino acid composition and inhibition by antibodies prepared in rabbits are also reported.

Factor XI (plasma thromboplastin antecedent)[1] is a plasma protein which participates in the early stage of intrinsic blood coagulation (Davie and Fujikawa, 1975). A deficiency of the protein in humans was first reported in 1953 by Rosenthal et al. as a hemorrhagic disease resembling, but differing from, factor IX deficiency (Christmas disease) and factor VIII deficiency (classic hemophilia). The absence of factor XI activity has also been detected in a cow by Kociba et al. (1969).

Factor XI is transmitted by an autosomal recessive gene, and a defect in this gene produces major factor XI deficiency in the homozygote and minor deficiency in the heterozygote (Rapaport et al., 1961). Recently, Forbes and Ratnoff (1972) reported that patients with hereditary factor XI deficiency lack the antigen related to factor XI, and these individuals may fail to synthesize adequate amounts of this plasma protein. In normal plasma, factor XI is present in a precursor form. In the early stage of intrinsic blood coagulation, factor XI is converted to an active form (factor XI_a) by other coagulation factors.

Factor XI and factor XI_a have been substantially purified from human plasma and serum (Ratnoff et al., 1961; Ratnoff and Davie, 1962; Schiffman et al., 1963; Kingdon et al., 1964; Wuepper, 1972; Saito et al., 1973; Schiffman and Lee, 1974). The human preparation has a mol wt of about 158 000 as estimated by sodium dodecyl sulfate gel electrophoresis (Wuepper, 1972) and 175 000–210 000 by gel filtration (Ratnoff, 1972; Saito et al., 1973; Schiffman and Lee, 1974). It is composed of two similar or identical chains with a mol wt of about 80 000 as indicated by sodium dodecyl sulfate gel electrophoresis, and these two chains are held together by a disulfide bond(s) (Wuepper, 1972).

The molecular mechanism for the conversion of factor XI to factor XI_a is not known at the present time. Wuepper (1972) and Saito et al. (1973) reported that their factor XI preparations were activated by factor XII_a or trypsin. Schiffman and Lee (1974) reported that their preparation was activated by trypsin, but not directly by factor XII_a. They suggested that factor XII_a and an additional component(s) distinct from Fletcher factor (prekallikrein) are required for the activation of factor XI. Recently, Saito et al. (1975) and Griffin and Cochrane (1976) have reported that Fitzgerald factor, as well as factor XII, prekallikrein, and kaolin, participates in the activation of factor XI.

The role of factor XI_a in blood coagulation has recently been clarified. Factor XI_a is a serine protease which converts factor IX to factor IX_a in the presence of calcium ions (Kingdon et al., 1964). The activation occurs in a two-step reaction (Fujikawa et al., 1974). In the first step, an internal peptide bond in factor IX is cleaved, leading to the formation of an intermediate containing two polypeptide chains held together by a disulfide bond(s). This intermediate, which has no enzymatic activity, is then converted to factor IX_a by a second factor XI_a catalyzed cleavage. This second step releases an activation peptide from the amino-terminal end of the heavy chain of the factor IX intermediate.

In order to study factor XI and its mechanism of activation in detail, we have developed a method for the isolation of milligram quantities of this coagulation factor from bovine plasma. The highly purified protein was then characterized as to its size, chemical composition, and immunological properties.

Materials

Heparin sodium salt (Grade I, 170 USP units/mg), soybean trypsin inhibitor (Kunitz, types I-S and II-S), bovine serum albumin, ovalbumin, bovine carbonic anhydrase, mannose, galactose, imidazole (grade I), dithiothreitol, ammonium persulfate, and Coomassie brilliant blue were obtained from Sigma Chemical Co., St. Louis, Mo. Centrolex-P was obtained from Central Soya, Chicago, Ill. Polybrene (hexadimethrine bromide), benzamidine hydrochloride, and diisopropyl phosphorofluoridate (iPr_2FP)[2] were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Barium sulfate (x-ray

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

[2] Abbreviation used is: iPr_2FP, diisopropyl phosphorofluoridate.

grade) was purchased from Picker Corp., Cleveland, Ohio. CM-Sephadex C-50 and DEAE-Sephadex A-50 were products 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, acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Kodak Co., Phillipsburg, N.J. Kaolin was obtained from Matheson Coleman and Bell, Norwood, Ohio, and guanidine hydrochloride (extreme purity) was purchased from Heico, Inc., Delaware Water Gap, Pa. Sodium dodecyl sulfate was obtained from British Drug House, Poole, England. Freund's adjuvants were purchased from Difco Laboratories, Detroit, Mich. All other chemicals were commercial preparations of the highest quality available. Phosphorylase *b* and glycogen debranching enzyme were kindly provided by Dr. Edmond Fischer in our department, and bovine factor XI deficient plasma was a generous gift of Dr. G. Kociba of Ohio State University. Human Fitzgerald factor deficient plasma (GK-1601) and human Fletcher factor deficient plasma (GK-1702) were purchased from George King Biochemicals, Salem, NH. Human factor XII deficient plasma from a Hageman trait patient in the Seattle area was kindly provided by Dr. Gottfried Schmer in our department.

Methods

Protein concentration was routinely estimated by absorption assuming an $E_{280}^{1\%}$ value of 10.0. A value of 12.6 was employed for highly purified factor XI. This value was determined for factor XI in the analytical centrifuge employing the methods of Babul and Stellwagon (1969) and Richards et al. (1968). Protein for carbohydrate analyses was determined by amino acid analysis after hydrolyzing the sample in 6 N HCl for 24 h at 110 °C in evacuated tubes.

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 Durrum Model D 500 amino acid analyzer. Samples were hydrolyzed in 6 N HCl at 110 °C for 24, 48, 72, and 96 h in evacuated sealed tubes. The values for threonine and serine were determined by extrapolation to zero-time hydrolysis. Tryptophan was determined after alkaline hydrolysis by the method of Hugli and Moore (1972) and half-cystine was determined as cysteic acid (Hirs, 1967) and *S*-pyridylethylcysteine (Friedman et al., 1970). The values for other amino acids were determined by the average of the values at four different hydrolysis times unless otherwise stated.

