Preparation of Highly Purified Prothrombin Complex

I. CRYSTALLIZATION, BIOLOGICAL ACTIVITY, AND MOLECULAR PROPERTIES*

(Received for publication, April 9, 1968)

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SUMMARY

Bovine prothrombin, an α2-glycoprotein clotting factor of plasma, has been isolated in high degree of purity in crystalline form as the barium glycoprotein product of interaction. Chemical determinations made on the product showed the following composition, in percentage of the dry weight: protein, 21.5%; barium, 32%; and citrate, 8.9%. That the crystals represent a true prothrombin-metal complex has been established by control studies, x-ray diffraction pattern, chemical composition, and recrystallization. The product showed constant solubility characteristics.

Extensive physical and chemical studies of the barium-free protein are presented. Further purification was achieved by gel filtration on Sephadex G-100. The peak fractions evidenced prothrombin activity of 3000 "Iowa" units (Seegers, W. H., Prothrombin, Harvard University Press, Cambridge, Massachusetts, 1962, p. 420) per mg (corresponding to a 600-fold purification), as well as appreciable Factors VII, IX, and X coagulant activities. This product is designated "prothrombin complex," in accordance with the nomenclature used by other investigators. The preparation was homogeneous by sedimentation velocity pattern, sedimentation equilibrium analysis, and gel filtration. Despite these indications of homogeneity, physical heterogeneity was shown by immuno- and disc electrophoresis. The multiple coagulant activities and protein subcomponents were found to be homogeneous with respect to molecular weight (70,477 ± 2,780), determined by sedimentation equilibrium in a dissociating solvent consisting of 6 M guanidine hydrochloride and 0.5% mercaptoethanol, and also with respect to Stokes radius. Physical constants, determined in various systems, include diffusion constant, sedimentation coefficient, frictional ratio, isoelectric point, isoionic point, electrophoretic mobility, and extinction coefficient. Physical data failed to show evidence of dissociation into subunits or fragmentation by dilution to below 0.1% protein or by prolonged exposure to a dissociating solvent. The latter observation suggests that the reduction in sedimentation constant noted in high ionic strength solvents in this study, and by other observers, is probably attributable to changes in shape and hydration of the kinetic unit. The product was also shown to undergo rapid, reversible association in solvents of low ionic strength (γ/2 = 0.15).

DEAE-Sephadex chromatography resulted in partial activation of the prothrombin complex, and several homogeneous protein species were isolated and partially characterized. The monomeric molecular weights determined by sedimentation equilibrium in a solvent of 6 M guanidine hydrochloride and mercaptoethanol were as follows: prothrombin (devoid of Factors VII and X), 65,530 ± 1,247; modified zymogen, 52,395 ± 3,449; Factor VII, 33,900 ± 3,390; and Factor X, 37,772 ± 1,234. The modified zymogen was inert in the two-stage assay and in 25% sodium citrate, but readily transformed to thrombin when Factor X was added. The results are consistent with the interpretation that the prothrombin complex, as isolated in this study, comprises a family of glycoproteins that can undergo a proenzyme-enzyme transformation and that have similar molecular properties but separate clotting activities.

The current concepts of blood coagulation stem from the classical theory of Morawitz, proposed in 1905. According to this theory four factors (thromboplastin, calcium, prothrombin, and fibrinogen) interact to form an insoluble gel or fibrin. Thromboplastin is now considered to arise during the first phase of physiological clotting and is apparently a highly specific protease capable of splitting prothrombin to a smaller fragment, thrombin (1). It is now also recognized that the biological pathway of prothrombin activation to thrombin requires the presence of certain plasma cofactors, referred to as Factors V, VII, and X.1

1 With the exception of prothrombin, the designations of clotting activities conform to the recommendations of the Interna-
At a molecular level, the mechanisms of cofactor interactions involved in prothrombin activation are complex and poorly understood. Seegers (2) ascribes Factor VII and X activities as arising from prothrombin intermediates or derivatives ("autothrombins"). He considers the end products of prothrombin activation to be thrombin and another enzyme, autoprothrombin C. Thrombin and autoprothrombin C are also considered to be enzymatic activators of prothrombin conversion. Both thrombin and autoprothrombin C can be isolated in the form of precursor molecules, designated protomin and autoprothrombin III, respectively. Seegers considers both precursors to be sub-units derived from the parent prothrombin molecule (3). Other investigators (4-8) have presented physiological, biochemical, and immunochemical evidence that the plasma cofactors VII and X, although chemically similar to prothrombin, are distinct entities. Moreover, Factor X isolated chromatographically from purified prothrombin, and free of prothrombin activity, evidences autoprothrombin C and esterase activity when activated by tissue thromboplastin and calcium (9). A critical appraisal of the clotting factor versus prothrombin derivative concepts of blood coagulation has been presented by Kline (10).

It has also been recognized that the biosynthesis in mammalian species of these plasma cofactors (VII and X), together with prothrombin and Factor IX, are vitamin K dependent. The vitamin K-dependent clotting activities have been alluded to as the "prothrombin complex." They also share in common the chemical property of a high affinity for the insoluble salts of the alkaline earth metals. The latter property has made it possible to isolate the prothrombin complex in crystalline form as a barium glycoprotein product of interaction. A description of the isolation procedure and some of the physical and chemical properties of the purified prothrombin product follows. The isolation and comparative properties of certain individual coagulant proteins that comprise the prothrombin complex are also described.

**EXPERIMENTAL PROCEDURE**

**Materials**

*Starting Materials—* Bovine plasma was generously supplied by Swift and Company, Los Angeles. Thromboplastin was a 0.9% sodium chloride extract of acetone-dehydrated human brain (11). The brain powder was stored at 4° until used, and the saline extract was made at 47°. Oxalated human plasmas devoid of specific clotting activities were obtained from human subjects congenitally deficient in Factors VII, X, or IX. This material was stored at -20°. Because of the intrinsic lability of Factor V activity, this factor was supplemented in the assay procedures. A source of Factor V was provided by adsorbing oxalated human plasma with 100 mg of BaSO4 (3). Other Clotting Activities—Factors VII and X activities were involved in prothrombin activation are complex and poorly understood. Seegers (2) ascribes Factor VII and X activities as arising from prothrombin intermediates or derivatives ("autothrombins"). He considers the end products of prothrombin activation to be thrombin and another enzyme, autoprothrombin C. Thrombin and autoprothrombin C are also considered to be enzymatic activators of prothrombin conversion. Both thrombin and autoprothrombin C can be isolated in the form of precursor molecules, designated protomin and autoprothrombin III, respectively. Seegers considers both precursors to be sub-units derived from the parent prothrombin molecule (3). Other investigators (4-8) have presented physiological, biochemical, and immunochemical evidence that the plasma cofactors VII and X, although chemically similar to prothrombin, are distinct entities. Moreover, Factor X isolated chromatographically from purified prothrombin, and free of prothrombin activity, evidences autoprothrombin C and esterase activity when activated by tissue thromboplastin and calcium (9). A critical appraisal of the clotting factor versus prothrombin derivative concepts of blood coagulation has been presented by Kline (10).

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**Special Reagents—** All chemicals used were regent grade. Sephadex G-100, 40- to 120-μ particle size, bead form (Lot TO 2946), and DEAE-Sephadex A-50, 40- to 120-μ particle size, bead form (Lot 0012), were purchased from Pharmacia Fine Chemicals, Inc. Dowex 50W-X8, 20 to 50 mesh, Na+ form, was analytical reagent grade from Baker. AG 50W-X8, 100 to 200 mesh, was prepared in Na+ form by Bio-Rad Laboratories, Richmond, California. N-Acetyl-Lcarnitine acid was a synthetic crystalline product obtained from Sigma. Guanidine hydrochloride was a product of Baker. Ovalbumin was a two times crystallized product of Mann Research Laboratories. Rabbit antiserum to purified prothrombin and bovine plasma were prepared by Hyland Laboratories, Los Angeles, by the Freund technique (14). Human Factor VII-deficient plasma was generously supplied by Dr. O. Egeberg of the Rikshospitalet, Oslo, Norway.

**Assay of Blood Clotting Factors**

**Prothrombin—** The method of prothrombin assay consists of the conversion of prothrombin to thrombin in the presence of biological activators by a "two-stage" procedure initially described by Warner, Brinkhous, and Smith (15). The procedure used in this study was essentially that of Ware and Seegers (16), with slight modifications. The composition of the reaction mixture was as follows: 12.5 ml of 15% gum acacia solution, 25 ml of Veronal-buffered 0.85% sodium chloride, 4.5 ml of Veronal buffer, and 3.0 ml of thromboplastin extract. Oxalated bovine serum and BaSO4-adsorbed bovine plasma were used as sources of accelerator activities in a final dilution in the clotting mixture equivalent to 1:250 and 1:750 dilution of normal plasma, respectively. Fibrinogen used in the assay was a 1.5% solution of bovine Fraction I (Armour Pharmaceutical Company, Kenneke, Illinois, containing 40 to 50% sodium citrate) in distilled water.

