Structural Studies on Bovine Prothrombin. Isolation and Partial Characterization of the Ca^{2+} Binding and Carbohydrate Containing Peptides of the N-Terminus Region†

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ABSTRACT: Three peptides, one of which binds Ca^{2+} (calcium binding fragment, CBF) but contains no carbohydrates and two of which bind no Ca^{2+} but contain carbohydrates, have been isolated from the N-terminus region of bovine prothrombin. The preparation of these peptides involved (a) thrombin cleavage of prothrombin to intermediate 1 (thrombinogenic) and fragment 1 (nonthrombinogenic), (b) tryptic attack on fragment 1, and (c) separation of the CBF from the latter reaction by addition of a phosphatidylcholine–phosphatidylserine dispersion in the presence of Ca^{2+}. Further study on the non-calcium-binding peptides from the tryptic digest of fragment 1 revealed the presence of two low molecular weight glycopeptides, GP-1 and GP-2. A detailed examination of the chemical characteristics of CBF provided some insight into this unusual peptide. Whereas fragment 1, as well as prothrombin, exhibited two classes of Ca^{2+} binding sites (one of high affinity, 3–4 mol/mol of peptide and the other of low affinity, 10–12 mol/mol of peptide), CBF bound only 3–4 mol of Ca^{2+}/mol of peptide. This indicated the presence of only the high affinity sites of the parent molecule. CBF contained an unusually high level of glutamic acid (~30% of the total amino acids as determined in an acid hydrolysate) and had an N-terminal glycine. Most likely these glutamyl residues were present originally as the γ-carboxyglutamyl residue as proposed by Stenflo et al. (Stenflo, J., Ferlund, P., Egan, W., and Roepstorff, P. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71*, 2730). The CBF contained no detectable carbohydrate. Its molecular weight varied inexplicably according to the procedure used and gave the following values: 8500, by gel filtration; 5200, by 6 *M* guanidine·HCl gel chromatography; 3490, by analytical ultracentrifugation. The glycopeptides, GP-1 and GP-2, were distinguished from each other by differences in their behavior on ion exchange chromatography and in their amino acid composition, and from CBF by their inability to bind calcium under any conditions. On the other hand, GP-1 and GP-2 had nearly identical levels of carbohydrate, 45.1 and 48.0 wt %, and possessed essentially the same percent distribution of carbohydrates: sialic acid, 16.5 ± 0.5; mannose, 10.3 ± 0.4; glucosamine, 11.2 ± 0.1; galactose, 7.9 ± 0.3. Their molecular weights were as follows: GP-1, 7000, by gel filtration; 6500, by 6 *M* guanidine·HCl gel chromatography; 4600, by ultracentrifugation; GP-2, 6500 by gel filtration; 6900, by 6 *M* guanidine·HCl gel chromatography; 1960, by analytical ultracentrifugation. Though there are some obvious variations depending on method, this could be attributable to a probable error in $\bar{\nu}$ measurement on these carbohydrate containing peptides. The significance of these findings as they relate to prothrombin to thrombin conversion is discussed.

The importance of calcium ions in the enzymatic conversion of prothrombin to thrombin in mammalian blood coagulation is well established. Until recently, however, the location and chemical characteristics of the binding sites on prothrombin had not been clearly defined. However, evidence published from several laboratories (Nelsestuen and Suttie, 1974; Benson et al., 1973; Gitel et al., 1973) supports a preferential positioning of these sites in the N-terminus region of the prothrombin molecule. Furthermore, on the basis of data supplied by Stenflo et al. (1974) it appears most likely that the newly discovered γ-carboxyglutamic acid residues are the primary binding sites in bovine prothrombin.

More precise location and characterization of the calcium binding sites are necessary in order to elucidate the mode of action of factor Xa, a protease, on prothrombin and to establish the mechanism by which factor V and phospholipid so significantly accelerate this proteolytic attack. A particularly effective route to the establishment of the location of the metal ion binding sites is through use of limited proteolysis with thrombin (Benson et al., 1973). This latter procedure leads to the formation of two products, one of which is thrombinogenic intermediate 1[1] (mol wt 55,000) which is derived from the C-terminus region and the other of which is nonthrombinogenic, fragment 1[1] (mol wt 25,000), which is derived from the N terminus. As noted above, the latter component, fragment 1, contains the Ca^{2+} binding sites and its ready availability in highly purified form allowed a detailed study of its physical and chemical characteristics. To this end, fragment 1 was subjected to tryptic digestion and the resulting peptides were subjected

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[1] The nomenclature used here for identification of the conversion products of prothrombin is that proposed by Owen et al. (1974).

to a procedure which allowed isolation of a Ca^{2+} binding peptide and at the same time two non-calcium-binding, carbohydrate-containing peptides. The results of these experiments are reported here and provide some insight into the structure of the prothrombin molecule.

Experimental Section

Materials. DEAE-cellulose, Whatman DE-52, pre-swollen, 1.0 mequiv g, was precycled according to manufacturer's instructions (H. Reeve Angel and Co., Clifton, N.J.). Sephadex G-10, 25, 75, 150, 200, DEAE Sephadex A-50, Sephadex SE and SP-C50, and Dextran Blue 2000 were obtained from Pharmacia Fine Chemicals, Piscataway, N.J.

Neuraminidase (*Clostridium perfringens,* 1.1 units/mg), bovine serum albumin, cytochrome *c,* bovine carbonic anhydrase, myoglobin, insulin, mannose, galactose, glucose, *N*-acetylneuraminic acid, glucosamine, acetylacetone, *p*-dimethylaminobenzaldehyde, 2-thiobarbituric acid, sodium lauryl sulfate, glycine, Trizma base, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)[2] were purchased from Sigma Chemical Co., St. Louis, Mo.

Urea and guanidine hydrochloride were obtained from Schwarz/Mann, Van Nuys, Calif. *N,N'*-Methylenebisacrylamide and acrylamide, recrystallized from acetone and chloroform, respectively, according to Loening (1967), and *N,N,N',N'*-tetramethylenediamine were purchased from Eastman Chemicals, New York, N.Y. Dialysis tubing was obtained from Van Waters and Rogers, Los Angeles, Calif., and treated according to McPhie (1971) before use. Trypsin, pre-treated with tosylphenylalanyl chloromethyl ketone (Tos-PheCH_2Cl), 189 units/mg, was purchased from Worthington Biochemicals, Freehold, N.J. Fetuin was obtained from Grand Island Biological Co., Huntington Beach, Calif.

Phosphatidylserine and phosphatidylcholine were isolated from bovine brain and hens eggs, respectively, and purified by Dr. Masami Gamo in this laboratory. Cyclohexanone was purchased from Mallinckrodt Chemical Co., St. Louis, Mo. Cyanogen bromide was obtained from Aldrich Chemical Co., San Leandro, Calif. Agarose was supplied by Bio-Rad Laboratories, Richmond, Calif. Bovine topical thrombin was purchased from Parke-Davis, Los Angeles, Calif. The dansyl amino acid standards were obtained from Calbiochem, San Diego, Calif. Barium chloride and calcium chloride standards for atomic absorption analysis were obtained from Beckman Instruments, Palo Alto, Calif.

Methods. Prothrombin concentration was measured by absorption at 280 nm using an $E_{280nm}^{1\%}$ of 13.6, correcting for Rayleigh light scattering according to Shapiro and Waugh (1966).

Amino acid analyses were performed according to Spackman et al. (1958) using a Beckman 120 amino acid analyzer. Peak areas were determined by manual integration. The protein solutions were desalted by dialysis and lyophilized prior to hydrolysis in constant boiling HCl at 110° for 24, 48, and 72 hr. (Norleucine was incorporated as an internal standard into all of the hydrolyses.) The peptides released by Tos-PheCH_2Cl tryptic digestion were hydrolyzed for 48 hr. All acid hydrolyses were carried out in evacuated tubes. The values for serine and threonine were determined by semilogarithmic extrapolation to zero hydrolysis time while valine and isoleucine were determined by their value at 72 hr of hydrolysis. Tryptophan was estimated spectrophotometrically by the method of Goodwin and Morton (1946).

Neutral sugars were determined by the anthrone procedure described by Spiro (1966), using an equimolar mixture of mannose and galactose as a standard. Hexosamines were assayed according to a modification of the procedure of Elson and Morgan as reported by Gatt and Berman (1966) using galactosamine and glucosamine as standards. The sialic acid content of prothrombin as well as of its conversion fragments and peptides was assessed by the 2-thiobarbituric acid assay of Warren (1959). In early studies fetuin was used to calibrate the assay.

Neutral sugars and amino sugars were quantitated by gas–liquid chromatography of the alditol acetates of the sugars according to the method reported by Grimes (1974). A Hewlett Packard gas–liquid chromatograph equipped with $\frac{1}{8}$ in. glass columns containing 3% OV^{225} on Supelcon 80–100 mesh (Supelco) was used. This unit was programmed for 8 min at 170° and the temperature then increased at 2°/min to 220°. The responses and retention times were determined with a Hewlett-Packard 3370b integrator.

Analytical ultracentrifugation was performed in a Beckman Model E ultracentrifuge, equipped with a photoelectric scanner. Sedimentation equilibrium determinations were all scanned at 280 nm using absorption optics. Attainment of equilibrium was assessed by reading the absorbance from the scanner at a given radial position at various time intervals after 24 hr. When the optical density at a given radial position remained unchanged for over 4 hr it was assumed that equilibrium had been reached. The rotor speed for the tryptic fragments was 3400 rpm at 20°. A partial specific volume for factor II of 0.700 ml/g determined by pycnometry (Cox and Hanahan, 1970) was used in the calculation of the weight average molecular weight by the equation:

$$M_w = [(2.303)2RT/(1 - \bar{\nu}\rho)\omega] \, (d \log A/dr^2)$$

where A refers to the absorbance at 280 nm, ρ is the density of the buffer, $\bar{\nu}$ is the partial specific volume of the protein in milliliters/gram, ω is the angular velocity in radians/second, r is the distance from the axis of rotation in centimeters, R is the gas constant, and T is the absolute temperature.