Hexosamine was determined after hydrolyzing samples in 2 N HCl for 22 h at 110 °C by the method of Elson and Morgan as described by Gardell (1957) using galactosamine as a standard. Neutral sugar was determined by the phenol–sulfuric acid method of Dubois et al. (1956) using a 1:1 mixture of galactose and mannose as a standard. Neuraminic acid was determined by the method of Warren (1959) using *N*-acetylneuraminic acid as a standard.

Polyacrylamide disc gel electrophoresis was performed at pH 9.4 according to the method of Davis (1964). The gels were stained for protein with Coomassie brilliant blue by the method of Chrambach et al. (1967) or for carbohydrate by the method of Zacharius et al. (1969).

Sodium dodecyl sulfate gel electrophoresis was performed by the method of Weber and Osborn (1969). Samples (7–10 μg in 10–20 μL) were subjected to electrophoresis at room temperature for 3–4 h in 5% acrylamide gels at a current level of 4 mA/gel. Electrophoresis was carried out in 0.1 M Tris–H_3PO_4 buffer (pH 7.0), containing 0.1% sodium dodecyl sulfate. Gels were stained for protein with Coomassie brilliant blue according to Fairbanks et al. (1971). The molecular weight of factor XI was obtained by interpolation from a linear semilogarithmic plot of apparent molecular weight vs. migration distance using the following proteins as standards: glycogen debranching enzyme (160 000 daltons), phosphorylase *b* (100 000 daltons), bovine serum albumin (68 000 daltons), ovalbumin (45 000 daltons), and bovine carbonic anhydrase (29 000 daltons).

Ultracentrifugation was performed with a Beckman Model E analytical ultracentrifuge equipped with an electronic speed control. Sedimentation equilibrium experiments were performed according to Yphantis (1964) employing a six-channel Kel-F centerpiece. Rayleigh patterns were recorded on Kodak II-G photographic plates, and the plates subsequently read on a Nikon microcomparator automated as described by DeRosier et al. (1972). By this procedure, the entire fringe envelope is read at 50-μm intervals and the calculated Fourier transform parameter employed to compute the actual fringe displacement using the program developed by DeRosier et al. (1972). Point-by-point molecular weight averages were then calculated from the data utilizing a computer program developed by Teller (1973).

Sedimentation equilibrium measurements with factor XI were performed at three different concentrations (1.0, 0.75, and 0.50 mg/mL) in 0.05 M sodium acetate–6 M guanidine hydrochloride (pH 5.5), in the presence and absence of 0.1 M dithiothreitol. The salt-free lyophilized sample was dissolved in the above-mentioned buffer and dialyzed against this buffer for 48 h at room temperature prior to ultracentrifugation. The appropriate sample concentration was obtained by dilution with the diffusate. Ultracentrifugation was carried out at 20 °C at a rotor speed of 18 000 rpm for factor XI in the absence of 0.1 M dithiothreitol and 26 000 rpm for factor XI in the presence of 0.1 M dithiothreitol or S-pyridylethylated factor XI. A partial specific volume of $\bar{v}$ = 0.707 mL/g was determined for factor XI from its amino acid and carbohydrate content (Cohn and Edsall, 1943; Gibbons, 1966) and corrected for 6 M guanidine hydrochloride (Lee and Timasheff, 1974). The solvent density at 20 °C was obtained from its index of refraction at 23.5° measured with an Abbe refractometer (Kielley and Harrington, 1960).

Heparin–agarose was prepared by the cyanogen bromide method as described by Fujikawa et al. (1973). The heparin–agarose was used repeatedly for purification of factor XI. After use, the heparin–agarose (1 L) was unpacked from the column and stirred with 0.1 N NaOH for 20 min at 4 °C. Following a rapid filtration by suction, the gel was extensively washed with cold water.

After packing into the column, heparin–agarose was washed with 4 L of 0.1 M Tris base (pH 10.5), containing 4.0 M NaCl, and further washed with 10 L of 0.02 M Tris-HCl buffer (pH 7.2) containing 0.05 M NaCl. Polybrene was not added to the equilibration buffer. Heparin–agarose employed for the second and third heparin–agarose columns was used only after repeated washing with the buffer containing 4 M NaCl followed by equilibration with the starting buffer.

Antibody against factor XI was obtained by injection of the purified protein (330 μg), emulsified in Freund's complete adjuvant, into rabbits. Two and three weeks after the first injection, the same amount of factor XI, emulsified in Freund's incomplete adjuvant, was injected into each rabbit. One week after the last injection, the rabbits were exsanguinated and the blood was allowed to clot and retract overnight at 4 °C. The

serum was treated with $BaSO_4$ (100 mg/mL) for 30 min at room temperature and centrifuged. Solid ammonium sulfate was added to the supernatant to 35% saturation, and the precipitate was collected by centrifugation. It was then dissolved in half of the original volume of 0.05 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and dialyzed against the same buffer for 24 h. Ammonium sulfate was added again to the dialyzed sample to 35% saturation and the same procedure was repeated as described above.

For immunoelectrophoresis, the samples were run in 1% agarose on microscope slides in a buffer prepared by mixing equal volumes of 0.02 M sodium barbital buffer (pH 8.6) and 0.036 M Tris-borate buffer (pH 9.1). Electrophoresis was carried out in 0.02 M sodium barbital buffer (pH 8.6) in the anode compartment and 0.036 M Tris–borate buffer (pH 9.1) in the cathode compartment. Electrophoresis was continued for 60 min at 150 V per 6.5 cm. Antibody was added to the center trough and allowed to diffuse for 24 h. The slides were then allowed to soak in 0.15 M NaCl for 24 h and in distilled water for 12 h at room temperature and photographed by indirect lighting (Schmer et al., 1972).