One unit of prothrombin (or thrombin) is defined as the amount which, when fully converted to thrombin, clots 0.1 ml of fibrinogen at 25° in 15 sec. This unit has been designated the "Iowa" unit, as defined by Seegers (18). The fibrinogen reagent was also standardized against a reference thrombin obtained from the National Bureau of Standards (Lot 3B, specific activity 21.7 units mg⁻¹, dissolved in Veronal-buffered oxalated sodium chloride). In our assay system, 1 NIH unit was equivalent to 1.73 Iowa units. Normal bovine plasma assayed 300 to 350 units per ml. Aliquots of the solution to be assayed were appropriately diluted to yield 0.3 to 1.5 units of thrombin. Two different dilutions were used for each assay; the standard error of measurement over this range of concentration was ±3%.

**Other Clotting Activities—** Factors VII and X activities were measured by a one-stage method (17). In the one-stage method the factor to be assayed is incubated with thromboplastin, calcium, and a constant amount of a reference standard, and the clotting time is measured. The clotting time is defined as the time required for 0.1 ml of fibrinogen to clot from a 1:250 dilution in the clotting mixture to a 1:750 dilution of normal plasma.

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assayed by a modification of the one-stage procedure of Quick (19). The procedure consists of examining the corrective effect of the test material on the prolonged prothrombin time of plasma congenitally deficient in either Factor VII or Factor X. Barium sulfate-adsorbed human plasma was added as source of Factor V activity. The assay system was as follows: 0.2 ml of Factor VII- or X-deficient plasma, 0.1 ml of BaSO₄-adsorbed human plasma, 0.1 ml of thromboplastin, 0.1 ml of test material, and 0.2 ml of 0.04 M calcium chloride. Factor V activity was assayed by the same method, except that plasma congenitally deficient in Factor V was used as substrate plasma and BaSO₄-adsorbed human plasma was omitted from the system. Factor IX activity was assayed by a slight modification of the procedure of Proctor and Rapaport (20). The assay was based on the corrective ability of the test material on the prolonged partial thromboplastin time of plasma congenitally deficient in Factor IX. Barium sulfate-adsorbed human plasma was added as source of labile factors (Factors V and VIII). The results of the above assays were expressed as a percentage obtained by interpolation of a reference curve prepared from normal bovine plasma (equivalent to 100%). Diluted in Veronal-buffered 0.85% sodium chloride. Factor V activity was assayed by the same method, except that plasma congenitally deficient in Factor V was used as substrate plasma and BaSO₄-adsorbed human plasma was omitted from the system. Factor IX activity was assayed by a slight modification of the procedure of Proctor and Rapaport (20). The assay was based on the corrective ability of the test material on the prolonged partial thromboplastin time of plasma congenitally deficient in Factor IX. Barium sulfate-adsorbed human plasma was added as source of labile factors (Factors V and VIII). The results of the above assays were expressed as a percentage obtained by interpolation of a reference curve prepared from normal bovine plasma (equivalent to 100%) diluted in Veronal-buffered 0.85% sodium chloride.

**Physical Measurements**

**Electrophoresis**—Immunoelectrophoresis was performed on microscope slides (1 X 3 inches) by the technique of Grabar and Williams (21) as modified by Scheidegger (22). The gel matrix was 2% agarose (Brauch and Lomb, Rochester, New York) in barbital buffer, pH 8.6, (v/v). Gels were also stained by the periodic acid-Schiff reaction for localization of carbohydrate.

**Sedimentation Velocity Studies**—Sedimentation velocity analyses were done with the Spinco model E ultracentrifuge at 20°. Samples were concentrated by ultrafiltration and dialyzed against 0.1% Coomassie Brilliant Blue R-250 (Mann Research Laboratories, New York) in methanol-water-acetic acid (1:1:0.2, v/v). Gels were also stained by the periodic acid-Schiff reaction for localization of carbohydrate.

**Sedimentation Equilibrium Analysis**—Weight average molecular weights (Mₐ) were determined in the Spinco model E ultracentrifuge at 20° by the high speed equilibrium method described by Yphantis (25). A centerpiece with three double channels was utilized, with column heights of approximately 2.7 mm, and with the use of interference optics for the study of the samples in 0.42 M phosphate buffer, pH 5.9, (v/v). The sedimentation coefficients were plotted to a solvent with the density and viscosity of water at 20° (24).

**X-ray Diffraction Studies**—X-ray powder diffraction patterns were made with a Phillips-Norelco x-ray diffractometer with the use of copper radiation (Kα, Kα); β radiation was filtered by nickel foil. The runs were made at 50 kv and 40 ma. The samples were prepared by centrifuging saturated aqueous suspensions of prothrombin-barium complex or barium citrate, removing the water, and drying the solids at 58°. The powder was ground and packed into a standard x-ray diffraction sample holder.

**Ultrafiltration Experiments**—Protein solutions were concentrated by ultrafiltration through collodion membrane filters. The all glass apparatus and collodion membranes were manufactured by Membranfiltergesellschaft, GmbH, Göttingen, Germany (Schleicher and Schuell Company, Keene, New Hampshire). The capacity of the membrane bag was 8 ml. Filtration was carried out at room temperature against phosphate-NaCl buffer (pH 6.0), with a negative pressure of 130 mm of Hg applied to the suction flask.

**Solubility Measurements**—Constant solubility curves were determined by the method of Northrop and Kunitz (27). The preparation was washed twice with cold solvent and centrifuged at 2,000 × g at 5°. A suspension of the solid was prepared in solvent at room temperature and the protein content was determined by the Kjeldahl method. This stock suspension of known nitrogen content was then used to prepare serial dilutions with solvent in 15-ml graduated centrifuge tubes. Three 3-mm glass beads were added to each tube and equilibration was then made in a rotator (27 rpm) for 4 hours or for 15 hours at room temperature. The suspensions were decanted into Corex tubes and centrifuged at 34,800 × g. The supernatant was assayed for protein by the method of Lowry et al. (28) and for prothrombin activity by a two-stage method (16).

**Column Chromatography**—Sephadex G-100 chromatography was performed by the method of Andrews (29), as modified by Whitaker (30) and by Leach and O'Shea (31). A Sephadex G-100 column was prepared as described by Flocin (32); the glass column dimensions were 2.5 × 45 cm. The column was packed under a hydrostatic pressure of 20 cm to a bed height of 38.4 cm (bed volume, 194 ml). The packing of the column was checked and void volume (V₀) was determined by use of colored dextran (blue dextran 2000, Pharmacia). Experiments were performed with (a) phosphate-NaCl buffer, pH 6.0, (v/v), (b) 0.11 M sodium citrate, pH 8.4, (v/v); and (c) 0.42 M phosphate buffer, pH 5.9, (v/v). All experiments were done at room temperature (25°). The flow rate was 15 ml per hour (0.06 ml min⁻¹ cm⁻² cross-section of column) with a constant hydrostatic pressure of 20 cm relative to the drip point of the column. Fractions (3 ml) of effluent were collected with a siphon fraction collector. Proteins were estimated spectrophotometrically in a cuvette with a 1-cm light path at a wavelength of 280 μg. Measurements of prothrombin and Factors VII, X, and IX activities were also made on the eluents (see "Assay of Blood Clotting Factors"). The effluent volume corresponding to maximum concentration of protein (elution

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4 These studies were kindly performed by Mr. J. Kington Lou, Hyland Laboratories, Los Angeles.

6 These studies were performed by Dr. R. D. Sloan, Sloan Research Industries, Inc., Santa Barbara, California.
volume, $V_2$ was estimated to the nearest 0.1 ml by extrapolation of both sides of the distribution curve to an apex (29). The standard error of measurement of $V_2$ determined on different days was $\pm 0.7\%$.

DEAE-Sephadex chromatography was performed with columns of the same dimensions as used for G-100 chromatography. The resin was rinsed through acid and base with 0.5 N hydrochloric acid and 0.5 N sodium hydroxide, washed with water, and brought to pH 5.9 by the addition of 85% phosphoric acid. The resin was allowed to settle and washed several times with 0.05 M phosphate buffer, pH 5.92, until constant pH was attained. The column was packed under a hydrostatic pressure of 20 cm.

**Analytical Methods**

**Protein Determination**—Protein content was estimated from Kjeldahl nitrogen determination by the method of Ma and Zuazaga (33). Protein was calculated on the basis of a nitrogen to protein ratio of 0.145. Specific activity is expressed as units of activity as assayed by methods described in "Experimental Procedure."