Calcium and barium ion levels were quantitated at 427 and 553 nm, respectively, in a Beckman 440 atomic absorption spectrophotometer. Samples for atomic absorption measurement were placed in 15-ml platinum crucibles and heated on a hot plate until the contents were dry. Blanks containing only water were treated in similar manner. The crucibles were then placed in a muffle furnace at 600° for at least 5 hr to ash the samples. After cooling, the crucible contents were transferred quantitatively into acid-washed test tubes with 1% lanthanum chloride in 0.1 *N* HCl. These solutions were subjected to analysis. Standards of calcium and barium ions containing 1 to 20 μg/ml were used to calibrate the instrument.

Polyacrylamide disc gel electrophoresis was performed by the technique of Ornstein (1964) in the Tris-HCl–glycinate buffer system devised by Davis (1964). Polyacrylamide gels were also run in the presence of sodium dodecyl sulfate according to Weber and Osborn (1969) or in the 8 *M* urea–sodium dodecyl sulfate system reported by Swank and

[2] Abbreviations used are: CBF, calcium binding fragment; GP-1, glycopeptide-1; GP-2, glycopeptide-2; PC, phosphatidylcholine; PS, phosphatidylserine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tos-PheCH_2Cl, tosylphenylalanyl chloromethyl ketone; Gdn-HCl, guanidine hydrochloride; Dnp, dinitrophenyl; dansyl, 8-dimethylamino-1-naphthalenesulfonate.

Munkres (1971). Subsequent to electrophoresis the gels were stained with either Amido Schwartz or in Coomassie Brilliant Blue by the system described by Fairbanks et al. (1971). Some of the gels were also stained for carbohydrate after electrophoresis by the periodate–Schiff reagent method reported by Segrest and Jackson (1972).

Molecular weights of proteins and peptides were also estimated under denaturing conditions in the presence of 6 *M* guanidine hydrochloride (Gdn·HCl). Columns (1.6 × 40 cm) of 8% agarose were equilibrated, packed, and run in the denaturant as described by Mann and Fish (1972). The columns were calibrated with bovine serum albumin, bovine carbonic anhydrase, myoglobin, cytochrome *c*, and insulin. The void volume of the column was determined with Dextran Blue 2000 while the inclusion volume of the column was determined with dinitrophenylalanine (Dnp-Ala). The standards as well as all of the samples were equilibrated for 24 hr prior to application to the column. The columns were packed by gravity and then a constant flow rate of 3–4 ml/hr was maintained with a Buchler polystaltic pump. Fractions were collected by drop counting and weighed to determine the elution positions. This method was chosen instead of collecting the fractions by a timer to ensure that fluctuations in the flow rate, if any, would not be reflected in the molecular weight determinations.

Gel filtration experiments were performed according to the procedure of Andrews (1964). The gels were treated according to manufacturer's specifications.

The affinity of prothrombin, intermediate 1, and fragment 1, as well as the peptides released by tryptic digestion, for calcium ions was investigated by equilibrium dialysis according to Hughes and Klotz (1956) and by the gel filtration method of Hummel and Dreyer (1962) as modified by Price (1972). In ligand binding studies by the gel filtration method, approximately 10 mg of protein was applied to 1.6 × 60 cm columns of Sephadex G-25 previously equilibrated at 25° with 0.04 *M* Hepes–0.1 *M* KCl buffer mixture at the desired calcium ion concentration and pH. Calcium ion concentrations ranged from 0.01 to 10 m*M;* pH was varied between 6 and 9. Specific activity of $^{45}Ca^{2+}$ in the equilibrating and elution buffer was maintained at 500 cpm/nmol. Equilibrium dialysis experiments were performed at 4° in a CRC multicavity dialysis cell for 12 hr, and calcium binding was investigated over the same range of calcium ion concentrations, pH, and buffer conditions employed in the gel filtration experiments. Protein concentrations were 1–2 mg/ml in the equilibrium dialysis studies. Aliquots of gel filtration chromatographic fractions and the retentates and diffusates from the equilibrium dialysis cells were suspended in 10 ml of scintillation cocktail (80 g of naphthalene, 4 g of 2,5-diphenyloxazole, and 400 ml of methanol diluted to 1 l. with 1,4-dioxane) and radioactivity was determined in a Beckman LS-250 scintillation spectrometer using the ^{14}C window.

Amino-terminal analysis was performed according to Seiler (1970). Approximately 0.5–1.0 mg of lyophilized protein was dissolved in 1.0 ml of 0.5 *M* sodium bicarbonate in 8 *M* urea. To this solution was added 1.0 ml of an acetone solution of dansyl chloride (50 mg/ml). The mixture was stirred occasionally and kept at 37° for 10 hr. The reaction tube was protected from the light during the incubation. The sample was then dialyzed overnight in 1 l. of 0.1 *N* ammonium hydroxide at 4° and subsequently lyophilized. When dry, 1.0 ml of constant boiling HCl was added to the residue and the tube was sealed and placed in an oven for 18 hr at 105°. After cooling the contents were blown dry under a stream of nitrogen and then the residue was washed twice with water and blown dry with a stream of nitrogen. The material was then redissolved in 0.2 ml of acetone–0.1 *N* acetic acid (3:2, v/v), and 50–100 μl aliquots of the samples were then chromatographed on 0.5 mm thin-layer plates of silica gel G. The solvent system was toluene–2-chloroethanol–25% NH_3 (6:10:4, v/v). Various dansyl amino acid standards were also run on the same plate to identify the unknown N terminus.

Preparative Procedures. (A) BOVINE PROTHROMBIN. This protein was isolated essentially by the procedure described by Benson (1974), which involves barium citrate adsorption, DEAE-cellulose chromatography, and preparative gel electrophoresis. The prothrombin, isolated in 40–50% yield, had a mol wt of 67,800 ± 1500. This protein contained 8.5% (by weight) carbohydrate which consisted of glucosamine, mannose, galactose, and sialic acid.

(B) BOVINE THROMBIN. Thrombin was purified by a modification procedure published by Lundblad (1971). Parke-Davis topical thrombin (10,000 units/vial) was reconstituted in 10 ml of 0.1 *M* sodium phosphate buffer (pH 6.5) and then dialyzed for 12 hr against the same buffer containing 0.001 *M* ε-aminocaproic acid. The retentate after dialysis was then centrifuged at 30,000*g* at 4° for 15 min in a Sorvall RC2-B centrifuge. The supernatant from this centrifugation was then applied to a 2.6 × 20 cm column of sulfopropyl (SP) Sephadex C-50 at a flow rate of 0.75 ml/min. After application the column was washed with 200–300 ml of the 0.1 *M* sodium phosphate equilibrating buffer (pH 6.5) until all the unadsorbed proteins had been washed off the column. The ionic strength of the eluting buffer was increased to 0.25 *M* sodium phosphate buffer (pH 6.5) which eluted the thrombin. The thrombin containing fractions were checked for homogeneity by polyacrylamide disc gel electrophoresis and those judged pure by this criterion were pooled. The activity of the pooled samples varied from 300 to 400 NIH units/ml. One-milliliter aliquots were then placed in plastic tubes and stored at −25°.

(C) FRAGMENT 1 AND INTERMEDIATE 1. In a typical preparation, 50 mg of purified prothrombin was incubated in 20 ml of 0.05 *M* Tris-HCl buffer (pH 7.5) with 300–350 units of thrombin for 1 hr at 25°. In other experiments in which the preparation of larger amounts of fragment 1 was necessary 200 and 250 mg of prothrombin were incubated with 750–1000 units of thrombin at 37° for 2 hr in 0.05 *M* Tris-HCl buffer (pH 7.5). At the end of the incubation period, the mixture was made 5% in sucrose and applied to a preparative polyacrylamide gel electrophoresis system. The preparative disc gel system for this separation was identical with the system described for the isolation of prothrombin (Benson, 1974; Kisiel and Hanahan, 1974) except that the resolving gel volume was reduced to 80 ml. All other conditions were exactly the same. The highly anionic character of fragment 1 allowed it to be separated very well from the more cationic intermediate 1 (Benson et al., 1973). The elution profile from such an electrophoresis indicated that all of the original prothrombin had been converted to fragment 1 and intermediate 1 by thrombin. Those fractions containing fragment 1 and intermediate 1 were tested for homogeneity both by polyacrylamide disc gel electrophoresis and by sodium dodecyl sulfate gel electrophoresis prior to pooling and storing.

(D) LIPID DISPERSIONS. Dispersions of phosphatidyl-

choline (PC) and of phosphatidylcholine (PC)/phosphatidylserine (PS) were generated in the following manner: 25 mg each of PC and PS in chloroform–methanol (2:1) were taken to dryness under a stream of nitrogen. The residue was dissolved in anhydrous diethyl ether and again blown dry under a nitrogen stream. The addition of diethyl ether with subsequent drying was repeated three more times to remove all traces of chloroform and methanol. The appearance of the phospholipid at this time was that of a smooth, very pale yellow oil. The phospholipids were redissolved in about 2 ml of diethyl ether and then 2.5 ml of 0.1 *M* Tris-HCl–0.1 *M* sodium chloride buffer mixture (pH 7.5) was added. The tube was swirled vigorously to mix the phases and then the diethyl ether was blown off under nitrogen. Then, an additional 2.5 ml of the same buffer was added. Nitrogen was then bubbled through the emulsion for 10 min. The emulsion appeared to be smooth and milky in appearance at this time. This sample was sonicated at 4° under nitrogen for 45 min at power level 5 on a Branson sonifier equipped with a microtip. After sonication was completed, the mixture appeared slightly opalescent. This preparation was centrifuged in a Beckman Model L2-65 ultracentrifuge at 4° using a TY-65 rotor, for 1 hr at 100,000*g*. The contents of the tube after centrifugation were nearly clear with only a slight opalescent cast. There was a small yellow-gray precipitate containing undispersed phospholipid and bits of titanium from the sonifier probe. The solution was stored at 4° and checked by thin-layer chromatography to be certain that no destruction of the phospholipids had occurred. There was consistently between 9.2 and 9.4 mg of phospholipid/ml (theoretical = 10 mg/ml) in all the preparations.