Factor XI activity was routinely measured in siliconized tubes by the one-stage kaolin-activated partial thromboplastin time using bovine factor XI deficient plasma. Samples to be assayed for factor XI activity were routinely diluted 100- to 400-fold with 0.02 M Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl and 1 mg/mL bovine serum albumin. Samples were diluted to give a clotting time between 40 and 100 s. A 0.1-mL aliquot of the diluted sample was then incubated at 37 °C for 10 min with 0.05 mL of bovine factor XI deficient plasma and 0.1 mL of 0.5% kaolin suspension. After a 10-min preincubation, 0.1 mL of 0.2% phospholipid suspension and 0.1 mL of 0.05 M $CaCl_2$ solution were added to the mixture and the time required for clot formation was recorded. Clot formation was followed by the tilting method. The amount of factor XI activity was estimated from a calibration curve made by serial dilution of normal bovine plasma. The log of the clotting time was plotted against the log of the concentration of platelet-poor normal bovine plasma. One unit of factor XI is defined as that amount of activity present in 1.0 mL of normal bovine plasma. A sample which contains 0.1 unit/mL of factor XI gives a clot in about 56 s. Specific activity is expressed as units per milligram of protein. For the assay of factor XI_a, the kaolin suspension was replaced by 0.02 M Tris-HCl buffer (pH 7.4), containing 0.1 M NaCl and 1 mg/mL bovine serum albumin, and the sample mixture was recalcified without preincubation. Fitzgerald factor, Fletcher factor, and factor XII activities were measured by a procedure identical with that of factor XI, except that the respective deficient plasma was employed and the preincubation was 2 min at room temperature instead of 10 min at 37 °C. Clotting assays for prothrombin, factor VII, factor IX, and factor X were carried out as previously described (Fujikawa et al., 1973).

Purification of Bovine Factor XI. Bovine blood was collected in buckets, each containing 0.1 vol of anticoagulant solution (13.4 g of sodium oxalate, 100 mg of heparin (17 000 units), and 100 mg of crude soybean trypsin inhibitor/L of solution). The plasma was isolated at room temperature with a continuous flow separator (De Laval Model BLE 519). All subsequent steps were performed at 4 °C employing plastic columns and tubes. The plasma (15 L) was mixed and stirred with barium sulfate (20 g/L) for 30 min, and the slurry was centrifuged for 8 min at 7800*g* in a Sorvall RC 3 centrifuge. The supernatant was made 0.1 mM in ethylenediaminetetraacetic acid and then brought to 20% saturation by the slow addition of solid ammonium sulfate. After stirring for 15 min, the precipitate was removed by centrifugation for 15 min at 7800*g*. The supernatant was then brought to 50% saturation with solid ammonium sulfate, and after stirring for 30 min the suspension was centrifuged for 60 min at 7800*g*. The precipitate was redissolved in 4.5 L of cold distilled water containing polybrene (50 mg/L), iPr_2FP (0.2 mM), and soybean trypsin inhibitor (100 mg). The solution was then dialyzed for 15 h against 100 L of cold distilled water followed by dialysis against 80 L of 0.02 M Tris-HCl buffer (pH 7.2), and 0.05 M NaCl for an additional 7–8 h.

After dialysis, the conductivity of the sample solution was adjusted to 7 $m\Omega^{-1}$ at 4 °C by the addition of cold distilled water. iPr_2FP (final concentration of 0.2 mM) and polybrene (final concentration of 50 mg/L) were added to the sample, which was then applied at a flow rate of less than 200 mL/h to a heparin–agarose column (8 × 20 cm). After application of the sample, the column was washed with 2.5–3 L of 0.02 M Tris-HCl buffer (pH 7.2) containing 0.05 M NaCl and 0.2 mM iPr_2FP. Factor XI was eluted with a linear gradient formed with 3 L of 0.02 M Tris-HCl buffer (pH 7.2), containing 0.15 M NaCl, and 3 L of 0.02 M Tris-HCl buffer (pH 7.2), containing 0.6 M NaCl. Each solution also contained 0.2 mM iPr_2FP and 50 mg/L polybrene. Fractions (200 mL) were collected at a flow rate of 300 mL/h. Polybrene (20 mg) and soybean trypsin inhibitor (40 mg) were added to each bottle prior to collection of the eluate.

Fractions containing factor XI activity were pooled and polybrene, benzamidine, soybean trypsin inhibitor, and iPr_2FP were added to the pooled fractions to a final concentration of 200 mg/L, 1 mM, 50 mg/L, and 0.2 mM, respectively. The solution was then dialyzed overnight against 20 L of 0.02 M phosphate buffer (pH 6.6), containing 0.07 M NaCl. After dialysis, a small precipitate was removed by centrifugation. Polybrene (100 mg/L), benzamidine (final concentration of 1 mM), and iPr_2FP (final concentration of 0.2 mM) were then added to the dialyzed sample.

CM-Sephadex C-50 (200 mL of settled volume) was equilibrated with 0.02 M phosphate buffer (pH 6.6), containing 0.07 M NaCl, and added to the dialyzed sample from the heparin–agarose column. The suspension was stirred slowly for 2 h at 4 °C and allowed to settle, and the supernatant was removed by siphon. The CM-Sephadex was then poured into a 4.5 × 13 cm column, and the column was washed with 500 mL of 0.02 M phosphate buffer (pH 6.6) containing 0.07 M NaCl and polybrene (100 mg/L). Protein was eluted with a linear gradient formed with 1 L of 0.02 M phosphate buffer (pH 6.6), containing 0.1 M NaCl, and 1 L of 0.02 M phosphate buffer (pH 6.6), containing 0.55 M NaCl. Both solutions also contained iPr_2FP (0.2 mM) and polybrene (100 mg/L). The flow rate was 100 mL/h. Fractions containing factor XI were combined (approximately 460 mL) and 2 mL of 0.5 M benzamidine, 20 mg of soybean trypsin inhibitor, 100 mg of polybrene, and 0.2 mL of 1 M iPr_2FP were added to the pooled fraction.