**Barium and Calcium Determinations**—The prothrombin-barium complex (approximately 50 mg of protein) was dissolved in 12.5 ml of 0.11 M sodium citrate and passed through a column (0.9 × 4.5 cm) of AG 5OW-X8 resin, 100 to 200 mesh (Na+ form), for the removal of metal ions. Protein recovery for this step was 100%. Barium was eluted from the column with 4 M NaCl and estimated turbidimetrically as the sulfate at an optical density of 435 and 385 nm. Recovery of a known quantity of barium was also estimated simultaneously from an identical resin column. A correction factor based on the recovery value was used in the calculations. Additional analyses for barium, calcium, nitrogen, moisture, and ash were performed by the Schwarzkopf Micro-analytical Laboratories, Woodside, New York.

**Citrate Determination**—Citrate was assayed by the Furr-Hermann reaction as described by Hartford (35). The protein-barium complex was dissolved in 0.1 M EDTA, and any insoluble residue was discarded. Negligible chromogen yields were obtained from an EDTA blank or from several reference proteins (albumin and $\gamma$-globulin) at the wave lengths, 435 and 385 nm.

**Tyrosine and Tryptophan Contents**—The content and molar ratio of tyrosine and tryptophan were determined spectrophotometrically by the method of Beneze and Schmid (36). Absorbances in the ultraviolet wave lengths were determined for a 0.2% protein solution in 0.1 M NaOH (pH 13). The molar tyrosine to tryptophan ratios were estimated from the calculated $s$ value (with the use of the cited values of Beneze and Schmid).

**Carbohydrate Analyses**—Total hexose was determined by the anthrone method of Morris (37) with a galactose-mannose standard. Hexosamine analysis was performed by the Eison-Morgan procedure (38). Sialic acid was determined by the thiobarbituric acid method of Warren (39) after preliminary heating of the protein in 0.1 N H2SO4 for 1 hour at 80° to release bound sialic acid. Crystalline N-acetylneuraminic acid was used as reference standard.

**RESULTS**

**Preparation of Prothrombin Complex**

A representative fractionation of bovine plasma is described below and summarized in Table I. The procedure has been repeated many times with similar results. Prothrombin was adsorbed from citrated bovine plasma onto barium citrate according to the modification by Goldstein and Zonderman of the procedure of Lewis and Ware (40). All operations were carried out at room temperature except for centrifugations, which were done in a refrigerated centrifuge at 4°. Bovine blood was collected into siliconized plastic buckets containing

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### Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Recovery</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>units</td>
<td>%</td>
<td>units/mg</td>
<td>-fold</td>
</tr>
<tr>
<td>Bovine plasma&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1,700</td>
<td>119,000</td>
<td>573,000</td>
<td>100</td>
<td>100</td>
<td>4.8</td>
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<tr>
<td>1. Barium citrate adsorption and elution</td>
<td>354</td>
<td>354</td>
<td>576,000</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Dialysis</td>
<td>384</td>
<td>556</td>
<td>595,000</td>
<td>0.47</td>
<td>104</td>
<td>1,070</td>
</tr>
<tr>
<td>3a. Mother liquor</td>
<td>384</td>
<td>253</td>
<td>79,000</td>
<td>0.29</td>
<td>13</td>
<td>32</td>
</tr>
<tr>
<td>3b. Once crystallized&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23</td>
<td>187</td>
<td>364,000</td>
<td>0.16</td>
<td>64</td>
<td>1,940</td>
</tr>
<tr>
<td>4. Twice crystallized&lt;sup&gt;d&lt;/sup&gt;</td>
<td>36</td>
<td>97</td>
<td>220,000</td>
<td>0.08</td>
<td>42</td>
<td>2,450</td>
</tr>
<tr>
<td>5. Sephadex G-100 gel filtration&lt;sup&gt;e&lt;/sup&gt;</td>
<td>106.2</td>
<td>250,000</td>
<td>250,000</td>
<td>0.09</td>
<td>44</td>
<td>3,000&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Units of activity are expressed in Iowa units (18) as assayed by methods described in "Experimental Procedure."

<sup>b</sup> Bovine blood was collected in 0.11 M sodium citrate in the ratio of 9:1 (v/v). Normal bovine plasma assayed at 300 to 350 units of prothrombin activity per ml. Specific activity is uncorrected for dilution by anticoagulant.

<sup>c</sup> Protein and activity assays were done on material that was dissolved in 0.11 M sodium citrate and passed through a column (0.9 × 4.6 cm) of AG 5OW-X8 resin (Na+ form, 100 to 200 mesh), and the eluate was concentrated by ultrafiltration to one-fifth the original volume. The specific activity of triplicate preparations made from a single plasma pool exhibited a variability of $\pm 5\%$.

<sup>d</sup> Sephadex gel filtration was performed on material from Step 3b prepared according to Footnote c above. Further details are given in the text.

<sup>*</sup> Specific activity of the peak fraction obtained on gel filtration (see Fig. 10). Protein was estimated from the $E_{254}^1$ cm (280 nm) as 15.3.
3.1% sodium citrate (0.11 M) as anticoagulant, in a ratio of 9:1 (v/v). The blood was processed within 2 hours after collection.

Step 1: Barium Citrate Adsorption and Elution—To 1.7 liters of centrifuged plasma were added 170 ml of 1.0 M barium chloride. The suspension was stirred gently for 80 min and centrifuged for 30 min at 2500 \( \times g \), and the supernatant plasma was discarded. The yellowish precipitate was suspended in 850 ml of distilled water and stirred gently with 35 g of Dowex 50W-X8 resin (Na\(^+\) form, 20 to 50 mesh) to elute the prothrombin. The resin was removed by filtration through a coarse, sintered glass filter and then washed with 25 ml of 0.11 M sodium citrate. The total volume of the combined filtrates was 920 ml, and the solution appeared slightly cloudy. Prothrombin adsorption was repeated by adding 92 ml of 1.0 M barium chloride, stirring for 20 min, and removing the precipitate by centrifugation. The slightly yellowish precipitate was suspended in 225 ml of distilled water and stirred with 20 g of Dowex 50W-X8 resin. The resin was removed by filtration and washed with 25 ml of 0.11 M sodium citrate, and the washings and filtrate were combined.

Step 2: Dialysis—The combined filtrates from Step 1 were placed in Visking casing and dialyzed against running tap water for 16 to 18 hours. The insoluble globulins were removed by centrifugation for 40 min at 2500 \( \times g \) and discarded. The volume of the slightly cloudy supernatant was 295 ml. The protein concentration was 1.1 to 1.8 mg per ml, and the barium concentration was 0.6 mg per ml. The pH was approximately 8.2. The specific activity of this solution was 1070; yield of prothrombin, 100%.

Step 3: Crystallization—The solution from Step 2 was divided into 50-ml aliquots and frozen at -20°C. On thawing, a well-defined crystalline sheen was evident. The crystals appeared as flat, transparent plates or striated cylinders of variable size (Fig. 1), evidenced low order birefringence, and were insoluble in water and isotonic NaCl but readily soluble in 0.11 M sodium citrate. The specific activity of the crystals was 1890; yield of prothrombin, 64%.

Step 4: Recrystallization—A suspension of crystals from a single aliquot of Step 3 was centrifuged and the supernatant was discarded. The precipitate was dissolved in 12.5 ml of 0.11 M sodium citrate to give a protein concentration of approximately 3.5 mg per ml; any insoluble residue was discarded. The supernatant was dialyzed with stirring against 1 liter of a freshly prepared saturated solution of barium citrate at room temperature (25°C) for 4 to 5 hours. During this time a heavy cloudiness appeared in the dialysis bag. The prothrombin solution was then dialyzed against 2 liters of distilled water for 22 hours at 4°C and centrifuged at 2500 \( \times g \) for 30 min, and the supernatant was frozen at -20°C. Crystals appeared on thawing. Occasional attempts at recrystallization of these preparations were not successful. The specific activity of two times crystallized material was 2460; yield of prothrombin, 42%.

Step 5: Sephadex G-100 Gel Filtration—Gel filtration procedure with the use Sephadex G-100 was carried out on Step 3 material. The crystals from a single aliquot of Step 3 (30 to 45 mg of protein) were collected by centrifugation for 30 min at 2500 \( \times g \) and dissolved in 12.5 ml of 0.11 M sodium citrate. This solution was centrifuged and the precipitate was discarded. The supernatant was passed through a column (0.9 \( \times \) 4.6 cm) of AG 50W-X8 resin (Na\(^+\) form, 100 to 200 mesh) followed by 5 ml of distilled water. The eluate (18 ml) was concentrated by ultrafiltration to 2 or 3 ml against phosphate-NaCl buffer, pH 6.0 (Buffer A). The concentrated eluate was then applied to a Sephadex G-100 column equilibrated with 0.42 M phosphate buffer, pH 5.9 (Buffer B), and eluted with the same buffer. The peak tubes (44% of activity) were pooled, dialyzed against Buffer A, and concentrated by ultrafiltration against the same buffer. This material comprised Step 5 fraction and is referred to as "purified prothrombin." Chemical and physical studies were made on this fraction. Details of the chromatographic behavior of prothrombin on gel filtration are described in the section "Properties of Purified Prothrombin."