(E) ASIALO PROTHROMBIN. Purified bovine prothrombin (30 mg) was dissolved in 12 ml of 0.1 *M* Tris-phosphate buffer (pH 6.0). Two milliliters was removed as a control and also aliquots were removed for measuring free sialic acid, total bound sialic acid, and for sodium dodecyl sulfate electrophoresis. To the remainder of the original sample was added 1.0 mg of neuraminidase (1.1 unit/mg), and the incubation mixture was placed at 37°. Aliquots were removed at 0, 10, 20, 30, 45, and 60 min and transferred to conical centrifuge tubes and heated in a boiling water bath for 2–3 min to stop the reaction. Aliquots were removed to determine the amount of free sialic acid liberated by this procedure. A control prothrombin solution (not enzyme treated) was also placed in a boiling water bath. Finally, the incubation was allowed to proceed for 120 min at 37°; the mixture was then concentrated to 1.0 ml and diluted to 10 ml. The buffer concentration was now 0.01 *M* Tris-phosphate buffer (pH 6.0) after tenfold dilution. This 10-ml sample was then charged onto a 1.6 × 60 cm column of DEAE-cellulose which had been equilibrated and packed in the 0.01 *M* Tris-phosphate buffer (pH 6.0). The prothrombin was eluted with a linear gradient of 0.0–0.5 *M* sodium chloride in the equilibrating buffer. Those fractions containing prothrombin activity were pooled, concentrated, and assayed for calcium ion binding ability, prothrombin activity compared to untreated control, and also the amount of sialic acid still associated with the neuraminidase treated prothrombin.

(F) DERIVATIZATION OF CARBOXYL GROUPS ON PROTHROMBIN. This treatment was conducted according to Carraway and Koshland (1969). Ten milligrams of purified prothrombin was dialyzed against water and subsequently concentrated to 3 ml. The latter was made 1.0 *M* in glycine methyl ester and the pH of the solution was adjusted to 4.75. This solution was made 0.1 *M* in cyclohexylmorpholinocarbodiimide and stirred at room temperature, and the pH continually monitored and maintained at 4.75. After 1 hr the same amount of glycine methyl ester and cyclohexylmorpholinocarbodiimide was added, and the reaction continued for 3 hr at pH 4.75. The reaction was stopped by addition of 1.0 *M* acetate buffer (pH 4.75) and the mixture then dialyzed against 1 l. of 10^{-3} *N* HCl overnight. The derivatized protein was checked for prothrombin activity and for the ability to bind calcium ions.

(G) BINDING OF PROTHROMBIN AND CONVERSION PRODUCTS TO PHOSPHOLIPID DISPERSIONS. Purified prothrombin was incubated in the presence of a PC/PS dispersion (protein:lipid, 1:1, w/w) in the presence of 10 m*M* calcium chloride in 0.05 *M* Tris-HCl–0.1 *M* sodium chloride buffer mixture (pH 7.5). After 15 min, the mixture was chromatographed on a 2.6 × 50 cm column of Sephadex G-150 equilibrated in the same buffer. The void volume material was pooled, concentrated, and assayed for prothrombin, phosphorus, and protein. Any unbound prothrombin eluted later from this column. The pooled and concentrated (to 2 ml) void volume material was then treated with purified thrombin for 1 hr and the mixture chromatographed on a 1.6 × 50 cm column of Sephadex G-150 in the same buffer as used above. Each peak resulting from this chromatographic separation was assayed for protein concentration, prothrombin activity, and phosphorus, and also subjected to sodium dodecyl sulfate electrophoresis.

Results

(A) *Calcium Ion Binding of Prothrombin and Conversion Polypeptides.* Prior to measurement of prothrombin binding capabilities, its calcium content was determined by atomic absorption spectrophotometry. Since the prothrombin purification involves an initial precipitation with barium ions, the level of this cation was also determined, but the value was so low as to be insignificant. The calcium ion level in purified prothrombin varied from preparation to preparation, ranging between 0.17 and 7.0 μmol of calcium ions per μmol of prothrombin. This associated calcium could be removed easily by dialysis of the prothrombin for 12 hr against 0.05 *M* Tris-HCl–0.001 *M* EDTA buffer mixture (pH 7.5). This treatment effectively decreased calcium ion levels to less than 1 μmol of calcium ions per μmol of protein.

Calcium ion binding experiments using the radionuclide $^{45}Ca^{2+}$ were carried out by both gel filtration equilibrium experiments and equilibrium dialysis. The concentration range of Ca^{2+} was 0.01–10 m*M*. The buffer used in all of the experiments was 0.04 *M* Hepes–0.1 *M* potassium chloride (pH 7.0). The binding profile of prothrombin (at 1 m*M* calcium chloride), as obtained by the gel filtration procedure, is shown in Figure 1. The amount of Ca^{2+} bound at a particular concentration was calculated by determining the level of radioactivity above the base-line radioactivity value and dividing this value by the amount of protein in that fraction. In this way the amount of Ca^{2+} bound per mole of protein can be determined over many protein concentrations in a single experiment. The base-line concentration for radioactivity was determined by averaging the radioactivity (counts per minute) in those fractions containing no protein.

The reversibility of Ca^{2+} binding to prothrombin was explored as follows. Fractions containing protein and radioac-

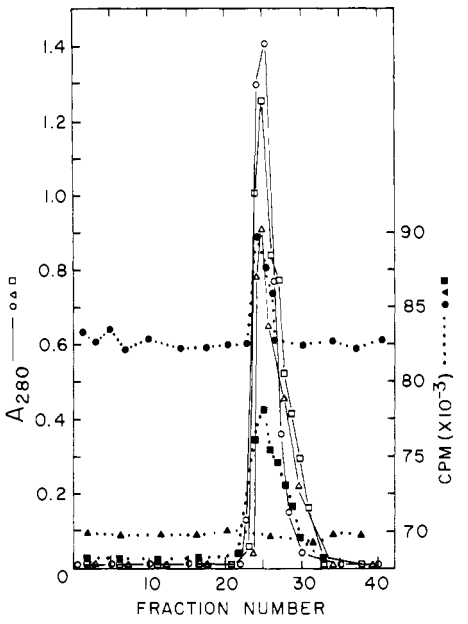

FIGURE 1: Calcium ion binding by prothrombin, fragment 1, and intermediate 1 as shown by gel filtration chromatography. In each experiment 10 mg of protein was applied to a 1.6 cm × 50 cm column of Sephadex G-25 equilibrated in 0.04 *M* Hepes–0.1 *M* potassium chloride–0.001 *M* calcium chloride at 500 cpm/nmol. The flow rate was 24 ml/hr and 2.0-ml samples were collected. The absorbance at 280 nm (○,□,△) was monitored and 0.1 ml from each sample was assayed for radioactivity (●,■,▲). The individual runs are designated as follows: prothrombin (●,○); fragment 1 (■,□); and intermediate 1 (▲,△).

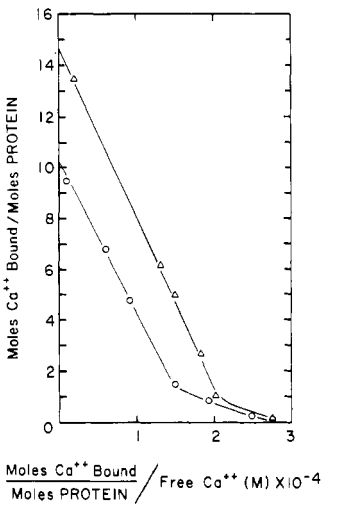

FIGURE 2: Scatchard plot of calcium ion binding to prothrombin and fragment 1. Each point on the curves represents the mean value obtained from four experiments with different preparations of prothrombin (○) and fragment 1 (△) in each experiment.

tive calcium were concentrated to a small volume (usually 1.0 ml) and applied to a Sephadex G-25 column of the same dimensions as given in Figure 1. The buffer and calcium ion concentrations were identical except no radiolabeled calcium ions were included. No radioactivity above background could be detected in the prothrombin peak in this instance, indicating that the calcium ions were freely exchangeable and that the binding was reversible. Furthermore, it was found that the initial calcium ion content of the prothrombin molecule (as determined by atomic absorption spectrophotometry) had no effect on the amount of Ca^{2+} bound either in the gel filtration or equilibrium dialysis experiments. Both EDTA treated and nontreated prothrombin preparations bound identical amounts of Ca^{2+}.

The calcium ion binding activity of intermediate 1 and fragment 1 was determined both by gel filtration equilibrium and equilibrium dialysis under exactly the same conditions as those utilized for prothrombin. Typical elution profiles from gel filtration experiments, using 1 m*M* calcium chloride, for fragment 1 and intermediate 1 are given in Figure 1. Whereas fragment 1 showed significant binding capacity for Ca^{2+}, intermediate 1 exhibited no binding.