The sample was then applied directly to a second heparin–agarose column (3.2 × 12 cm) previously equilibrated with 0.05 M phosphate buffer (pH 6.6) containing 0.15 M NaCl. The column was washed with 200 mL of 0.05 M phosphate buffer (pH 6.6), containing 0.20 M NaCl, and 0.2 mM iPr_2FP. Factor XI was eluted from the column with a linear gradient formed with 300 mL of 0.05 M phosphate buffer (pH 6.6), containing 0.25 M NaCl, and 300 mL of 0.05 M phosphate buffer (pH 6.6), containing 1.0 M NaCl. Both solutions also contained 0.2 mM iPr_2FP and polybrene (100 mg/L). The

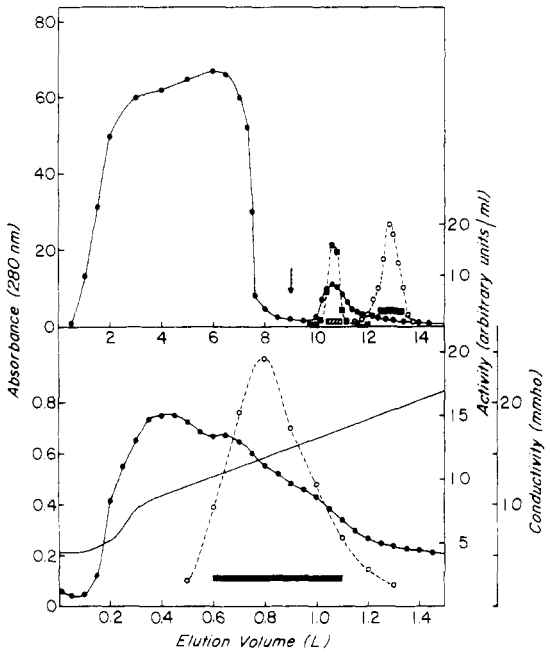

FIGURE 1: Elution pattern for bovine factor XI from the first heparin-agarose column and the CM-Sephadex column. (Top panel) The ammonium sulfate precipitate was dialyzed and applied to a heparin-agarose column (8 × 20 cm) as described under Methods. Proteins were eluted from the column with a linear gradient formed with 3 L of 0.02 M Tris-HCl buffer (pH 7.2), containing 0.15 M NaCl and 3 L 0.02 M Tris-HCl buffer (pH 7.2), containing 0.6 M NaCl. Each solution also contained 0.2 mM iPr_2FP and 50 mg/L polybrene. Fractions (200 mL) were collected at a flow rate of 300 mL/h. Factor XI activity was determined as described under Methods: (●—●) absorbance at 280 nm; (○- - -○) factor XI activity; (■- - -■) factor XII activity. The arrow shows where the gradient was started. The solid bar shows the factor XI fractions which were combined. The hatched bar shows fractions pooled for the purification of factor XII (Fujikawa et al., 1977). (Bottom panel) The factor XI solution obtained from the first heparin-agarose column was dialyzed overnight and mixed with CM-Sephadex C-50 as described under Methods. The suspension was poured into a 4.5 × 13 cm column and washed with 500 mL of 0.02 M phosphate buffer (pH 6.6), containing 0.07 M NaCl and 100 mg/L polybrene. Factor XI was eluted from the column with a linear gradient formed with 1 L of 0.02 M phosphate buffer (pH 6.6), containing 0.1 M NaCl, and 1 L of 0.02 M phosphate buffer (pH 6.6), containing 0.55 M NaCl. Each solution also contained 0.2 mM iPr_2FP and polybrene (100 mg/L). The eluate was collected in 10-mL fractions at a flow rate of 100 mL/h: (●—●) absorbance at 280 nm; (○- - -○) factor XI activity (units/mL). Fractions shown by the solid bar were combined.

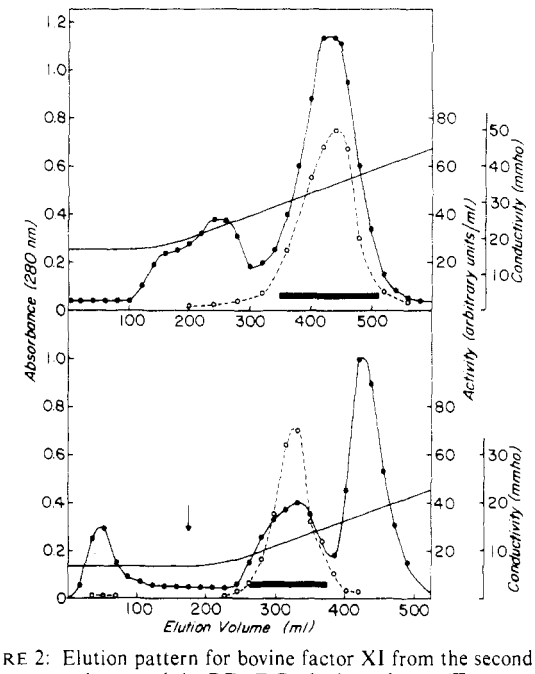

FIGURE 2: Elution pattern for bovine factor XI from the second heparin-agarose column and the DEAE-Sephadex column. (Top panel) The pooled fractions from the CM-Sephadex column were applied directly to the second heparin-agarose column (3.2 × 12 cm) previously equilibrated with 0.05 M phosphate buffer (pH 6.6), containing 0.15 M NaCl as described under Methods. Protein was eluted from the column with a linear gradient formed with 300 mL of 0.05 M phosphate buffer (pH 6.6), containing 0.25 M NaCl and 300 mL of 0.05 M phosphate buffer (pH 6.6), containing 1.0 M NaCl. Each solution also contained 0.2 mM iPr_2FP and polybrene (100 mg/L). The eluate was collected in 4-mL fractions at a flow rate of 80 mL/h: (●—●) absorbance at 280 nm; (○- - -○) factor XI activity. Fractions shown by the bar were combined. (Bottom panel) The fractions from the second heparin-agarose column were dialyzed and applied to a DEAE-Sephadex column (1.6 × 25 cm), previously equilibrated with 0.05 M Tris-HCl (pH 8.4), containing 0.08 M NaCl as described under Methods. Protein was eluted from the column with a linear gradient formed with 200 mL of 0.05 M Tris-HCl buffer (pH 8.4), containing 0.08 M NaCl, and 200 mL of 0.05 M Tris-HCl buffer (pH 8.4), containing 0.4 M NaCl. Both solutions also contained 0.2 mM iPr_2FP. The eluate was collected in 4-mL fractions at a flow rate of 20 mL/h: (●—●) absorbance at 280 nm; (○- - -○) factor XI activity. The arrow shows where the gradient elution was started. Fractions shown by the solid bar were combined.