Protein recoveries from the ultrafiltration step were incomplete in many experiments and ranged from 68 to 80%; recovery of prothrombin activity was 100%. Therefore, ultrafiltration resulted in a uniform increase in specific activity. Folin-Ciocalteu reaction performed on the ultrafiltrate confirmed the presence of protein, but no attempt was made to isolate and characterize this material. Control studies performed with ovalbumin (mol wt 45,000) and lysozyme (mol wt 14,000), prepared and ultrafiltered under identical conditions, yielded recoveries greater than 88%. Prothrombin preparations at this stage of purification were shown to have trans-a-glucosylase activity. The enzyme was first shown as a contaminant in some prothrombin preparations by Miller (41). Specific activity of typical preparations ranged from 1.8 to 9.0 \( \times 10^4 \) unit of enzyme per mg of protein, with an amylolytic substrate (42).

All preparations at this stage of purification evidenced significant Factor VII, IX, and X coagulant activities (see "Properties of Purified Prothrombin"). In accordance with the nomenclature used by other investigators, the terms "purified prothrombin" and "prothrombin complex" are used interchangeably in this paper to refer to the "water-soluble, barium-free product." The term "crystalline prothrombin-barium complex" refers to the "water-insoluble barium glycoprotein product of interaction."
Properties of Crystalline Barium Complex

Control Studies—Efforts were made to exclude the possibility that the crystals represent an insoluble form of barium citrate with adsorbed prothrombin. Initially, we carried out the isolation from equivalent volumes of 0.85% NaCl containing citrate in the same concentration as the anticoagulant used in plasma. These studies failed to yield crystals. A stoichiometric relationship could be shown between crystal yield and plasma protein fractions adsorbed by the barium citrate. Bovine plasma was diluted with variable amounts of 0.90% sodium chloride solution and identical preparations were made of 0.90% sodium chloride solution alone, diluted plasma, and native plasma. Citrate concentration was constant in all experiments. A linear relationship was observed between the protein concentration of the dialyzed solution (Step 2, comprising adsorbed and eluted, water-soluble protein) and the crystal yield (Fig. 2). We concluded from these experiments that protein is an essential constituent of the crystalline complex. Additional evidence that the prothrombin-barium complex was distinct from barium citrate was obtained from powder x-ray diffraction data. Comparison of the x-ray diffraction pattern taken from the dried prothrombin complex with that of barium citrate illustrates that the samples have entirely different structures and are in no way similar (Fig. 3).

Solubility Studies—The results of constant solubility tests on one time and two times crystallized complex are shown in Fig. 4. A plot of solubility versus amount of solid phase in two different solvents (water and unbuffered NaCl, P/2 = 0.15) exhibits experimental points that fall close to the theoretical curve for the solubility of a one-component system. The barium-glycoprotein interaction product readily crystallizes in the presence of a very low concentration of barium citrate. Hence, it is not surprising that the complex shows low solubility in water. Studies performed in phosphate-NaCl buffer, pH 6.0, P/2 = 0.15, showed the physical structure to be unstable, with the appearance of amorphous material in the solid phase; constant solubility was not observed in this system. It is seen from Fig. 4 that the specific activity of the dissolved zymogen was not constant and decreased appreciably with increasing amounts of solid phase. We have interpreted this behavior to suggest that either protein denaturation or inhibition of prothrombin activity by excess barium ion has occurred during equilibration.

Chemical Composition—The gross composition of the crystalline prothrombin-barium complex is given in Table II. Also included, for comparison, in Table II is the composition of barium-free prothrombin further purified by gel filtration (Step 5). The crystalline barium complex evidences an unusually high ash content, which reflects the metal content of 38%. Barium comprises the largest fraction of metal present. Citrate is an integral component of the complex, and is present to the extent of approximately 9%. Preliminary spectrographic data also
indicate significant quantities of a phosphorus-containing moiety that may account for part of the undetermined mass, amounting to 30%. The molar barium to citrate ratio was 4: or 5: 1 on several determinations. Analytical analyses have also shown that much of the barium was removed by passage of a solution of the material dissolved in dilute citrate through an AG 50W-X8 resin column; however, appreciable citrate remained in complex with protein.

Homogeneity of Purified Prothrombin (Step 5)

1. Sedimentation Velocity Studies—The sedimentation pattern of purified prothrombin (Step 5, Table I) obtained with a Spinco model E ultracentrifuge revealed a single peak with an $s_{20,w}$ value of 3.88 S (Fig. 5).

2. Sedimentation Equilibrium Pattern—Step 5 fraction, equilibrated with 6 M guanidine hydrochloride containing 0.5% mercaptoethanol, was examined by sedimentation equilibrium analysis according to the method of Yphantis (25). After equilibration was reached, the photographs were analyzed for fringe displacement, and log c (concentration) was plotted against the square of the radius of rotation. The protein concentration was 0.8 mg per ml in a solvent consisting of 6 M guanidine hydrochloride plus 0.5% mercaptoethanol at pH 7.0. The rotor speed was 26,000 rpm, and the duration of the run was 46 hours.

Table II

<table>
<thead>
<tr>
<th>Component</th>
<th>Prothrombin-barium complex</th>
<th>Purified prothrombin (Step 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>3.4 (4)</td>
<td>14.5</td>
</tr>
<tr>
<td>Protein (calculated)$^a$</td>
<td>21.5 (4)</td>
<td>90.6</td>
</tr>
<tr>
<td>Barium</td>
<td>32.0 (4)</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td>Calcium</td>
<td>6.1 (2)</td>
<td>0.08</td>
</tr>
<tr>
<td>Citric acid</td>
<td>8.9 (2)</td>
<td></td>
</tr>
<tr>
<td>Moisture</td>
<td>3.8 (4)</td>
<td>4.1</td>
</tr>
</tbody>
</table>

$^a$ Step 5 material was dialyzed exhaustively against three changes of 200 volumes of distilled water and lyophilized.

$^b$ Calculated on the basis of a nitrogen to protein ratio of 0.16.

Fig. 5. Sedimentation schlieren patterns of purified bovine prothrombin (Step 5 material) in Spinco model E ultracentrifuge at 59,780 rpm and 20°C. The runs were made in 0.42 M phosphate buffer, pH 5.9, $\Gamma / 2 = 0.6$, and the photographs were taken at 16-min intervals after maximum speed was attained. The upper patterns were obtained at a protein concentration of 0.84 mg per ml. For this run, the $s_{20,w}$ value was calculated to be 3.88 S. The lower patterns were made at a protein concentration of 4.92 mg per ml. The $s_{20,w}$ value was calculated to be 3.76 S. Sedimentation was from left to right.

Fig. 6. Sedimentation equilibrium centripetalation of purified prothrombin (Step 5 material) according to the method of Yphantis (25). The logarithm of the concentration is presented as a function of the square of the radius from axis of rotation. The protein concentration was 0.8 mg per ml in a solvent consisting of 6 M guanidine hydrochloride plus 0.5% mercaptoethanol at pH 7.0. The rotor speed was 26,000 rpm, and the duration of the run was 46 hours.

Evidence of heterogeneity was also noted on disc electrophoresis. The electrophoretic pattern of Step 5 material showed two major components in close approximation as a doublet zone (which merged into a single zone at high protein concentrations) and two minor components (Fig. 7B, tube 1). The two major components were present in approximately the same concentration, as evidenced by the staining characteristics. The major and minor zones gave a positive periodic acid-Schiff reaction,
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Fig. 7. Electrophoresis of purified prothrombin complex. A, immunoelectrophoresis of Step 5 material (protein, 17%; specific activity, 2200); tube 2 to 6, fractions obtained from DEAE-Sephadex A-50 chromatography as shown in Fig. 14A. Well a contained rabbit antihuman plasma; Well b contained rabbit antibovine prothrombin. B, polyacrylamide gel electrophoresis. The materials analyzed were tube 1, Step 5 material (specific activity, 2200); tubes 2 to 6, fractions obtained from DEAE-Sephadex A-50 chromatography as shown in Fig. 14A. All fractions were concentrated to uniform A280 by ultrafiltration prior to electrophoresis. Migration rates varied with individual tubes, and therefore relative zone positions are not comparable.

Fig. 9. Electrophoretic mobility as a function of pH. All buffers were 0.1 ionic strength. Buffers used were pH 2.3, glycine-HCl; pH 4.1 to 5.6, acetate; pH 6.9 to 7.3, acetate-barbital; pH 8.2, barbital; and pH 9.6, glycine-NaOH.

Properties of Purified Prothrombin

Spectral Studies—The spectral absorbance curves for purified prothrombin (Step 5) at neutral and alkaline pH are shown in Fig. 8. The absorbance of a 1% solution of prothrombin at 280 mµ in 0.1 N NaOH was 14.8, based on a protein nitrogen content of 14.5%. Under identical conditions, Shulman and Hearon (45) reported a 1% extinction coefficient of 10.8 for purified bovine prothrombin. We observed a 1% extinction coefficient value in phosphate-NaCl buffer, pH 6.0, of 13.5; a value of 13.3 was calculated from the data of Lamy and Whugh (46) at the same pH value.