The affinity of prothrombin and fragment 1 for calcium ions is presented in the form of a Scatchard plot in Figure 2. The points on each of these curves represent the average of at least four different binding experiments at a given Ca^{2+} concentration, each on a different preparation of the respective protein. Prothrombin maximally binds 10 mol of Ca^{2+}/mol of protein with a dissociation constant of 6.3×10^{-4} *M*. Similarly, fragment 1 binds 12–15 mol of Ca^{2+}/mol of protein with a dissociation constant of 6.8×10^{-4} *M*. The biphasic nature of both curves indicates that not all of the binding sites are equivalent. The lower part of each curve represents the high affinity sites and these appear responsible for binding from 3 to 4 mol of Ca^{2+}, while the upper portions of the curves represent the lower affinity binding sites.

As an initial means of determining the type of functional group or groups on prothrombin and fragment 1 responsible for Ca^{2+} binding, the affinity of these two components for Ca^{2+} was measured as a function of pH. Prothrombin was found to bind identical amounts of calcium ions between pH 6 and 8 while the fragment 1 bound identical amounts of calcium ions over a range of pH values from 5 to 9. If the binding were due to normal carboxyl functions on the proteins, varying the pH would be expected to influence the binding; for example, the carboxylate ions would become protonated as the pH was decreased.

In order to test further the hypothesis of carboxylate ion involvement in the calcium ion binding, the carboxyl groups on prothrombin were derivatized with glycine methyl esters according to Carraway and Koshland (1969). When the calcium ion binding of this derivatized prothrombin was determined, the results were equivocal. Though the derivatized molecule bound no calcium ions, it could not be converted to thrombin in the prothrombin assay. These data could indicate that not only are carboxyl functions involved in calcium ion binding but also a prothrombin molecule that does not retain the ability to bind calcium ions cannot be converted to thrombin. Alternatively, the prothrombin may have undergone a gross conformational change during the derivatization reaction rendering it incapable of binding calcium ions, and/or altering it such that it was not a substrate for the "prothrombinase" enzyme.

The derivatized prothrombin displayed significant alterations in its net charge as evidenced by the observation that it would not migrate in the disc gel electrophoresis system of Davis (1964). This result indicates that most probably a large majority of the carboxyl groups in the prothrombin molecule have been derivatized by the procedure.

Since the net negative charge inherent in many glycoproteins is a function of the terminal sialic acids in the sugar chains, a series of experiments was initiated to determine the involvement of the sialic acids in calcium ion binding. Approximately 30 mg of prothrombin was incubated with 1 mg of neuraminidase (1.1 unit/mg of protein) as described

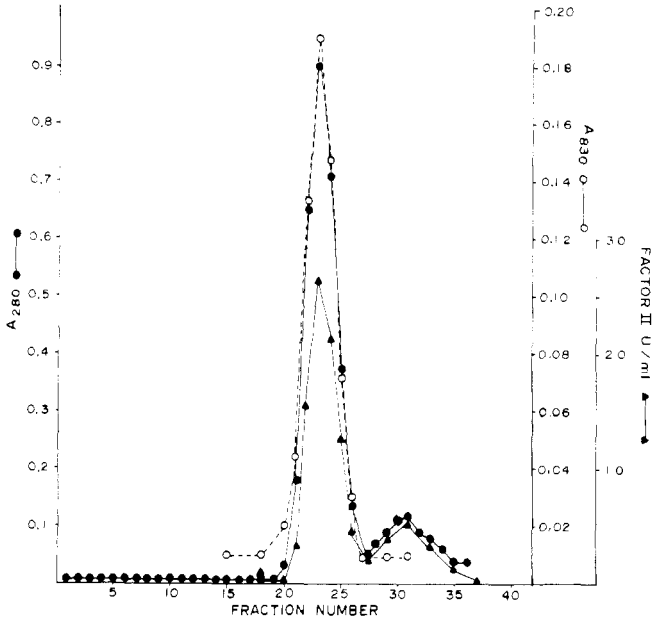

FIGURE 3: Plot of the elution profile obtained by passage of a mixture of factor II and a PC/PS dispersion incubated in the presence of calcium ions through Sephadex G-150. Approximately 25 mg of factor II was incubated in the presence of a 10-mg dispersion of equimolar PC/PS in the presence of 10 m*M* calcium chloride. Two milliliters of the incubate was then passed through a 2.6 cm × 60 cm column of Sephadex G-150 in 0.05 *M* Tris-HCl-0.1 *M* sodium chloride-0.010 *M* calcium chloride. The flow rate was 30 ml/hr. The fractions obtained from this chromatography were monitored for absorbance at 280 nm (●), phosphorus content (absorbance at 830 nm (○)), and factor II activity (▲).

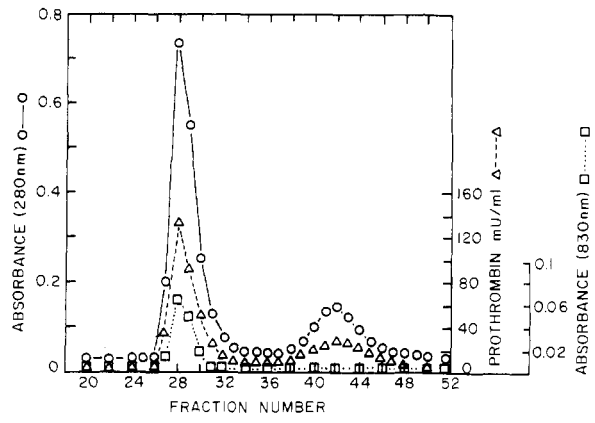

FIGURE 4: Gel filtration chromatography of the products formed by treatment of prothrombin-Ca^{2+}-phospholipid complex with thrombin. A prothrombin-Ca^{2+}-phospholipid complex (see text for details) was treated with 20 NIH units of thrombin in 0.1 ml for 1 hr. After incubation the mixture was applied to a 1.6 cm × 50 cm column of Sephadex G-150 in 0.05 *M* Tris-HCl-0.1 *M* sodium chloride-0.010 *M* calcium chloride buffer (pH 7.5). The flow rate was 14 ml/hr. The fractions from this experiment were monitored for absorbance at 280 nm (○), phosphorus content (□), and prothrombin activity (△).

in the Experimental Section. This treatment was found to liberate nearly 90% of the sialic acid compared to that released by acid hydrolysis alone. When the reaction was completed, the asialo prothrombin was separated from the neuraminidase by DEAE-cellulose ion exchange chromatography. The isolated asialo prothrombin was then assayed for its ability to bind Ca^{2+} and compared with a native (untreated) prothrombin control. No difference in Ca^{2+} binding activity could be detected between these two preparations. Also, the specific activity of the asialo prothrombin was identical with that of the native prothrombin control.

(B) *Phospholipid Binding of Prothrombin, Fragment 1, and Intermediate 1.* The conversion of prothrombin to thrombin is markedly accelerated in the presence of phospholipid aggregates (Barton et al., 1967; Jobin and Esnouf, 1967). Both prothrombin (Barton and Hanahan, 1969) and activated factor X (Papahadjopolous and Hanahan, 1964) bind to phospholipid vesicles in the presence of calcium ions. The binding is reversible as shown by removal of the divalent metal ions. In contrast to prothrombin, thrombin does not bind to phospholipid surfaces (Barton and Hanahan, 1969).

Since fragment 1 avidly binds Ca^{2+}, whereas intermediate 1 does not, it would be quite possible that fragment 1 would bind to phospholipid while intermediate 1 would not be similarly associated. It is also possible that intermediate 1 had lost its ability to bind calcium ions by undergoing some conformational alteration during generation of the intermediate from prothrombin or during its subsequent purification. Therefore, the following series of experiments was undertaken to determine these possibilities.

Twenty-five milligrams of prothrombin and a phospholipid dispersion of equimolar quantities of PS/PC in 0.05 *M* Tris-HCl-0.025 *M* calcium chloride buffer mixture (pH 7.5) were incubated at 23° for 30 min. The entire incubation mixture was then applied by upward flow to a 2.6 × 40 cm column of Sephadex G-150 equilibrated in the same buffer. The elution profile from this chromatography gave two peaks; the first tube 23, or void volume, contained both phospholipid phosphorus and prothrombin clotting activity (Figure 3). The second peak (tube 31) contained only prothrombin clotting activity, with an approximate molecular weight of 99,000–100,000. This latter material was considered to be prothrombin in excess of the amount capable of being absorbed to the phospholipid, and as noted above (Experimental Section), it gave an anomalously high molecular weight in Sephadex gel columns.

The void volume peak (fractions 22–24) was pooled and concentrated to 2 ml and treated with 20 NIH units of thrombin for 1 hr. A prothrombin sample without added phospholipid and Ca^{2+} was treated in a similar manner and served as a control. An aliquot of the prothrombin-Ca^{2+}-phospholipid after thrombin treatment was subjected to gel filtration in a 1.6 × 50 cm column of Sephadex G-150 in the same buffer as noted in Figure 3. The elution profile is presented in Figure 4. The absorbance at 280 nm, the prothrombin clotting activity, and phosphorus were determined on each fraction. While there was a small amount of prothrombin activity in the void volume (fraction 28), most of the activity resided in the second peak (fraction 42).[3] The molecular weight of this latter component was approximately 55,000, and it contained no (phospholipid) phosphorus. The fractions in the respective peaks were pooled and treated with diethyl ether–ethanol (3:1, v/v) to remove phospholipid.

Aliquots from the thrombin-treated as well as the control prothrombin were treated with sodium dodecyl sulfate and

[3] Though there appears to be more prothrombin activity in the first peak (Figure 4), a gel electrophoresis pattern of the total reaction mixture showed a faint prothrombin band and a heavy intermediate 1 band. The latter component has a much lower specific activity than prothrombin in the assay system; hence this explains the conclusion that there was more "prothrombin" activity in fraction 42 than in fraction 28.

subjected to gel electrophoresis in the presence of sodium dodecyl sulfate. The control prothrombin sample had stained bands with values corresponding to molecular weights of 70,000, 54,000, 40,000, and 25,000, respectively. The void volume peak from the gel filtration separation had stained bands corresponding to molecular weights of 70,000 (faint) and 25,000 (strong). The second peak had a very darkly stained band corresponding to a molecular weight of 54,000 and a very faint band corresponding to a molecular weight of 40,000. These data indicate that the void volume peak contained mostly fragment 1 with some unreacted prothrombin while the second peak contained primarily intermediate 1 with a small amount of thrombin (40,000 daltons). The control contained exactly the same molecular weight species indicating that the fragment 1 and intermediate 1 are formed by thrombin treatment of prothrombin in the presence or absence of phospholipid and calcium ions. Also, these data showed that fragment 1 binds calcium ions and subsequently absorbs to phospholipid aggregates while the intermediate 1 does not.