flow rate was 80 mL/h. Fractions containing factor XI were combined (approximately 180 mL) and 2 mL of 0.5 M benzamidine, 100 mg of polybrene, 1 mg of pure soybean trypsin inhibitor, and 0.2 mL of iPr_2FP were added. The protein solution was then concentrated to a volume of about 50 mL by ultrafiltration employing an Amicon Diaflo concentrator with a PM-30 membrane. The concentrated sample was dialyzed for 2 h against 2 L of 0.05 M Tris-HCl buffer (pH 8.4), containing 0.08 M NaCl, and dialysis was then continued for an additional 4 h against two changes of fresh buffer (2 L each). The dialyzed sample was applied to a 1.6 × 25 cm DEAE-Sephadex A-50 column which was previously equilibrated with 0.05 M Tris-HCl buffer (pH 8.4), containing 0.08 M NaCl. After application of the sample, the column was washed with 150 mL of the equilibration buffer containing 0.2 mM iPr_2FP. Factor XI was eluted from the column with a linear gradient formed with 200 mL of 0.05 M Tris-HCl buffer (pH 8.4), containing 0.08 M NaCl, and 200 mL of 0.05 M Tris-HCl buffer (pH 8.4), containing 0.4 M NaCl. Both solutions also contained 0.2 mM iPr_2FP. The flow rate was 20 mL/h. Fractions containing factor XI were pooled (approximately 120 mL) and 1 mL of 0.5 M benzamidine, 1 mg of pure soybean trypsin inhibitor, 0.2 mL of 1 M iPr_2FP, and 50 mg of polybrene were added. The sample was then dialyzed overnight against 2 L of 0.05 M phosphate buffer (pH 6.6), containing 0.15 M NaCl.

The sample was applied to a third heparin-agarose column (3.2 × 12 cm) previously equilibrated with 0.05 M phosphate buffer (pH 6.6), containing 0.15 M NaCl. The column was then washed with 100 mL of 0.05 M phosphate buffer (pH 6.6) containing 0.15 M NaCl, and 150–200 mL of 0.05 M phosphate buffer (pH 6.6), containing 0.2 M NaCl. Purified factor XI was eluted with 200 mL of 0.05 M phosphate buffer (pH 6.6), containing 0.4 M NaCl. Fractions containing factor XI were pooled (approximately 100 mL) and iPr_2FP was added to a final concentration of 0.2 mM. The solution was then concentrated to about 10 mL and stored at −20 °C.

Results

Preparation of Bovine Factor XI. The various steps in the purification of bovine factor XI from 15 L of plasma are shown in Table I. The purification was about 28 000-fold with an

TABLE I: Purification of Bovine Factor XI.

Purification Step	Vol (mL)	Total Protein[a] (mg)	Total Act. (Units)	Sp Act. (Units/mg)	Recovery (%)	Purification (Fold)
Plasma	15 000	10.5×10^5	15 000	0.014	100	1
20–50% $(NH_4)_2SO_4$	6 000	4.6×10^5	13 900	0.030	93	2.1
First heparin–agarose	1 200	2 190	13 300	6.08	89	430
CM-Sephadex	460	232	9 660	41.6	64	2 970
Second heparin–agarose	180	111	7 580	68.3	51	4 880
DEAE-Sephadex	120	26.4	5 400	204	36	14 600
Third heparin–agarose	100	9.1	4 500	494	30	27 900

[a] Protein concentration was determined by absorption employing $E_{280}^{1\%} = 10.0$ for plasma and subsequent steps up to the third heparin–agarose column for which $E_{280}^{1\%} = 12.6$ was employed.

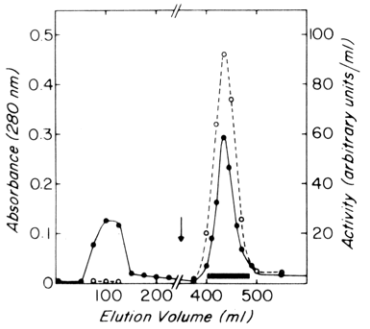

FIGURE 3: Elution pattern for bovine factor XI from the third heparin–agarose column. The pooled fractions from the DEAE-Sephadex column were dialyzed and applied to the third heparin–agarose column (3.2 × 12 cm) previously equilibrated with 0.05 M phosphate buffer (pH 6.6), containing 0.15 M NaCl as described under Methods. The column was washed with 100 mL of 0.05 M phosphate buffer (pH 6.6), containing 0.15 M NaCl, and 150–200 mL of 0.05 M phosphate buffer (pH 6.6), containing 0.2 M NaCl. Factor XI was eluted from the column with 200 mL of 0.05 M phosphate buffer (pH 6.6), containing 0.4 M NaCl. The eluate was collected in 4-mL fractions at a flow rate of 80 mL/h: (●—●) absorbance at 280 nm; (○- - -○) factor XI activity. The arrow shows where the factor XI elution buffer was started. Fractions shown by the solid bar were combined.

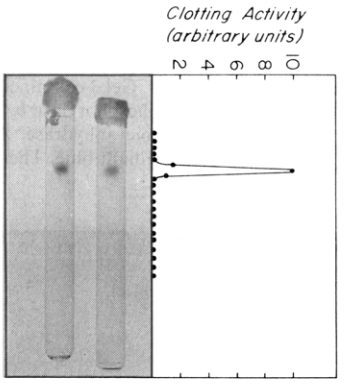

FIGURE 4: Polyacrylamide disc gel electrophoresis of bovine factor XI. All samples contained 50 μg of protein. The gel on the left was stained for protein with Coomassie brilliant blue, and the gel on the right was stained for carbohydrate with the periodic acid–Schiff base reagent. A third gel was sliced into 2-mm segments, soaked with 0.02 M Tris-HCl (pH 7.4), containing 0.1 M NaCl, and assayed for factor XI activity. The anode was at the bottom of the gel.

overall yield of 30%. The isolation procedure involves a barium sulfate adsorption of contaminants, ammonium sulfate precipitation, and chromatography on heparin–agarose, CM-Sephadex C-50, and DEAE-Sephadex A-50. The barium sulfate adsorption of the plasma was employed to remove prothrombin, factor X, factor IX, and factor VII prior to ammonium sulfate precipitation. The first heparin–agarose was designed for the simultaneous purification of factor XII (Fujikawa et al., 1977) as well as factor XI. The elution profile of factor XI on the first heparin–agarose is shown in the top panel of Figure 1. More than 95% of the proteins passed through the column before the gradient was started. Factor XI appeared in the trailing edge of the second peak at a conductivity of 23–24 $m\Omega^{-1}$ as measured at 4°C. Factor XII appeared in the middle of the second peak at a conductivity of 12–13 $m\Omega^{-1}$ and trailed only slightly into the factor XI peak. Factor XI was purified 300- to 600-fold at this stage over the starting plasma (Table I).