Isoelectric and Isoionic Points—Electrophoretic mobility measurements were made by the method of Waldmann-Meyer...
A plot of mobility as a function of pH is shown in Fig. 9 over the pH range, 2 to 10. The isoelectric point obtained from this plot, 4.1, is in close agreement with previously reported values for bovine prothrombin (48). Measurement of isoelectric point was as described for fumarase (49). A solution (1 ml) of prothrombin (0.1 mg per ml) in deionized water was passed through a column (0.9 x 3 cm) of a mixed bed resin (Amberlite MB-1). The eluate contained 1.8 mg of protein per ml. Recovery of prothrombin activity of the deionized zymogen was 30%. The pH of the isoelectric solution was determined at 25°C with a Beckman model 70 pH meter equipped with a Beckman glass electrode, No. 39183. The meter was calibrated with National Bureau of Standards reference buffers. The pH of an isoelectric solution of bovine prothrombin was 7.6 at 25°C. This value is considerably higher than the isoelectric point, and suggests that there is no anionic binding at neutral or slightly alkaline pH.

Assay of Coagulant Activities—Purified prothrombin (Step 5), isolated chromatographically from Sephadex G-100 columns and eluted with relatively low ionic strength solvents, showed specific activity in the peak fractions of 3,000 to 4,000 luoia units per mg of protein. Columns eluted with greater ionic strength solvents (i.e., $\Gamma/2 = 0.6$) resulted in a significant decrease of prothrombin specific activity, to approximately 2,000 luoia units per mg of protein. All prothrombin preparations at this stage of purification (Step 5) evidenced significant Factors VII, IX, and X coagulant activities. Traces of Factor V were also detected. Typical preparations of Step 5 material assayed as follows: prothrombin, 16,100%; (calculated on the basis of 100% activity as equivalent to 330 luoia units per ml); Factor VII, 28,800%; Factor IX, 5,180%; Factor X, 8,500%; Factor V, 54%. The preparations appeared to be relatively free of thrombin activity as assayed by clotting of a standard fibrinogen solution, and no thrombin activity evolved on storage for several months at -20°C. The zymogen was readily activated to thrombin in good yields with 25% sodium citrate in 48 hours at room temperature (25°C).

Gel Filtration Studies—A typical elution pattern for Step 3 material is shown in Fig. 10 for a Sephadex G-100 column eluted with phosphate-NaCl buffer, pH 6.0, $\Gamma/2 = 0.15$ (Buffer A). Two protein peaks were evident in the elution profile. The first component emerged with the void volume and was inactive; the second contained the prothrombin activity. Considerable purification of Step 3 material was achieved by gel filtration as shown by a 2- to 3-fold increase of specific activity of the peak fraction (3000 to 4000 units per mg) compared with the original activity. Specific activities of the ascending and descending limbs of the prothrombin curve were not constant. Factors VII and X activities emerged with the same elution volumes as the prothrombin-active fractions. The protein solution applied to the column and the individual effluents were free of thrombin activity.

Somewhat divergent behavior of prothrombin was observed in experiments performed with Step 3 material on gel filtration in systems of high ionic strength. A typical run done with 0.42 M phosphate buffer, pH 5.9, $\Gamma/2 = 0.6$ (Buffer B), as eluting solvent is shown in Fig. 11. Two protein components were again noted, as with the lower ionic strength buffer, and the prothrombin activity was associated with the retarded component. The first component emerged with the void volume and was inactive; however, the protein content of this component was significantly reduced and comprised about 20% of the applied protein. Since the relative amount of the excluded material decreased in solvents of high ionic strength, we concluded that at least some of this material in the void volume contained aggregates of the retarded protein. The specific activity of the peak prothrombin fraction was appreciably lower than that of the corresponding fraction obtained on chromatography at lower ionic strengths. Rechromatography of the active, pooled fractions in the higher ionic strength systems removed the coagulant-inactive component, but further decline in specific activity of the peak prothrombin fraction was noted (Fig. 12). Experiments performed with Step 3 material in 0.11 M sodium citrate, pH 8.4, $\Gamma/2 = 0.66$, gave similar protein elution patterns, as noted with the higher ionic strength phosphate buffer system. On occasion a small quantity of an inactive, slow, third component was present. Although not reflected in the protein elution pattern, a dissociation of activity was associated with the retarded component. The first component emerged with the void volume and was inactive; however, the protein content of this component was significantly reduced and comprised about 20% of the applied protein. Since the relative amount of the excluded material decreased in solvents of high ionic strength, we concluded that at least some of this material in the void volume contained aggregates of the retarded protein. The specific activity of the peak prothrombin fraction was appreciably lower than that of the corresponding fraction obtained on chromatography at lower ionic strengths. Rechromatography of the active, pooled fractions in the higher ionic strength systems removed the coagulant-inactive component, but further decline in specific activity of the peak prothrombin fraction was noted (Fig. 12). Experiments performed with Step 3 material in 0.11 M sodium citrate, pH 8.4, $\Gamma/2 = 0.66$, gave similar protein elution patterns, as noted with the higher ionic strength phosphate buffer system. On occasion a small quantity of an inactive, slow, third component was present. Although not reflected in the protein elution pattern, a dissociation of activity was associated with the retarded component. The first component emerged with the void volume and was inactive; however, the protein content of this component was significantly reduced and comprised about 20% of the applied protein. Since the relative amount of the excluded material decreased in solvents of high ionic strength, we concluded that at least some of this material in the void volume contained aggregates of the retarded protein. The specific activity of the peak prothrombin fraction was appreciably lower than that of the corresponding fraction obtained on chromatography at lower ionic strengths. Rechromatography of the active, pooled fractions in the higher ionic strength systems removed the coagulant-inactive component, but further decline in specific activity of the peak prothrombin fraction was noted (Fig. 12).
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Fig. 11 (left). Sephadex G-100 gel filtration of partially purified prothrombin eluted with solvents of higher ionic strength. The column was equilibrated and eluted with 0.45 M phosphate buffer, pH 5.9, r/2 = 0.6 (Buffer B). Total protein (Step 3) applied to column was 22.5 mg (specific activity, 1340) in a volume of 1.5 ml. Column dimensions, conditions of elution, and symbols are the same as given in the legend to Fig. 10. Inset also includes Factor IX activity, (------). Recoveries of protein and prothrombin activity were 81% and 91%, respectively. Solid bar on the abscissa indicates fractions which were pooled and rechromatographed.

Fig. 12 (right). Rechromatography on Sephadex G-100 of fractions with coagulant activity from previous chromatography on Sephadex G-100. Conditions were the same as in Fig. 11.

Fig. 13. Protein concentration dependence characteristics of prothrombin and ovalbumin eluted from a Sephadex G-100 column at 24°. Elution volume is plotted on the ordinate as percentage of the bed volume (V/Vo) and protein concentration is plotted on the abscissa. The volume of protein applied to the column was 2 ml. O, ovalbumin eluted with phosphate-NaCl buffer, pH 6.0, r/2 = 0.15; O, prothrombin eluted with phosphate-NaCl buffer, pH 6.0, r/2 = 0.15; o, prothrombin eluted with 0.42 M phosphate buffer, pH 5.9, r/2 = 0.6.

Fig. 14. Stokes radius—An attempt to estimate the molecular weight of prothrombin by previously described techniques with the use of calibrated molecular sieve columns (29-31) gave anomalous values when compared to previously reported values for bovine prothrombin estimated by sedimentation-diffusion. Similar anomalous behavior of glycoproteins on Sephadex gels has been observed by other investigators (29, 50, 51). Nevertheless, the physical size of the biologically active unit could be estimated from dextran gels by relating the elution volume to the Stokes radius of the effluent molecule and the effective pore radius of the molecular sieve.

The basic equation of this relationship has been given by Ackers (52). The gel pore parameter, r, was determined from the elution volumes and Stokes radii, a, of bovine plasma albumin and ovalbumin. Gel pore radii calculated from the data for these proteins on typical Sephadex G-100 columns at 24° gave an average pore size of 11.6 ± 0.4 nm.

Molecular sieve characteristics for bovine prothrombin at 24° showed protein concentration dependence over the concentration range, 0.4 to 14 mg per ml. A plot of elution volume of prothrombin and ovalbumin, expressed as percentage of the bed volume (i.e., the ratio of the volume of solvent required for elution of the peak, Vp, to the total volume of column, Vo), versus the protein concentration was rectilinear in a system of phosphate buffer of ionic strength 0.15 (Fig. 13). The negative slope of prothrombin, compared with the positive slope of a nonassociating protein, ovalbumin, indicates that prothrombin behaves as a chemically reacting system on Sephadex and suggests that the molecule undergoes rapid, reversible association under these conditions (53). Similar studies of prothrombin made in a system of phosphate buffer of ionic strength 0.6 did not show protein concentration dependence characteristics (Fig. 13). Presumably, protein interactions are minimized in a system of increased ionic strength.
The unaggregated molecular size of prothrombin was established by extrapolating the rectilinear plot for protein concentration dependence depicted in Fig. 13 to infinite dilution. It was then possible to use the extrapolated elution volume, $V_e$, in Equation 9 and Table III of Acker's Table (52) to arrive at an experimental value of $a/r$. The Stokes radius of prothrombin was found to be 4.15 m in phosphate-NaCl buffer (pH 6.0, $\Gamma/2 = 0.15$), 3.75 m in 0.42 M phosphate buffer (pH 5.9, $\Gamma/2 = 0.6$), and 4.08 m in 0.11 M sodium citrate (pH 8.4, $\Gamma/2 = 0.66$).