(C) *Isolation and Characterization of the Calcium Binding and Carbohydrate Containing Peptides in Fragment 1.* Since fragment 1 contains all of the calcium ion and lipid binding sites present in prothrombin, further fractionation of fragment 1 was undertaken to localize the carbohydrate and calcium binding region.[4]

(a) TRYPTIC DIGESTION. The effect of trypsin on fragment 1 was determined next in a preliminary experiment to ascertain whether peptides could be liberated without first denaturing and reducing the molecule. Consequently, 19.7 mg of fragment 1 in 3 ml of H_2O (pH 8.0) and 300 μg of Tos-PheCH_2Cl-trypsin in 50 μl of 10^{-3} *N* HCl were mixed and incubated for 3 hr. The pH was maintained at 8 by addition of 0.5 *N* NaOH. Another 30 μg of Tos-PheCH_2Cl-trypsin was added and digestion allowed to continue overnight. Then the solution was made to pH 4.5 with 0.5 *N* HCl and the entire mixture was applied to a 1.6 × 50 cm column of Dowex 50-X8 (200–400 mesh), with a flow rate of 18 ml/hr at 38°. The elution of peptides was attained by using the pyridine acetate buffer system described by Schroeder (1967), with 250 ml of pyridine acetate (pH 3.1) (0.2 *M* in pyridine) placed in the mixing chamber of the gradient box and 250 ml of pyridine acetate (pH 5.0) (2.0 *M* in pyridine) in the reservoir. One-milliliter fractions were collected and 0.1-ml aliquots were removed from each fraction and hydrolyzed with 13.5 *N* NaOH. This hydrolysate was then allowed to react with the ninhydrin reagent prepared according to Hirs (1967). After the ninhydrin treatment the absorbance of each fraction was read at 570 nm. It was apparent that the fragment 1 was hydrolyzed by trypsin into many peptides (~18) without the necessity of first reducing and denaturing the molecule.

(b) ISOLATION OF CALCIUM BINDING PEPTIDE (CBF) AND GLYCOPEPTIDES (GP-1 AND GP-2). Inasmuch as fragment 1 binds Ca^{2+} and subsequently binds to phospholipid vesicles, it seems likely that after proteolysis of the molecule with trypsin in the presence of lipid and Ca^{2+} those peptides containing the Ca^{2+} binding sites would remain bound to the phospholipid vesicles while the non-calcium-ion binding peptides would remain in solution.

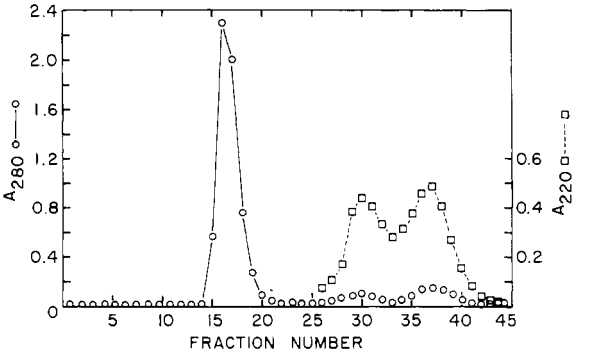

FIGURE 5: Chromatographic behavior of fragment 1-Ca^{2+}-phospholipid complex after trypsin treatment. Fragment 1-Ca^{2+}-phospholipid complex (see text for details) was treated with Tos-PheCH_2Cl-trypsin and the entire hydrolysate was applied to a 2.5 × 35 cm column of Sephadex G-150 in 0.05 *M* Tris-HCl–0.1 *M* sodium chloride–0.01 *M* calcium chloride buffer (pH 7.5). The flow rate was 30 ml/hr and 2-ml fractions were collected. The fractions were then monitored for absorbance at 280 nm (○) and 220 nm (□).

In one experimental approach, 20 mg of purified fragment 1 was incubated with 2 ml of a sonicated dispersion of PS/PC (10 mg/ml). After a 15-min incubation the mixture was chromatographed on a 2.5 × 35 cm column of Sephadex G-150 in 0.05 *M* Tris-HCl–0.1 *M* NaCl–10 m*M* calcium chloride buffer mixture (pH 7.5). The elution profile of this chromatographic procedure was similar to that given in Figure 3 and showed two peaks: (1) one at the void volume, which contained phosphorus and fragment 1, which was detected by disc gel electrophoresis, and (2) a later eluting small peak which contained (excess) fragment 1 only as detected by disc gel electrophoresis. The void volume fractions were pooled, concentrated to 2 ml, and treated with 0.4 mg of Tos-PheCH_2Cl-trypsin. After 3 hr, 0.4 mg of Tos-PheCH_2Cl-trypsin was added again and the digestion was continued for 12 hr. The incubation mixture was then charged onto the same Sephadex G-150 column (as above) in the same buffer still containing 10 m*M* calcium chloride. The elution profile for this separation is shown in Figure 5. In addition to the void volume peak (fraction 16), two additional peaks of low molecular weight appear after trypsin proteolysis (fractions 30 and 37, respectively). The void volume peak (fractions 15–18) was concentrated to 2 ml and made 0.01 *M* in EDTA. The Sephadex G-150 column was reequilibrated with 4 column volumes of 0.5 *M* Tris-HCl–0.1 *M* sodium chloride buffer mixture (pH 7.5) without calcium chloride. After the column had been reequilibrated, the EDTA-treated phospholipid mixture was passed onto the column and the subsequent elution profile is shown in Figure 6. Fraction 16 is the void volume containing the phospholipid only. There are two protein peaks, fractions 26 and 37, respectively, and analytical disc gel electrophoretic analysis indicated that the second peak contained only fragment 1. The material in the third peak (fraction 38) did not stain with Coomassie Blue. The latter was considered to be a peptide liberated from the phospholipid vesicles with EDTA and therefore contained Ca^{2+} binding sites.

These data showed that some of the peptides generated from tryptic digestion do contain intact Ca^{2+} binding sites. It can also be seen that not all of the fragment 1 was digested by the trypsin treatment in the presence of phospholipid. A likely explanation of this phenomenon might be hindered

[4] Inasmuch as fragment 1 contained only one methionine residue/mole (Esmon et al., 1974), it was hoped that cleavage with cyanogen bromide would liberate two peptides, one of which might contain only the calcium binding sites. However, no peptides were released by the cyanogen bromide treatment of Jackson et al. (1973) unless there was subsequent reduction of the sample with β-mercaptoethanol. Thus, this approach was not considered suitable for further study.

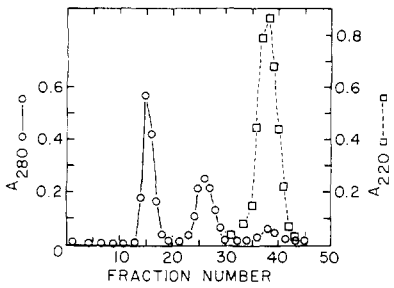

FIGURE 6: Chromatographic isolation of CBF from tryptic digest of fragment 1-Ca^{2+}-phospholipid complex. The phospholipid containing peak (void volume) from Figure 5 was treated with 0.01 *M* EDTA and then passed through a 2.5 × 35 cm column of Sephadex G-150 packed in 0.05 *M* Tris-HCl-0.1 *M* sodium chloride buffer (pH 7.5) without added calcium chloride. The flow rate was 30 ml/hr and 2-ml fractions were collected and the fractions were monitored for absorbance at 280 nm (O—O) and 220 nm (□---□).

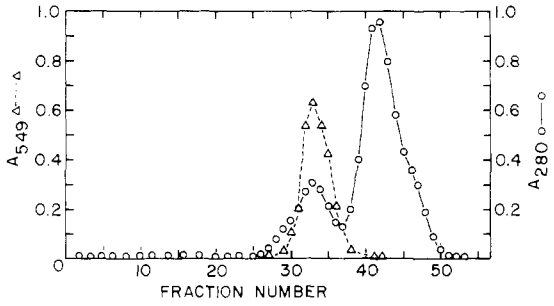

FIGURE 7: Chromatographic behavior of non-calcium-ion binding peptides from tryptic digest of fragment 1. Fifty milligrams of fragment 1 was treated (twice) with Tos-PheCH_2Cl-trypsin. After 16 hr, 2 ml of a sonicated dispersion of equimolar PC/PS (10 mg/ml) was added and the mixture was made 0.025 *M* in calcium chloride. After separation of the phospholipid-Ca^{2+}-peptide complex by centrifugation, the supernatant was applied to a 1.6 cm × 40 cm column of Sephadex G-75. The flow rate was 45 ml/hr and 5 ml fractions were collected. The column was equilibrated at 20–25° in 0.05 *M* Tris-HCl-0.1 *M* sodium chloride buffer (pH 7.5). The fractions were all monitored for absorbance at 280 nm (O—O) and aliquots from each fraction were hydrolyzed in 0.1 *N* sulfuric acid and assayed for sialic acid at 549 nm (Δ---Δ).

access to susceptible bonds in fragment 1 by trypsin when it is bound to the phospholipid vesicles, since fragment 1 was completely cleaved when treated with trypsin in the absence of phospholipid. The fact that unreacted fragment 1 and another peptide of low molecular weight were released into solution only after EDTA treatment indicated that this small peptide was binding Ca^{2+} and being adsorbed to the phospholipid vesicles.