The chromatography profile for factor XI on CM-Sephadex is shown in the lower panel of Figure 1. About 50% of the protein (not shown) passed through the column before the gradient was started. Factor XI was eluted on the trailing edge of the protein peak at a conductivity of 14–16 $m\Omega^{-1}$.

The top panel of Figure 2 shows the protein elution profile from the second heparin–agarose column after the gradient was started. Factor XI was eluted from this column in the second major peak at a conductivity of 33–36 $m\Omega^{-1}$. The chromatography profile for factor XI on DEAE-Sephadex is shown in the lower panel of Figure 2. Factor XI was eluted from this column in the second protein peak at a conductivity of 14–15 $m\Omega^{-1}$. Factor XI was purified 14 000- to 15 000-fold at this stage of the isolation procedure.

The final purification of bovine factor XI was performed by chromatography on heparin–agarose. As shown in Figure 3, factor XI was eluted from this column in the second protein peak in which the coagulant activity eluted in parallel with the protein.

The final preparation of factor XI has a specific activity of 494 units/mg of protein which corresponds to about a 28 000-fold purification over the starting plasma (Table I). The purified factor XI at a concentration of 0.1 mg/mL had no effect on the clotting time of plasmas deficient in Fitzgerald factor, Fletcher factor, factor XII, factor IX, factor VII, and factor X. Furthermore, it was completely free of prothrombin and thrombin. The activation of factor XI during the isolation procedure was effectively inhibited by the addition of soybean trypsin inhibitor, iPr_2FP, polybrene, and benzamidine to the samples and elution buffers.

Polyacrylamide Disc Gel and Sodium Dodecyl Sulfate Gel Electrophoresis of Bovine Factor XI. A single band was obtained for bovine factor XI by polyacrylamide disc gel electrophoresis at pH 9.4 (Figure 4). The gel on the left was stained for protein with Coomassie brilliant blue, and the gel on the

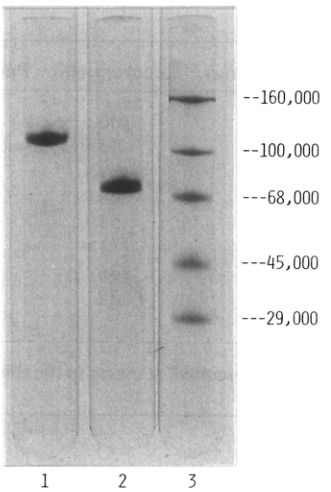

FIGURE 5: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of bovine factor XI. Gel 1 contained 10 μg of unreduced factor XI and gel 2 contained 20 μg of reduced factor XI. Gel 3 contained from top to bottom: glycogen debranching enzyme, phosphorylase *b*, bovine serum albumin, ovalbumin, and bovine carbonic anhydrase. All samples were stained for protein with Coomassie brilliant blue. The anode was at the bottom of the gel.

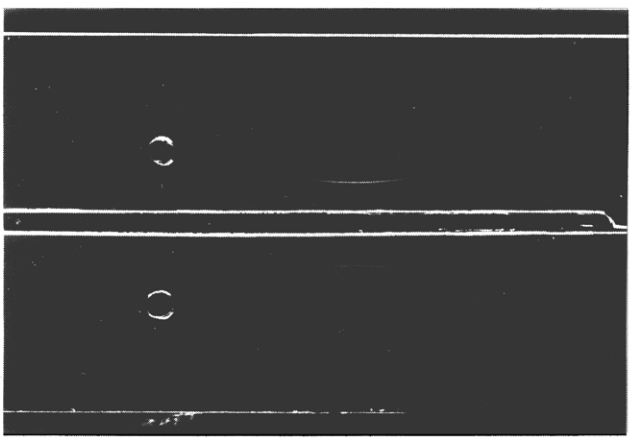

FIGURE 6: Immunoelectrophoresis of bovine factor XI. Immunoelectrophoresis was carried out for 60 min as described under Methods. Ten microliters of sample containing approximately 15 μg of highly purified factor XI from two different preparations was added to each well. Upon completion of electrophoresis, 50 μL of rabbit antibody to bovine factor XI was applied to the center trough. Photographs were taken by indirect lighting. The anode is at the right of the photograph.

right was stained for carbohydrate with the periodic acid–Schiff reagent. Identification of factor XI coagulant activity with the corresponding bands present in the two gels was shown in an experiment in which a third gel run in parallel with the first two gels was sliced into 2-mm segments, soaked with 0.02 M Tris-HCl buffer (pH 7.4), containing 0.1 M NaCl, and assayed for factor XI activity. As shown in the panel on the right side of Figure 4, factor XI coagulant activity corresponded directly to the protein and carbohydrate bands.