**Diffusion Coefficient**—The free diffusion coefficient, $D$, was calculated from the Stokes radius by use of the Stokes-Einstein equation (54)

$$ D = \frac{kT}{6\pi\eta a} $$

where $D$, the diffusion coefficient, is given in terms of the Boltzmann constant, $k$; absolute temperature, $T$; the system viscosity, $\eta$; and the Stokes radius, $a$, of the protein molecule.

The values of $D_{0.03}$ of purified prothrombin determined on calibrated Sephadex G-100 were found to be $1.77 \times 10^{-10}$ cm$^2$ sec$^{-1}$ and $0.10 \times 10^{-10}$ cm$^2$ sec$^{-1}$ in solvents of phosphate-NaCl buffer (pH 6.0, $\Gamma/2 = 0.15$), 0.42 m phosphate buffer (pH 5.9, $\Gamma/2 = 0.6$), and 0.11 m sodium citrate (pH 8.4, $\Gamma/2 = 0.66$), respectively.

**Molecular Weight**—The molecular weight of prothrombin was calculated by means of the Svedberg equation (24) from the values of $s$ and $D$. The values of the sedimentation constant were those of Lamy and Waugh (46) determined in phosphate buffer (pH 7.0, $\Gamma/2 = 0.15$), and our estimations made in 0.42 m phosphate buffer and 0.11 m sodium citrate at ionic strengths of 0.6 and 0.66, respectively. A value of 0.719 ml per g was used for the partial specific volume of prothrombin, calculated from the amino acid composition (55) and a carbohydrate content of 13%. With values of 4.85 $\times$ 0.719 ml per g and 5.17 $\times$ 10$^{-7}$ cm$^3$ sec$^{-1}$ for the sedimentation coefficient, partial specific volume, and diffusion coefficient, respectively, the upper limit of the hydrated molecular weight of prothrombin was calculated to be 81,500 in a solvent of ionic strength 0.15. Similar calculations based on sedimentation-diffusion parameters, summarized in Table III, for systems of higher ionic strengths gave values of 59,200 and 54,500 for the molecular weight of prothrombin in 0.42 m phosphate buffer and 0.11 m sodium citrate, respectively.

Molecular weight was also determined by equilibrium sedimentation by the method of Yphantis (25) in the higher ionic strength system consisting of 0.42 m phosphate buffer (pH 5.9, $\Gamma/2 = 0.6$) at three solute concentrations. These results gave a value for the weight average molecular weight ($M_w$) of 66,032 $\pm$ 1,462. Under these conditions a relatively small percentage of a higher molecular weight component was present with $M_w$ less than 84,000. There was good agreement in molecular weight values obtained at three solute concentrations, indicating absence of concentration dependence in this system. In a dissociating solvent composed of 6 m guanidine hydrochloride and 0.5% mercaptoethanol, purified bovine prothrombin was found to have an apparent molecular weight of 70,477 $\pm$ 2,780, assuming no change in partial specific volume or degrees of hydration and solvation in this solvent. This assumption appears justified from studies of Ullmann et al. (57) on the molecular weights of proteins in 6 m guanidine hydrochloride. Three preparations examined under these conditions were found to be molecularly homogenous without evidence of higher molecular weight material (Fig. 6).

**Frictional Coefficient**—The frictional coefficient ratio, $f/f_0$, was calculated from the equation (58)

$$ f/f_0 = \frac{1}{\left(\frac{3\pi M}{4N\pi}\right)^{1/2}} $$

where $f$ is the partial specific volume; $M$, the molecular weight; and $N$ is Avogadro's number. The results of these calculations are summarized in Table III.

**Chemical Composition**—The gross composition of lyophylized Step 5 purified prothrombin is given in Table II. This material assayed 14.5% nitrogen, calculated on a moisture- and ash-free basis. The polypeptide content was 89.1%, determined by the biuret procedure. The carbohydrate moiety comprised 10.1% of the total weight of the protein. Values for hexose, sialic acid (expressed as the N-acetyl form), and hexosamine were estimated as 4.0%, 2.8%, and 3.3%, respectively. The carbohydrate composition is in agreement with the values given by Magnusson (59) and Schwick and Schultze (6) for bovine prothrombin. The tyrosine to tryptophan molar ratio, determined by the method of Benze and Schmid (36), was 1.05 and the combined tyrosine and tryptophan content was 7.9% on a dry weight basis. Seegers, McLaughry, and Fabe (60) have reported a total tyrosine and tryptophan content of 7.9% for bovine prothrombin.

**Fractionation of Bovine Prothrombin Complex**

**DEAE-Sepharoid A-50 Chromatography**—Prothrombin preparations, judged to be highly purified by certain criteria (elution...
from Sephadex columns and ultracentrifugation analysis), nevertheless appeared to be heterogeneous with respect to electrophoretic behavior and multiple coagulant activities. The dissociation of coagulant activities (Factors VII and X) noted on gel filtration when elution was with high ionic strength solvents (Fig. 11), as well as the apparent segregation of zones when individual Sephadex effluents containing either Factor VII or Factor X activity were examined by disc electrophoresis, suggested the possibility of isolating individual coagulant activities and protein subcomponents from purified prothrombin by methods based in part on the charge structure of the protein.

The pooled Sephadex fractions corresponding to Step 5 material (18 mg of protein of specific activity 2200) were applied to a column (2.5 × 40 cm) of DEAE-Sephadex A-50 that had been equilibrated with 0.05 M phosphate buffer, pH 5.92. The proteins were eluted with an exponential gradient produced with an exponential gradient (see the text for details). Fractions of bovine prothrombin complex (Type II pattern) were collected for 100 tubes, and then 1.5 ml fractions were combined and further characterized. Fractions indicated by stippled area in A above. Conditions of chromatography were the same as in A. The pooled fractions from A were concentrated by ultrafiltration to a volume of 2.5 ml and diluted to 10 ml with 0.05 M phosphate buffer, pH 5.92, and 18 mg were chromatographed on a column (2.5 × 40 cm) equilibrated with 0.05 M phosphate buffer, pH 5.92. Elution was carried out with 0.05 M phosphate-1 M NaCl buffer, pH 6.66, with the use of an exponential gradient (see the text for details). Fractions of 0.05 M contained for the first 100 tubes, and then 1.5 ml fractions were made at a flow rate of 20 ml per hour. —— protein determined by optical density (A280). Protein recovery was approximately 100%. Horizontal lines indicate fractions that were combined and further characterized. Fractions indicated by stippled area were pooled from three experiments and rechromatographed. Prothrombin activity was eluted at about 160 tubes in A, and 200 tubes in B. The elution pattern was similar to that of the starting material shown in tube 1, although the migration rate was somewhat reduced. Incubation of Fraction F in 25% sodium citrate for 96 hours at 25° did not generate thrombin activity. However, when F (containing Factor X activity) was added to the incubation mixture, prompt generation of thrombin activity occurred (Fig. 15). Since this fraction retained the potential for thrombin transformation under certain conditions, we have designated this material "modified zymogen." The specific activity of Fraction B was 1.84%. Fraction C contained the prothrombin activity and comprised 40.1% of the total protein. The specific activity was approximately 3000 units per mg. Examination by disc electrophoresis established the presence of two major components and trace amounts of two minor components (Fig. 7B, tube 8). Although not shown well in the figure, the major component was a doublet zone, and over 90% of the protein appeared to reside in the major component. The migration distance corresponded with the major components of the starting material but no correspondence was noted in the minor components. All Factor IX activity was located in this fraction. Trace amounts of Factors VII and X activities were also detected. The fraction was inert in 25% sodium citrate, and no thrombin evolved after 48 hours of incubation. The specific activity was 2.88%. Fraction D (8.1% of the total

250 ml of 0.05 M phosphate buffer, pH 5.92, in a constant volume mixing chamber and 0.05 M phosphate-1.0 M NaCl buffer, pH 6.61, in the reservoir. Flow rates were 10 to 20 ml per hour. Two types of elution patterns were obtained that were directly related to the protein concentration applied to the column. The results were consistently reproducible in many experiments. Type I pattern was obtained at protein concentrations of 0.1% and was characterized by a single protein peak with a small shoulder on the descending limb. Prothrombin activity was located in the major protein peak and comprised 80% of the total protein. Disc electrophoresis of this fraction revealed a doublet zone corresponding to the doublet zone noted in the starting material (Fig. 7B, tube 1). Coagulant activities VII and X, together with the minor electrophoretic components, were located in the effluents corresponding to the shoulder in the profile. However, a discrete physical separation of the major and minor protein components was not achieved under these conditions. Type II pattern was obtained in studies performed at a 10-fold increase in protein concentration (1%). The protein profile was characterized by five protein peaks (Fig. 14A). Individual eluates were pooled as indicated by the horizontal bars in Fig. 14A into a total of six fractions. Coagulant activities and characterization studies were performed on these fractions. Assay of coagulant activities were made after the fractions were dialyzed against distilled water. The fractions were then concentrated by ultrafiltration to 0.1% protein for physical and chemical studies. The results of these studies were as follows.