In order to circumvent the problem that all the fragment 1 could not be cleaved by trypsin when phospholipid was present, a second experimental approach was employed. Fragment 1 was hydrolyzed with trypsin prior to addition of phospholipid and calcium chloride. The experiment was conducted as follows. Fragment 1 (50 mg) was mixed with 3 ml of 0.01 *M* ammonium bicarbonate buffer (pH 8.1), 1.0–1.4 mg of Tos-PheCH_2Cl-trypsin was added, and the mixture was incubated for 3 hr. At this time an additional 1.0–1.4 mg of Tos-PheCH_2Cl-trypsin was introduced and the reaction was allowed to proceed overnight. After lyophilization and reconstitution in 1.5 ml of 0.05 *M* Tris-HCl-0.1 *M* sodium chloride buffer mixture (pH 7.5), 2 ml of a PC/PS dispersion (10 mg of each/2 ml) was added and sufficient 0.5 *M* calcium chloride added to make the mixture 0.025 *M* in Ca^{2+}. After a 15-min incubation the phospho-

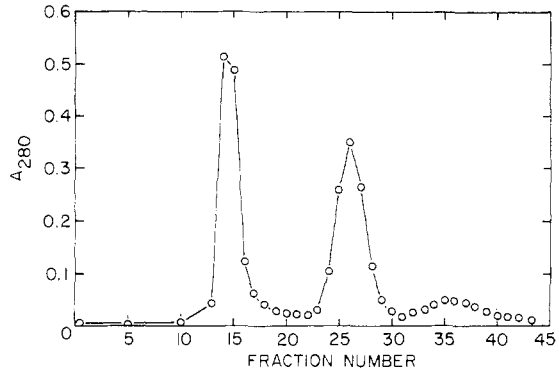

FIGURE 8: Elution profile of CBF released from the phospholipid-Ca^{2+} complex by EDTA. The phospholipid complex described in Figure 7 and in the text was treated with 0.025 *m* EDTA and, after a 15-min incubation, the mixture was applied to a 1.6 × 30 cm column of Sephadex G-75, previously equilibrated with 0.05 *M* Tris-HCl-0.1 *M* sodium chloride buffer (pH 7.5). The flow rate was 45 ml/hr and 5-ml fractions were collected. The absorbance at 280 nm (O) was monitored for each fraction.

lipid-Ca^{2+}-peptide aggregate was centrifuged at 30,000g. The phospholipid pellet (A) was saved and the supernatant (B) then chromatographed on a 1.6 × 40 cm G-75 Sephadex column, without any added Ca^{2+}. The elution profile is shown in Figure 7 and shows two protein peaks. Aliquots of each peak were subjected to mild acid hydrolysis and then assayed for free sialic acid by the thiobarbituric acid assay (Warren, 1959). The presence of this sugar was used as a marker for the location of the glycopeptides. All of the sialic acid containing peptides were found in the first peak only. No sialic acid was detected in the second peak.

The phospholipid pellet (A) was washed in buffer containing Ca^{2+} and then reconstituted in 0.05 *M* Tris-HCl-0.1 *M* sodium chloride-0.025 *M* EDTA buffer mixture (pH 7.5). After a 15-min incubation, the clear solution was chromatographed on the same 1.6 × 40 cm Sephadex G-75 column as used previously for the supernatant (B) separation. Results from this separation are shown in Figure 8. The first peak (fraction 14) is the void volume of the column and contains phospholipid. The second peak contained the Ca^{2+} binding peptide (CBF) and the last small peak eluted at the inclusion volume of the column and contained EDTA. The latter was confirmed in a separate experiment with EDTA only. There was no sialic acid present in any of these peaks.

The glycopeptides as well as the Ca^{2+} binding peptide were repeatedly concentrated and reconstituted in 0.01 *M* Tris-phosphate buffer (pH 6.0). Further fractionation of these peptides was achieved by ion exchange chromatography as illustrated below.

The glycopeptides were applied to a 1.0 × 30 cm column of DEAE-Sephadex A-25 equilibrated in 0.01 *M* Tris-phosphate buffer (pH 6.0). After the same was applied a linear gradient arising from 100 ml of 0.01 *M* Tris-phosphate buffer (pH 6.0) in the mixing chamber and 100 ml of 0.5 *M* sodium chloride in 0.01 *M* Tris-phosphate buffer (pH 6.0) in the reservoir was used to develop the column. The separation is shown in Figure 9. The first peptide peak which assayed for sialic acid (fraction 26) was labeled GP-1. The second carbohydrate containing peptide was labeled GP-2. These respective peaks were pooled, concentrated, and saved for study.

The calcium binding peptide or CBF, released from the

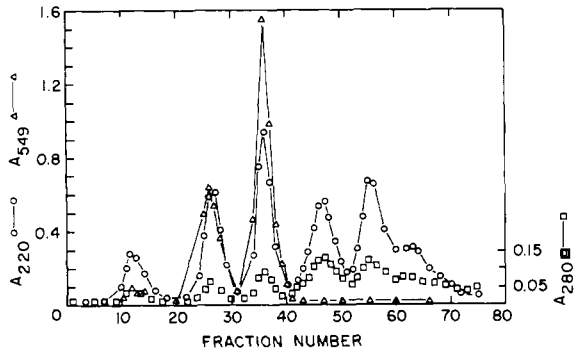

FIGURE 9: Fractionation of glycopeptides on DEAE-Sephadex A-50. The glycopeptide-containing fractions from Figure 7 were applied to a 1.0 × 30 cm column of DEAE-Sephadex A-50 equilibrated in 0.01 *M* Tris-phosphate (pH 6.0). The peptides were eluted with a linear gradient of sodium chloride from 0.0 to 0.5 *M*. The flow rate was 0.5 ml/min and 5-ml fractions were collected. Each fraction was then monitored for absorbance at 280 nm (●) and 220 nm (○). Aliquots (0.1 ml) from each fraction were hydrolyzed and treated with 2-thiobarbituric acid and subsequently monitored at 549 nm (▲) for sialic acid.

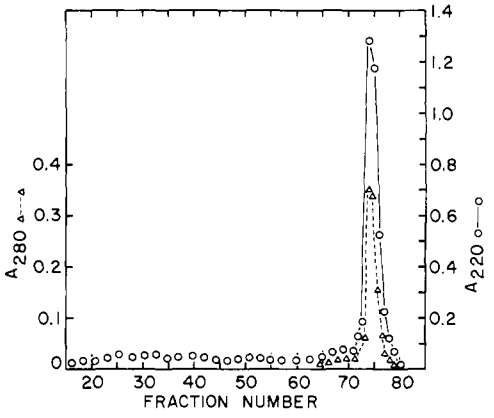

FIGURE 10: Elution profile of CBF on DEAE-Sephadex A-50. The CBF-containing fractions from Figure 8 were applied to a 1.0 × 30 cm column of DEAE-Sephadex A-50 in 0.01 *M* Tris-phosphate (pH 6.0) and the linear gradient of sodium chloride from 0.0 to 1.0 *M* was applied. The flow rate was 0.5 ml/min and 5-ml fractions were collected. Each fraction was then monitored for absorbance at 280 nm (△---△) and 220 nm (○—○).

phospholipid aggregate with EDTA, was applied to another 1.0 × 30 cm column of DEAE-Sephadex A-25 equilibrated with 0.01 *M* Tris-phosphate buffer (pH 6.0). CBF was significantly more anionic than either of the glycopeptides in that a linear gradient of 0.0–1.0 *M* sodium chloride was required to elute it from this column. The results of this chromatography are shown in Figure 10 and suggest that CBF is pure. Only one peak of absorbance was detected when the fractions were read at 280 or 230 nm.

(c) CHARACTERIZATION OF CBF, GP-1, AND GP-2. (1) Molecular Weight. The three peptides (GP-1, GP-2, and CBF) were chromatographed on a calibrated column of Sephadex G-75. Standard proteins of known molecular weight were used to calibrate the column. A standard curve of the elution volume–void volume ratio (V_e/V_o) vs. log molecular weight for bovine carbonic anhydrase, cytochrome *c*, and insulin was constructed. Interpolation of the V_e/V_o values gave apparent molecular weights of 8500, 7000, and 6500 for CBF, GP-1, and GP-2, respectively.

Further estimation of the molecular weights of these three peptides was attempted by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate–8 *M* urea (Swank and Munkres, 1971). After electrophoresis the gels were stained with Coomassie Blue according to Fairbanks et al. (1971). Upon destaining the gels, no bands of protein were apparent. Regardless of the amount of peptide electrophorized, no binding of Coomassie Blue was noted.

Due to the ineffectiveness of sodium dodecyl sulfate gels for determination of the molecular weights of these peptides, gel filtration in the presence of guanidine hydrochloride (Gdn·HCl) was employed. The procedure of Mann and Fish (1972) was followed in these molecular weight determinations. These authors reported that gel filtration in the presence of Gdn·HCl gave more accurate molecular weights for small peptides than any other procedure. A 1.6 × 30 cm column of 8% agarose equilibrated in 6 *M* Gdn·HCl was prepared at room temperature. After equilibration of the flow rate, the column was calibrated with molecular weight markers. Blue dextran was used to determine the void volume while dinitrophenylalanine was used to measure the inclusion volume. Bovine serum albumin, bovine carbonic anhydrase, cytochrome *c*, and insulin were used as standards for the molecular weight curve. Aliquots of all three peptides were also dissolved in 6 *M* Gdn·HCl and charged onto the column. Interpolation of the elution volumes of these three peptides on the standard curve gave molecular weights for CBF, GP-1, and GP-2 as 5200, 6500, and 5900, respectively.