The highly purified factor XI also showed a single protein band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 5, gel 1). After reduction with 2-mercaptoethanol, a single, faster moving protein band was obtained (Figure 5, gel 2). The apparent molecular weight for factor XI before and after reduction was estimated as 113 000 and 76 000, respectively. These data indicate that bovine factor XI is made up of two similar or identical polypeptide chains, and these two chains are held together by a disulfide bond(s). A mol wt of 113 000 for the intact protein is significantly lower than

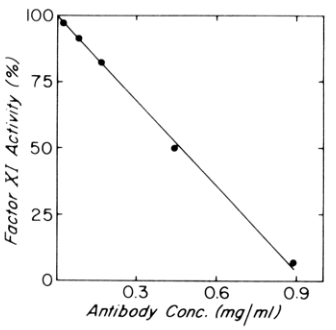

FIGURE 7: The inhibition of bovine factor XI by an antibody prepared in rabbits against bovine factor XI. Factor XI (60 μg/mL) was incubated at 37 °C for 1 h with increasing concentration of antibody and then diluted 50-fold with 0.02 M Tris-HCl buffer (pH 7.4), containing 0.1 M NaCl. The remaining factor XI activity was measured by the one-stage assay as described under Methods.

that obtained by sedimentation equilibrium as described below. The mol wt of 76 000 for the reduced protein, however, is slightly larger than that obtained by sedimentation equilibrium.

Immunoelectrophoresis of Bovine Factor XI. As further evidence of purity, factor XI was subjected to immunoelectrophoresis on microscope slides layered with 1% agarose. The agarose was suspended in a buffer solution containing 0.01 M sodium barbital and 0.018 M Tris–borate buffer (pH 8.9), as described under Methods. Electrophoresis of bovine factor XI followed by immunodiffusion with antibody prepared in rabbits against the purified bovine factor XI gave a single, sharp precipitin line (Figure 6). Immunoelectrophoresis of bovine factor XI in 0.05 M sodium barbital buffer (pH 8.6) also gave a single, sharp precipitin line. Under these conditions, however, the protein moves only a short distance from the starting well. This is consistent with factor XI being classified as a β globulin.

The factor XI antibodies employed in these experiments readily neutralized factor XI coagulant activity as measured in the two-stage coagulant assay (Figure 7). In these experiments, highly purified factor XI was incubated for 1 h at 37 °C with increasing concentrations of antibody and an aliquot was then diluted and assayed for clotting activity. These studies support the conclusion that the single protein precipitin line observed in the immunoelectrophoresis experiments shown in Figure 6 was due to the presence of bovine factor XI and not some contaminating protein.

Sedimentation Equilibrium Studies on Bovine Factor XI. Sedimentation equilibrium studies on bovine factor XI in 6 M guanidine-HCl in the presence and absence of 0.1 M dithiothreitol demonstrated a minimal mol wt of 54 900 and 124 000, respectively (Table II). The reduced S-pyridylethylated protein showed a minimal mol wt of 59 300.

Amino Acid and Carbohydrate Compositions of Bovine Factor XI. The amino acid and carbohydrate compositions for bovine factor XI are shown in Table III. Bovine factor XI is a glycoprotein composed of about 89% protein and 11% carbohydrate. The carbohydrate includes 5.4% hexose, 4.7% *N*-acetylhexosamine, and 1.0% *N*-acetylneuraminic acid. This corresponds to 110 000 g of protein and 14 000 g of carbohydrate per 124 000 g of glycoprotein.

Discussion

During the past 15 years, a number of investigators have attempted to isolate factor XI from human or bovine plasma (Ratnoff et al., 1961; Ratnoff and Davie, 1962; Schiffman et

TABLE II: Molecular Weight of Bovine Factor XI and Its Subunits.[a]

Sample	M_1	M_n	M_w	M_Z
Factor XI	124 000 ± 1700	133 000 ± 1300	141 000 ± 1200	131 000 ± 1700
Reduced factor XI	54 900 ± 390	54 900 ± 80	54 700 ± 270	54 200 ± 400
Pyridylethylated factor XI	59 300 ± 1300	62 900 ± 750	67 000 ± 630	66 300 ± 1200

[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 described by Kraemer (1940). A partial specific volume of 0.707 mL/g was employed for each sample as described under Methods.

al., 1963; Kingdon et al., 1964; Wuepper, 1972; Saito et al., 1973; Schiffman and Lee, 1974). The purification of this protein, however, has been difficult since its concentration in plasma is low and it tends to be activated spontaneously during purification. In the present procedure, large amounts of plasma (15 L) were fractionated in the presence of heparin, oxalate, polybrene, iPr_2FP, soybean trypsin inhibitor, and benzamidine to inhibit factor XII_a and other plasma proteases. Under these conditions, it has been possible to isolate factor XI in a precursor form with good recovery.

In the present procedure, prothrombin, factor VII, and factor IX were removed by adsorption to barium sulfate prior to ammonium sulfate precipitation. It was also important to remove factor XII from factor XI early in the isolation procedure, and this was achieved in the first heparin–agarose chromatography. In this column, factor XII and factor XI readily separated from each other. As previously reported by Gentry and Alexander (1973), factor XI was adsorbed to heparin–agarose. This also made it possible to concentrate factor XI from large amounts of plasma in the first major step in the isolation procedure. Factor XII was also bound to heparin–agarose under these conditions, but factor XII eluted prior to factor XI without detectable overlapping.

The addition of polybrene at various stages of the isolation procedure was important in obtaining factor XI in a precursor form. Polybrene changes the elution position of factor XI and factor XII from the first heparin–agarose column, and excess polybrene prevents these proteins from binding to heparin–agarose. Therefore, polybrene was not added to the reequilibration buffer of heparin–agarose after washing with 0.1 M NaOH and high salt solution. In the absence of polybrene, factor XI and factor XII were bound to heparin–agarose more strongly and were eluted in very broad protein peaks.

The bovine factor XI described in the present investigations had a specific activity of 494 units per mg of protein with a purification of about 28 000-fold from the starting plasma. This indicates that the factor XI content in bovine plasma is about 2 μg/mL of plasma. This value is in the same range as factor IX and factor X which are present in about 3.4 and 7.6 μg, respectively, per mL of plasma (Fujikawa et al., 1972, 1973).

Human factor XI has been reported to be a glycoprotein consisting of two similar or identical polypeptide chains (Wuepper, 1972). The molecular weight of human factor XI was estimated as 160 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Wuepper, 1972). This value is substantially larger than the 113 000 found for bovine factor XI in this study by the same technique.