Properties of Isolated Fractions—Fraction A comprised 5.5% of the total protein and was devoid of coagulant activity. No sialic acid was found in this fraction, and further characterization studies were not performed. Fraction B comprised 27.5% of the total protein, and was also devoid of coagulant activity. The quantity of protein varied with individual experiments, and there appeared to be an inverse relationship between the amount of protein contained in this fraction and Fraction C. Disc electrophoresis revealed two major components (appearing as a doublet zone) and two minor components (Fig. 7B, tube 8). The electrophoretic pattern was similar to that of the starting material shown in tube 1, although the migration rate was somewhat reduced. Incubation of Fraction B in 25% sodium citrate for 96 hours at 25° did not generate thrombin activity. However, when Fraction F (containing Factor X activity) was added to the incubation mixture, prompt generation of thrombin activity occurred (Fig. 15). Since this fraction retained the potential for thrombin transformation under certain conditions, we have designated this material "modified zymogen." The sialic acid content of Fraction B was 1.84%. Fraction C contained the prothrombin activity and comprised 40.1% of the total protein. The specific activity was approximately 3000 units per mg. Examination by disc electrophoresis established the presence of two major components and trace amounts of two minor components (Fig. 7B, tube 8). Although not shown well in the figure, the major component was a doublet zone, and over 90% of the protein appeared to reside in the major component. The migration distance corresponded with the major components of the starting material but no correspondence was noted in the minor components. All Factor IX activity was located in this fraction. Trace amounts of Factors VII and X activities were also detected. The fraction was inert in 25% sodium citrate, and no thrombin evolved after 48 hours of incubation. The sialic acid content was 2.88%. Fraction D (8.1% of the total

Properties of Isolated Fractions—Fraction A comprised 5.5% of the total protein and was devoid of coagulant activity. No sialic acid was found in this fraction, and further characterization studies were not performed. Fraction B comprised 27.5% of the total protein, and was also devoid of coagulant activity. The quantity of protein varied with individual experiments, and there appeared to be an inverse relationship between the amount of protein contained in this fraction and Fraction C. Disc electrophoresis revealed two major components (appearing as a doublet zone) and two minor components (Fig. 7B, tube 8). The electrophoretic pattern was similar to that of the starting material shown in tube 1, although the migration rate was somewhat reduced. Incubation of Fraction B in 25% sodium citrate for 96 hours at 25° did not generate thrombin activity. However, when Fraction F (containing Factor X activity) was added to the incubation mixture, prompt generation of thrombin activity occurred (Fig. 15). Since this fraction retained the potential for thrombin transformation under certain conditions, we have designated this material "modified zymogen." The sialic acid content of Fraction B was 1.84%. Fraction C contained the prothrombin activity and comprised 40.1% of the total protein. The specific activity was approximately 3000 units per mg. Examination by disc electrophoresis established the presence of two major components and trace amounts of two minor components (Fig. 7B, tube 8). Although not shown well in the figure, the major component was a doublet zone, and over 90% of the protein appeared to reside in the major component. The migration distance corresponded with the major components of the starting material but no correspondence was noted in the minor components. All Factor IX activity was located in this fraction. Trace amounts of Factors VII and X activities were also detected. The fraction was inert in 25% sodium citrate, and no thrombin evolved after 48 hours of incubation. The sialic acid content was 2.88%. Fraction D (8.1% of the total
protein) evidenced the major content of Factor VII activity. Disc electrophoresis showed a single zone and a slowly migrating trace component (Fig. 7B, tube 4). The major zone did not correspond to any zone in the starting material and evidenced more rapid migration. Trace quantities of Factor X activity were detected. This fraction was also inert in 25% sodium citrate. The sialic acid content was 3.45%. Fraction E (10.9% of the total protein) contained significant quantities of Factors VII and X activities. A large quantity of protein (50%) was not recovered during ultrafiltration of this fraction, suggesting that it may contain a small molecule. Two trace components were noted on disc electrophoresis (Fig. 7B, tube 5). The sialic acid content of the remaining protein was 1.54%. Fraction F (3.0% of the total protein) contained the major content of Factor X activity. Disc electrophoresis revealed two components in close approximation (Fig. 7B, tube 6). The relative positions corresponded approximately to the minor components in the starting material. This fraction was also inert in 25% sodium citrate. The sialic acid content was 1.52%.

Molecular Weights of Coagulant Proteins Isolated by DEAE-Sephadex Chromatography—The molecular weights and homogeneity of the coagulant proteins represented by Fractions B, C, D, and F were determined by the sedimentation equilibrium method of Yphantis (25). However, in order to enhance the separation and increase the yields of Factors VII and X activities, Fractions D, E, and F were combined from a total of three experiments in which purified prothrombin was chromatographed on DEAE-Sephadex columns. The combined fractions were concentrated by ultrafiltration and rechromatographed under conditions identical with those for the initial chromatography (Fig. 14B). This procedure resulted in a discrete separation of Factors VII and X coagulant proteins. The eluates indicated by the horizontal bars in Fig. 14B were pooled and used for molecular weight studies.

This study shows that coagulant activities comprising the “prothrombin complex” can be isolated in crystalline form in good yields. Bordet and Delange (61) first demonstrated that plasma treated with barium sulfate becomes incoagulable, and subsequent studies have shown the effectiveness of the insoluble salts of the alkaline earth metals as selective adsorbents of prothrombin from plasma as preliminary steps in isolation. From a study of the interaction of prothrombin and barium sulfate, Surgenor and Noetkier (92) postulated the formation of metal-protein complexes. They also investigated the behavior of certain carboxylic acids on the prothrombin-barium sulfate interaction, and confirmed the observations of others that citrate is a particularly effective anion for the elution of prothrombin from the insoluble alkaline earth metal salts.
Quick and Stefani (63) postulated the formation of a prothrombin-citrate complex from studies of inhibition by citrate of the interaction between prothrombin and calcium phosphate. Our findings have extended these observations and have shown that prothrombin has the property of interacting with both barium and anion (citrate) to form a stable interaction product of definite composition.

Attention has been directed in this investigation toward various studies to establish whether or not the crystals represent a unique form of barium citrate with adsorbed prothrombin or a true crystalline complex of prothrombin. That we have not isolated crystalline barium citrate with adsorbed protein may be summarized as follows: (a) control studies have shown protein to be an essential component of the prothrombin-metal complex (see Fig. 2), (b) the protein complex and barium citrate have different powder x-ray diffraction patterns (see Fig. 3), (c) the molar ratio of barium to citrate in the complex does not conform to the composition of barium citrate, (d) crystallization occurs from solutions that have been exhaustively dialyzed whereas barium citrate has been shown to be readily dialyzable (64), and (e) specific prothrombin activity of the crystalline complex exceeds that of the mother liquor by several fold and recrystallization enhances specific activity (Table I).

Among the properties of the prothrombin-barium complex that deserve comment are the unusually high content of metal in the complex and the nature of the protein-metal linkage. One possibility would consider the metal as "atomically dispersed" throughout the protein, thereby implicating a peptide structure as the binding site. This would be considered unlikely. The chemical composition data show 680 moles of barium and 140 moles of citrate for each mole of protein. Assuming that carbonyl groups function as univalent binding sites, calculations based on the amino acid and amide content given by Seegers (26) and a specific acid content of 3% indicate a total of 70 carbonyl groups available as possible binding sites. Thus, only 10% of the total barium could be bound to protein and the remainder would have to be linked through chelation to citrate residues.