The molecular weight of these peptides was also determined by analytical ultracentrifugation. The peptides were dissolved in 0.005 *M* Tris-HCl–0.1 *M* sodium chloride buffer mixture (pH 7.5) and centrifuged at 34,000 rpm to equilibrium. Scans were made and the absolute absorbance at various radial positions was determined. A value of $\bar{v}$ = 0.70 ml/g was assumed for CBF, while 0.696 ml/g = $\bar{v}$ was assumed for each of the glycopeptides. This assumption is based on data provided by Spiro (1960), who, using pycnometry, determined the $\bar{v}$ of fetuin, a bovine fetal serum protein containing 34% carbohydrate, to be 0.696 ml/g. The calculated molecular weight for the CBF was 3500.[5] Molecular weights of 4600 and 1960 were determined for GP-1 and GP-2, respectively. The log *A* vs. r^2 plots were linear for all three peptides. This was an indication of homogeneity of these peptides.

(2) Calcium Binding by CBF. The calcium ion binding parameters of CBF were next determined by equilibrium dialysis in the presence of 45 m*M* $CaCl_2$. The CBF was found to have between three and four binding sites for calcium ions based on a molecular weight of 3500. This number of sites is also approximately the number of high affinity binding sites found in prothrombin and fragment 1.

(3) Carbohydrate Content. The carbohydrate composition of GP-1 and GP-2 was determined as described in the Experimental Section. Table I shows the carbohydrate values for GP-1 and GP-2, each of which contain essentially the same level (40–45%) on a weight basis. Both GP-1 and GP-2 appeared to have the same carbohydrate distribution. CBF contained no detectable carbohydrate groups.

(4) Amino Acid Composition. After acid hydrolysis of the three peptides for 48 hr in constant boiling HCl, the amino acid composition of each peptide was determined using a Beckman 120 amino acid analyzer. The compositions of CBF, GP-1, and GP-2 are presented in Table II.

[5] This value agreed almost exactly with the minimum molecular weight calculated from the amino acid composition (Table II).

Table I: Carbohydrate Composition of Glycopeptide, GP-1, and GP-2 Derived from Fragment 1 by Tryptic Digestion.[a]

Residue	GP-1	GP-2
Sialic acid	17.1	15.9
Mannose	10.7	9.8
Galactose	8.1	7.6
Glucose	0.8	1.0
Glucosamine	11.3	11.0

[a] Values are given in weight percent. See text for further details on isolation and assay procedures.

Table II: Amino Acid Composition of CBF, GP-1, and GP-2 Derived from Fragment 1 by Tryptic Digestion.

	g of Amino Acid/100 g of Peptide		
Amino Acid	CBF	GP-1	GP-2
Lys	2.52	3.59	2.66
His	N.D.[c]	7.22	4.68
Arg	9.17	6.14	5.85
Asp	6.69	2.58	6.45
Thr[a]	2.61	6.61	5.30
Ser[a]	6.12	0.98	3.53
Glu	33.10	1.55	3.77
Pro	3.24	6.37	5.38
Gly	2.20	2.76	3.03
Ala[a]	8.15	1.27	4.40
Half-Cys	5.36	N.D.	N.D.
Val	N.D.	2.91	4.21
Met	N.D.	N.D.	N.D.
Ile	N.D.	3.05	2.11
Leu[a]	12.01	3.01	2.35
Tyr	N.D.	2.50	2.21
Phe	6.63	N.D.	N.D.
Trp[b]	4.91	1.56	1.30

[a] Value determined by ninhydrin reaction after 48 hr of hydrolysis. [b] Value determined spectrophotometrically. [c] Not detectable.

The N terminus of CBF was found to be glycine as determined by dansylation and thin-layer chromatography in chloroform–methanol–acetic acid (15:4:1) (Seiler, 1970) on 0.5-mm silica gel G plates.

Discussion

This investigation has provided a novel approach to the isolation of a significant segment of the calcium binding peptide region of the N terminus of bovine prothrombin and in addition has allowed the recovery of two glycopeptides not associated with the calcium binding sites. Cleavage of prothrombin with thrombin provided excellent yields of the N-terminus portion, or fragment 1, which contained the calcium binding sites (as well as the phospholipid binding sites) and two-thirds of the (total) carbodhydrate. Subsequent to tryptic digestion of this fragment the addition of a charged phospholipid dispersion plus calcium ions allowed easy removal of the calcium binding peptide as a complex. Further examination of the peptides which did not bind calcium and/or phospholipid permitted isolation of the carbohydrate containing regions of the N terminus in the form of two glycopeptides. The availability of these three fractions provided the basis of a detailed study on their biochemical characteristics.

A quantitative study of the calcium ion binding parameters of prothrombin and fragment 1 was undertaken, using (a) equilibrium dialysis and (b) gel filtration equilibrium. In theory, these two techniques are identical in that they both incorporate the use of separation of the macromolecular solution from the micromolecular solution by a semipermeable boundary. Earlier we reported that prothrombin possessed approximately ten Ca^{2+} binding sites (Benson et al., 1973). An examination of a Scatchard plot of this binding revealed a biphasic curve. Thus, all the binding sites were not identical, with possibly three to four of these sites having a higher affinity for calcium than the remainder. The biphasic nature of the curve is only an indication of the nonidentical nature of the calcium binding and no extrapolations from these data can be made as to evidence for cooperativity of binding as proposed by Stenflo and Ganrot (1973). The affinity constant calculated for the binding does not suggest a particularly tight binding of Ca^{2+} and this would be a reasonably logical conclusion since the process of coagulation in vivo is probably a fleeting, localized phenomenon. A strong binding of proteins to Ca^{2+} and phospholipid surfaces would have an anticoagulant effect. Bull et al. (1972) have shown that an increase in the anionic character of the phospholipid used in such instances would slow the prothrombin to thrombin conversion. In the current study, the Ca^{2+} binding to prothrombin phenomenon was shown to be reversible. This would also be a necessary requirement for these adsorption-desorption rate enhancement reactions. Cleavage of the prothrombin molecule with thrombin which yields the thrombinogenic intermediate 1 and the nonthrombinogenic fragment 1 allowed a more detailed examination of the calcium binding sites. Inasmuch as intermediate 1, in contrast to fragment 1, exhibited no uptake of calcium under any of our experimental conditions, attention was centered specifically on the behavior of fragment 1. The latter peptide, mol wt 25,000, was found to have approximately 12 Ca^{2+} binding sites, with an affinity constant essentially the same as noted for prothrombin. Also the apparent dissimilarity of binding sites noted with prothrombin is retained in fragment 1. The lower part of the fragment 1 binding curve idicated probably three to four sites with a high affinity for Ca^{2+}. Furthermore, the Ca^{2+} binding of fragment 1 and prothrombin was not pH dependent. Repeated experiments with pH values ranging from 5 to 9 and incorporating varying concentrations of Ca^{2+} failed to show any influence of pH on Ca^{2+} uptake. These data are in direct contrast to those published by Nelsestuen and Suttie (1972). These authors reported maximal binding between pH 8 and 9. Their data indicated that this pH maximum declined sharply on both sides and no binding occurred at pH 7.5 or below, or at pH 9.5 or higher. In a recent note, however, Nelsestuen and Buttie (1974) revised their earlier conclusion and now agree that there is no pH effect on calcium ion binding prothrombin.

The observation that intermediate 1 did not bind Ca^{2+} prompted a study to determine whether the "intermediate 1 portion" of the native prothrombin molecule retained the ability to bind Ca^{2+} prior to cleavage by thrombin. It was conceivable that, during generation and subsequent purification, the conformation of the intermediate 1 was altered such that it could not bind Ca^{2+} in the isolated state. Gitel et al. (1973) reported that fragment 1 would bind phospholipid in the presence of calcium ions while intermedate 1 would not. In contrast, Bajaj et al. (1974) reported that the intermediate 1 did contain calcium ion binding sites. Purified prothrombin was incubated with a mixed phospholipid dispersion, PS/PC, and Ca^{2+}. Upon gel filtration of this mixture, a single peak, containing PS/PC–Ca^{2+}–prothrom-

bin, was recovered in the void volume, and indicated a macromolecular complex. This latter complex was then treated with thrombin. Presumably, this would allow formation of fragment 1 and intermediate 1 in a more nearly "in vivo" environment. Barton and Hanahan (1969) and Hemker and Kahn (1967) have proposed that adsorption of the blood factors to phospholipid aggregate surfaces is probably an obligate occurrence in the "prothrombinase" reaction converting prothrombin to thrombin. Analysis of the fractions obtained after gel filtration of the thrombin-treated PS/PC-Ca^{2+}-prothrombin complex indicated that the fragment 1 was indeed bound to the phospholipid aggregates in the void volume, whereas the intermediate 1 eluted much later at a volume corresponding to a mol wt of 55,000. This result strongly suggested that intermediate 1 probably never possessed Ca^{2+} binding sites, while fragment 1 indeed did possess all of the Ca^{2+} binding sites originally present in the native factor II molecule. Further indications are that the fragment 1 is the portion of the prothrombin molecule which anchors it to the phospholipid surface and remains bound after conversion has taken place. These data further corroborate the "asymmetric" structure of prothrombin.

Further exploration of the nature of the metal ion binding sites on prothrombin was pursued through derivatization of the carboxyl groups with glycine methyl esters in the presence of a water-soluble carbodiimide. The derivatized prothrombin failed to migrate in a disc gel electrophoresis system, indicating severe alterations in the charge of the polypeptide compared with the native molecule. This phenomenon is indicative of the loss of most of the negative charges due to the carboxyl moieties on the aspartic and glutamic acid residues. This derivatized protein did not bind calcium ions under any conditions employed, and was completely refractory in the bioassay for prothrombin. An intriguing explanation for this result is that since the prothrombin can no longer bind Ca^{2+}, it can no longer be converted to thrombin by factor Xa in the assay. This would lend support to the concept that calcium ion binding capability is an absolute requirement for subsequent conversion. These results also could simply indicate that the Ca^{2+} binding is a function of the carboxyl groups on the protein which are masked after derivatization. Consequently, the conformation of the prothrombin could have been so severely disrupted during the derivatization reaction that it is no longer recognizable as a substrate for factor Xa in the assay.