Several attempts to determine the amino-terminal sequence of bovine factor XI in a Beckman sequenator were unsuccessful. In these experiments, 6–10 mg of S-pyridylethylated factor XI was employed. Pretreatment of the S-carboxymethylated protein with pyrrolidonecarboxylyl peptidase, kindly supplied by Dr. R. F. Doolittle of the University of California, San Diego, also failed to give any amino-terminal amino acid when analyzed in the sequenator. These negative results suggest that the amino-terminal residues in both peptide chains of bovine factor XI are blocked. These residues are probably not, however, pyrrolidonecarboxylic acid. Additional experiments are necessary to answer this question.

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

Component	Residues/124 000
Amino acid	
Lysine	55.6
Histidine	38.9
Arginine	40.9
Aspartic acid	82.9
Threonine	75.7[a]
Serine	79.9[a]
Glutamic acid	123.8
Proline	52.6
Glycine	68.7
Alanine	44.1
Half-cystine	42.3[b] (38.0[c])
Valine	46.8[d]
Methionine	11.6
Isoleucine	50.2[d]
Leucine	72.1
Tyrosine	29.4
Phenylalanine	41.6
Tryptophan	20.7[e]
Carbohydrate	
Hexose	37.2 (5.4%)
N-Acetylhexosamine	26.6 (4.7%)
N-Acetylneuraminic acid	4.3 (1.0%)
Protein (%)	88.9
Carbohydrate (%)	11.1

[a] Values extrapolated to zero time. [b] Determined as cysteic acid according to Hirs (1967). [c] Determined as *S*-pyridylethylcysteine according to Friedman et al. (1970). [d] Values from 48-h hydrolysate. [e] Determined after alkaline hydrolysis by the method of Hugli and Moore (1972).

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References

Babul, J., and Stellwagon, E. (1969), *Anal. Biochem. 28,* 216.

Chrambach, A., Reisfeld, R. A., Wyckoff, M., and Zaccari, J. (1967), *Anal. Biochem. 20,* 150.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N.Y., Reinhold, p 370.

Davie, E. W., and Fujikawa, K. (1975), *Annu. Rev. Biochem. 44,* 799.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

DeRosier, D. J., Munk, P., and Cox, D. J. (1972), *Anal. Biochem. 50,* 139.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28,* 350.

Fairbanks, H., Steck, A., and Wallach, E. E. (1971), *Biochemistry 10,* 2606.

Forbes, C. D., and Ratnoff, O. D. (1972), *J. Lab. Clin. Med.* 79, 113.

Friedman, M., Krull, L. H., and Cavins, J. F. (1970), *J. Biol. Chem. 245,* 3868.

Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972), *Biochemistry 11,* 4882.

Fujikawa, K., Legaz, M. E., Kato, H., and Davie, E. W. (1974), *Biochemistry 13,* 4508.

Fujikawa, K., Thompson, A. R., Legaz, M. E., Meyer, R. G., and Davie, E. W. (1973), *Biochemistry 12,* 4938.

Fujikawa, K., Walsh, K. A., and Davie, E. W. (1977), *Biochemistry* (preceding paper in this issue).

Gardel, S. (1957), *Methods Biochem. Anal. 6,* 289.

Gentry, P. W., and Alexander, B. (1973), *Biochem. Biophys. Res. Commun. 50,* 500.

Gibbons, R. A. (1966), *Glycoproteins 5,* 61.

Griffin, J. H., and Cochrane, C. G. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 2554.

Hirs, C. H. W. (1967), *Methods Enzymol. 11,* 59.

Hugli, T., and Moore, S. (1972), *J. Biol. Chem. 247,* 2828.

Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41,* 410.

Kingdon, H. S., Davie, E. W., and Ratnoff, O. D. (1964), *Biochemistry 3,* 166.

Kociba, G. J., Ratnoff, O. D., Loeb, W. F., Wall, R. L., and Heider, L. E. (1969), *J. Lab. Clin. Med. 74,* 37.

Koide, T., Kato, H., and Davie, E. W. (1977), *Methods Enzymol.* (submitted for publication).

Kraemer, E. O. (1940), in The Ultracentrifuge, Svedberg, T., and Petersen, K. O., Ed., Oxford University Press, London, p 345.

Lee, J. C., and Timasheff, S. N. (1974), *Arch. Biochem. Biophys. 165,* 268.

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.

Rapaport, S. I., Proctor, R. R., Patch, M. J., and Yettra, M. (1961), *Blood 18,* 149.

Ratnoff, O. D. (1972), *J. Lab. Clin. Med. 80,* 704.

Ratnoff, O. D., and Davie, E. W. (1962), *Biochemistry 1,* 677.

Ratnoff, O. D., Davie, E. W., and Mallett, D. L. (1961), *J. Clin. Invest. 40,* 803.

Richards, E. G., Teller, D. C., and Schachman, H. K. (1968), *Biochemistry 7,* 1054.

Rosenthal, R. L., Dreskin, O. H., and Rosenthal, N. (1953), *Proc. Soc. Exp. Biol. Med. 82,* 171.

Saito, H., Ratnoff, O. D., Marshall, J. S., and Pensky, J. (1973), *J. Clin. Invest. 52,* 850.

Saito, H., Ratnoff, O. D., Waldmann, R., and Abraham, J. P. (1975), *J. Clin. Invest. 55,* 1082.

Schiffman, S., and Lee, P. (1974), *Br. J. Haematol. 27,* 101.

Schiffman, S., Rapaport, S. I., and Patch, M. J. (1963), *Blood 22,* 733.

Schmer, G., Kirby, E. P., Teller, D. C., and Davie, E. W. (1972), *J. Biol. Chem. 247,* 2512.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.

Teller, D. C. (1973), *Methods Enzymol. 27,* 346.

Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N.Y. Acad. Sci. 164,* 66.

Warren, L. (1959), *J. Biol. Chem. 234,* 1971.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

Wright, I. (1959), *J. Am. Med. Assoc. 170,* 325.

Wuepper, K. D. (1972), in Inflammation: Mechanisms and Control, Lepow, I. H., and Ward, P. A., Ed., Academic Press, New York, N.Y., p 93.

Yphantis, D. A. (1964), *Biochemistry 3,* 297.

Zacharius, R. M., Zell, T. E., Morrison, J. H., and Woodlock, J. J. (1969), *Anal. Biochem. 30,* 148.