The procedure described for the preparation of highly purified bovine prothrombin, isolated as the barium-free, water-soluble product, differs radically in some respects from previous methods. Precipitation with ammonium sulfate was eliminated by subsequently increasing the protein concentration characteristics of human prothrombin on molecular sieve columns (Fig. 13). In a phosphate buffer solvent of ionic strength 0.15, the protein concentration dependence behavior of prothrombin on elution from Sephadex was found to be linear over the entire concentration range studied, including values less than 1 mg of protein per ml (the actual protein concentration is considerably less, since a 10-fold dilution occurs on the column). Our Sephadex studies indicate that prothrombin undergoes reversible association. Bovine thrombin has been shown to have similar behavior characteristics on molecular sieve columns by Winzor and Scheraga (67). Evidence for reversible prothrombin association has been demonstrated by Seegers (68) for bovine prothrombin and by Lanchantin, Friedmann, and Hart (69) for human prothrombin from protein concentration dependence behavior of the sedimentation coefficient in a system of low ionic strength. This study has also shown that reversible association is dependent on ionic strength. Protein interaction appeared to be inhibited in high ionic strength solvents from the elution behavior of prothrombin on Sephadex columns, and is in agreement with the protein concentration characteristics of human prothrombin in the ultracentrifuge under similar conditions (70). The results of this investigation indicate that, in aqueous buffers at pH 6.0 and low ionic strength, purified prothrombin consists of an associating system which at low concentrations dissociates to an apparent molecular weight of 70,500.

This study has established that, despite evidence of homogeneity by gel filtration, sedimentation velocity, and sedimentation equilibrium analysis, our purified prothrombin preparations of high specific activity were molecularly heterogeneous, judged from electrophoretic behavior (Fig. 7, A and B). Electrophoretic heterogeneity of purified bovine prothrombin has also been observed in preparations isolated by procedures different from that described in this study (71–74). Moreover, the present purified prothrombin (Step 5) evidenced multiple coagulant activities and was comparable in this respect to nonchromatographed preparations of Marciniak and Seegers (75) and other workers (8, 74, 76, 77). The single $R_{s}$ determination of Step 5 prothrombin in a dissociating solvent, together with information from disc electrophoresis of multiple molecular species, comprise presumptive evidence that the multiple components are of approximately the same mass $\sigma$ or are of variable molecular weight but...
present to the extent of less than 1% by weight (which represents the limit of detection by the sedimentation equilibrium method of Yphantis (25)). The latter possibility is considered unlikely, since the staining characteristics on polyacrylamide gels and direct elution from DEAE-Sephadex columns indicate that the minor components comprise about 20% of the total protein.

A fundamental question posed by the results of this study is whether the heterogeneity present in native prothrombin arises from the method of isolation. Marciniak and Seegers (78) ascribe the molecular heterogeneity and multiple coagulant activities to partial degradation of the prothrombin molecule as a result of autocatalytic interaction of traces of thrombin in the preparation and prothrombin. Lanchantin, Friedmann, and Hart (79) consider the heterogeneity to represent prothrombin activation fragments which evolved during chromatography on ion exchange celluloose resins. The homogeneity of our purified prothrombin preparations with respect to molecular weight invalidates the concept that electrophoretic heterogeneity arises from degradation of a single molecular species as a result of activation and the formation of relatively large peptide split products. The results, however, do not exclude the possibility that the subcomponents present in our preparations reflect a series of minor protein changes involving a single protein molecule.

The protein changes could result from loss of labile amide groups or terminal sialic acid residues, or from hydrolysis of a single peptide bond.

The ultimate answer to the problem of heterogeneity would demand the isolation and characterization of the individual molecular species and coagulant activities that comprise prothrombin complex in a native configuration. We endeavored to accomplish this by chromatography of purified prothrombin on DEAE-Sephadex A-50 columns. Two types of protein elution pattern were obtained that were reproducible in many experiments. Type I pattern, obtained in studies performed at 0.176 protein concentration, was characterized by a single elution peak that contained the prothrombin activity. The minor components, together with Factors VII and X activities, were eluted as a small shoulder on the descending limb without discrete separation from each other or from the major protein fraction. Type II pattern was obtained in studies performed at a 10-fold increase in protein concentration (1%). The elution profile showed five protein peaks (Fig. 14.1). Three fractions could be identified with coagulant activities; the remaining protein fractions were inert.

Evidence that partial activation had taken place under the conditions in which Type II pattern was obtained may be summarized as follows: (a) A protein fraction was isolated that could not be converted to thrombin in the two-stage assay system or by prolonged exposure to 25% sodium citrate (Fraction B). Ample documentation has been presented by other investigators that during the initial stages of prothrombin activation in 25% sodium citrate, a zymogen intermediate is formed which is inert to thrombin conversion in the two-stage assay system (70, 80). We have designated Fraction B a modified zymogen since under certain conditions the protein transformed to thrombin. (b) The recovery of prothrombin-active protein (Fraction C) was markedly reduced, and the yield was inversely related to the yield of modified zymogen. (c) A protein fraction was eluted which appeared to have a low molecular weight component (Fraction E). The formation of low molecular weight fragments from the parent zymogen during activation has been described (70, 81). (d) The protein components eluted from Type II profile showed heterogeneity with respect to molecular weight, indicating marked alteration in protein structures.

That partial activation occurred only with the Type II pattern of elution would exclude ion exchange chromatography per se as the sole cause of the activation. Activation also did not take place during protein concentration by ultrafiltration, since no thrombin activity evolved and specific activity remained unchanged. The mechanism is unexplained, but appears to involve both protein concentration and interaction with the high ionic strength surface density of the resin. Similar observations have been made by Shapiro and Waugh (82).

This study has given some insight into the molecular properties of the coagulant activities that comprise the prothrombin complex. Prothrombin, isolated by DEAE-Sephadex chromatography and completely devoid of Factors VII and X but still retaining Factor IX activity, was found to have a monomeric molecular weight of 65,500 and was homogeneous by sedimentation equilibrium analysis. We have been unsuccessful in separating Factor IX from prothrombin. A modified zymogen, with a monomeric molecular weight of 62,400, has also been isolated. This species was also homogeneous. The molecular properties suggest that the modified zymogen may be identical with the autoprothrombin II of Harmison and Seegers, which has been reported to have a molecular weight of 51,200 (56). Moreover, the clotting behavior of the modified zymogen shows a resemblance to the “prothrombin” of Marciniak and Seegers (83). The protein was inert in the two-stage assay and on prolonged exposure to 25% sodium citrate, but was readily activated in the latter system when pure Factor X was added. Similar behavior has been ascribed to prothrombin (83). The reduced sialic acid content of this protein species compared with that of prothrombin also suggests a possible relationship to Magnusson's Peak I fraction obtained on TEAE-cellulose chromatography of bovine prothrombin (74).

Comparison of the molecular properties of Factors VII and X throughout the purification procedure provides information to suggest that structural transformation of these proteins has taken place. Several lines of evidence indicate that prior to the DEAE-Sephadex step the native molecular weights of these activities are identical with or close to that of prothrombin. The distribution coefficients for Factors VII and X and prothrombin are identical when determined by molecular sieve chromatography with the use of low ionic strength systems for elution (Fig. 10). Thus, the molecular ratio of these activities must be the same. Furthermore, purified prothrombin, containing appreciable Factors VII and X activities, was found to have a single molecular weight by sedimentation equilibrium analysis. The changes observed in this study in the distribution coefficients for these activities on molecular sieve columns when eluted with higher ionic strength buffers (Fig. 11) probably reflect changes in hydration or frictional coefficients rather than alteration of molecular weights.

The data presented here on the molecular properties of Factors VII and X, isolated in a molecularly homogeneous form from DEAE-Sephadex, fit with the hypothesis that these activities are individual molecular species capable of undergoing a pro-enzyme-enzyme transformation similar to prothrombin. Structural transformation of Factors VII and X has been implied from the comparative properties of these coagulant proteins isolated from plasma and serum sources. Przydz (84) reported bovine Factor VII to have a molecular weight of approximately 63,000...
and 48,000 when isolated from plasma and serum, respectively. The molecular weight of bovine Factor X derived from plasma has been estimated as 85,000 by Eneouf and Williams (7), and values for activated Factor X have ranged from 22,500 to 70,000 (7, 85–87). In this study Factors VII and X were found to have monomeric molecular weights of 33,000 and 37,600, respectively. It is reasonable to assume that these values represent the activated forms of these molecules. It should be emphasized, however, that the nonactivated and activated forms would not be differentiated by the assay methods used in this study.

The results of this investigation are consistent with the interpretation that the prothrombin complex, as isolated by the methods of this study, is composed of a family of glycoproteins that can undergo a proenzyme-enzyme transformation and that have similar molecular properties but separate clotting activities. Information on the molecular properties of the nonactivated coagulant proteins (Factors VII, IX, and X), their possible relationship to the prothrombin molecule, the structural changes that occur on activation, and their role in the kinetics of prothrombin transformation must depend on their isolation in a native state. These studies are currently in progress.

Acknowledgments—We wish to express our appreciation to Dr. Emil L. Smith for many valuable discussions and critical comments during the preparation of this manuscript. We wish to thank Dr. Alexander N. Glazer, Dr. John G. Pierce, and Dr. Frank S. Markland for helpful advice during the course of this work. We also wish to thank Dr. Richard Riley and Mr. Monroe Coleman for the disc gel electrophoresis procedure. Finally, the competent technical assistance of Miss Jean Stueher is gratefully acknowledged.

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