Though rather remote as a possibility, the role of the terminal sialic acids of prothrombin as Ca^{2+} binding sites was explored. Subsequent to removal of approximately 90% of the sialic acid residues with neuraminidase, the prothrombin was resolved easily from the neuraminidase by application of DEAE-cellulose chromatography. The asialo prothrombin bound amounts of Ca^{2+} identical with that of the native molecule. Also, the biological (and specific) activity of the asialo prothrombin was unaffected and was the same as the normal prothrombin. This latter finding was at variance with other published reports (Tishkoff et al., 1960; Nelsestuen and Suttie, 1972). These investigators reported recovery of 60–85% of the starting activity after removal of the sialic acids. The data presented in the current study indicate that the sialic acids play no demonstratable role either in Ca^{2+} binding or in the activity of the molecule in its conversion to thrombin.

Even though the above experimental approach provided considerable valuable data on the calcium binding characteristics of prothrombin and fragment 1, a more precise definition of the calcium binding region was needed and was achieved through the combined use of tryptic digestion and mixed phospholipid dispersions. Previous results from this laboratory (Barton and Hanahan, 1969) reported the ability of prothrombin to bind charged phospholipid dispersions in the presence of Ca^{2+}. Also, it has been observed by Gitel et al. (1973) that fragment 1 can bind Ca^{2+} and charged phospholipids. Thus, it appeared highly likely that the isolation, by gel filtration or centrifugation, of a small molecular weight CBF, produced by tryptic action on fragment 1, could be achieved through this type of interaction. This approach was supported by the fact that trypsin liberated a large number of peptides from fragment 1 without the necessity of denaturing or reducing it prior to enzymatic attack. It was felt necessary to conduct this proteolysis on fragment 1 without denaturants since Nelsestuen and Suttie (1972) demonstrated that prothrombin would not bind any calcium ions when unfolded in urea.

Essentially, then, the major aim of the isolation of calcium binding peptides (CBF) was proven feasible through the use of two approaches, one of which involved (a) tryptic attack on a fragment 1-Ca^{2+}-phosphatidylcholine/phosphatidylserine (PC/PS) complex and the other of which involved (b) initial tryptic attack on fragment 1 and then subsequent isolation of the CBF through addition of a similar phospholipid dispersion to the digested sample in the presence of Ca^{2+}. In each instance, CBF with exactly the same characteristic was isolated, but the yield was significantly higher in procedure b and this was deemed the procedure of choice. The CBF-Ca^{2+}-PC/PS complex could be dissociated easily with EDTA and the CBF isolated in near quantitative yields. An unexpected bonus of this method allowed separation of the CBF, which contained no carbohydrate, from the carbohydrate containing as well as other peptides. In fact, two glycopeptides, GP-1 and GP-2, could be obtained from the latter mixture of peptides by virtue of their differences in behavior on ion exchange columns.

Binding studies on the CBF showed that it bound between 3 and 4 mol of Ca^{2+} per mol of peptide. It is interesting to note that CBF apparently binds the same number of calcium ions as noted with the higher affinity Ca^{2+} binding sites demonstrated in prothrombin and fragment 1. These data indicated that in all probability this CBF is the peptide responsible for the high affinity calcium ion binding sites on the native prothrombin. Recently, Nelsestuen and Suttie (1974), who were studying the mode of vitamin K action, reported the isolation of a small peptide released from normal prothrombin by tryptic digestion. This small peptide, which had an apparent mol wt of 10,000 on Sephadex G-50 columns, bound 3–4 mol of Ca^{2+} per mol of peptide. This peptide was not found in prothrombin molecules synthesized in the presence of vitamin K antagonists. The amino acid composition of this vitamin K dependent peptide indicated that its molecular weight based on amino acid composition is probably much lower. The amino acid composition of CBF in our study is very similar to that of their vitamin K dependent peptide. Of particular interest CBF, GP-1, and GP-2 did show certain distinct differences in amino acid content. Specifically, the CBF contained an unusually high content of glutamic acid residues (as analyzed in an acid hydrolysate), nearly 25% of the total amino acids in this peptide. On the basis of recent evidence presented by Stenflo et al. (1974), it is very probable that some of the "glutamic acid" residues are actually γ-carboxyglutamyl units, which have become decarboxylated under acid condi-

tions. Furthermore, it is likely that these γ-carboxyglutamyl residues are the calcium binding sites. On the other hand, the glycopeptides, GP-1 and GP-2, had a very low content of glutamic acid and were significantly different from each other not only in amino acid composition but in behavior on ion exchange columns. Thus, it is conceivable that these latter two peptides are derived from different segments of the native molecule.

The N-terminal amino acid of CBF was found to be glycine. The exact position of CBF in the prothrombin is unknown but it is proposed that it is located near the N terminus. Fujikawa et al. (1974) published a comparison of the 15 amino acids in the N termini of prothrombin, factor IX, and factor X to illustrate the similarities of this region in all three molecules. Three amino acids interior to the N-terminal alanine in prothrombin is a lysine residue. The fourth amino acid is glycine. It would seem logical that during trypsin digestion of this portion (fragment 1) of the molecule, this Lys-Gly bond would be cleaved readily since it is so near the end of the polypeptide chain. If the CBF were located near the N terminus of prothrombin, then it would be expected that the N-terminal amino acid in CBF would be glycine.

The carbohydrate content of GP-1 and GP-2 indicates that there are only two carbohydrate chains present in fragment 1. Thus, there are only three chains in the whole prothrombin molecule, with two present in fragment 1, and one in intermediate 1 which ultimately is found in thrombin. Magnusson (1970) reported the existence of one carbohydrate chain in thrombin.

All three of the peptides, CBF, GP-1, and GP-2, showed some unusual behavior as regards molecular weight analysis. Perhaps the most unique was the CBF which showed anomalously high molecular weight on Sephadex gel filtration columns. Though GP-1 and GP-2 did show some differences in molecular weight depending on the method used, the values are not as varied as noted with CBF. Interestingly, all the Ca^{2+} binding proteins or peptides investigated here exhibit anomalously high molecular weights on Sephadex gel filtration columns. Prothrombin had an apparent molecular weight of nearly 100,000 by gel filtration while, by sedimentation equilibrium analysis, the value was 68,000. Fragment 1, which also binds calcium ions, yielded a mol wt of 43,000 on gel filtration columns, whereas under denaturing conditions it was 25,000. In contrast, intermediate 1, which does not bind calcium ions, but does contain carbohydrate, has an apparent molecular weight of 55,000 both on gel filtration chromatography and by sodium dodecyl sulfate electrophoresis. The CBF also exhibits anomalies in its behavior in the various molecular weight procedures. It has a sedimentation equilibrium mol wt of 3500, a mol wt of 5100 on guanidine hydrochloride columns, which are supposed to be more accurate than the sodium dodecyl sulfate type, and a value of 8500 on gel filtration. In contrast, the two glycopeptides which contain some 40–45% carbohydrate did not display such a behavior, at least not to the degree as CBF. GP-1 and GP-2 exhibit molecular weights of 7000 and 6300 on gel filtration, respectively, and approximately 5700 each, by estimation on denaturing solvent columns. Their sedimentation equilibrium molecular weights were 4600 and 1950, respectively, which for GP-1 is not too dissimilar from the other estimations. (GP-2 has an anamolous molecular weight, but the error may be involved in the value of $\bar{v}$.) These data indicate that the unusual behavior of prothrombin and fragment 1 on Sephadex gel filtration columns cannot be attributed solely to the presence of carbohydrate chains on the molecule since a peptide, with no carbohydrate and contained in both of these proteins, displays very anomalous behavior. The variance in molecular weights noted in this study may be related to some unrecognized prosthetic group on prothrombin which is manifest in the CBF. Nelsestuen and Suttie (1974) also postulated some type of prosthetic group responsible for the vitamin K dependent calcium ion binding observed in the peptide they isolated from prothrombin. While the chemical character of this prosthetic group, if it exists at all, remains unknown, it appears not to be a carbohydrate.

In summary, this study has provided further insight into the structural characteristics of bovine prothrombin as revealed by examination of certain fragments derived from it by essentially sequential action of thrombin and then trypsin. Specifically it allows the possibility of a detailed investigation of the calcium binding peptide region, especially the various facets of its interaction with calcium and phospholipids. The latter will be of particular value in further defining the significant influence of factor V(Va) on the proteolysis of prothrombin to thrombin by factor Xa in the presence of calcium and phospholipids. One cannot help but be intrigued by the nature of these interactions since factor Xa exerts its action on prothrombin at a site considerably removed from the area where calcium and phospholipid appear to interact. The latter is especially provocative since it is now considered that the most active prothrombin converting principle in an in vitro system is prothrombinase, a large complex composed of factor Xa, factor V(Va), phospholipid, and calcium. Obviously other interpretations can be proposed but this shows the complexity of these particular reactions. Finally, the identification of two glycopeptides provides a focal point for further chemical study on their structure and behavior and of their importance to the activity of the prothrombin molecule.[6]

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[6] Subsequent to submission of this manuscript, Enfield et al. (1975) published the primary structure of most of the light chain of bovine factor X. The amino-terminal sequence of this coagulation factor was homologous to a large degree with the primary structure of the amino terminus of bovine prothrombin (Magnusson et al., 1974). The N-terminus regions of these two proteins appear to contain all the calcium binding sites.

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