Chem. 226, 497.
Johnson, D. A., Osaki, S., and Frieden, E. (1967), *Clin. Chem. 13,* 142.
Johnson, D. A., Osaki, S., and Frieden, E. (1970), *Clin. Chem. 16,* 65.
Lowry, O. H., Rosebrough, J. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Osaki, S. (1966), *J. Biol. Chem. 241,* 5053.
Osaki, S., Johnson, D. A., and Frieden, E. (1966), *J. Biol. Chem. 241,* 2746.
Sung, S. M., and Topham, R. W. (1973), *Biochem. Biophys. Res. Commun. 53,* 824.
Topham, R. W., and Frieden, E. (1970), *J. Biol. Chem. 245,* 6698.
Topham, R. W., and Johnson, D. A. (1974), *Arch. Biochem. Biophys. 160,* 647.
Topham, R. W., Sung, S. M., Morgan, F. G., Prince, W. D., and Jones, S. H. (1975), *Arch. Biochem. Biophys. 167,* 129.
Weber, K., and Kuter, D. J. (1971), *J. Biol. Chem. 246,* 4505.
Wharton, D. C., and Rader, M. (1970), *Anal. Biochem. 33,* 226.
Zak, B. (1957), *Am. J. Clin. Pathol. 27,* 583.

A Comparison of Human Prothrombin, Factor IX (Christmas Factor), Factor X (Stuart Factor), and Protein S†

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ABSTRACT: Human prothrombin, factor IX, and factor X have been isolated in high yield and characterized as to their amino-terminal sequence, molecular weight, amino acid composition, and migration in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. An additional human plasma protein, called protein S, has also been purified and its properties have been compared with those of prothrombin, factor IX, and factor X. Prothrombin (mol wt 72 000), factor IX (mol wt 57 000), and protein S (mol wt 69 000) are single-chain glycoproteins, while factor X (mol wt 59 000) is a glycoprotein composed of two polypeptide chains held together by a disulfide bond(s). The amino-terminal sequence of the light chain of human factor X is homologous with prothrombin, factor IX, and protein S. The heavy chain of human factor X is slightly larger than the heavy chain of bovine factor X and differs from bovine factor X in its amino-terminal sequence.

The formation of fibrin during the coagulation process involves the participation of a number of plasma proteins which require vitamin K for their biosynthesis (Davie and Fujikawa, 1975). These include prothrombin, factor VII, factor IX, and factor X.[1] Vitamin K is required for carboxylation of a number of specific glutamic acid residues located in the amino-terminal end of these proteins (Stenflo et al., 1974; Magnusson et al., 1974; Nelsestuen et al., 1974). This leads to the formation of γ-carboxyglutamic acid (Gla) which participates in the binding of calcium and the interaction of these coagulation factors with phospholipid. Stenflo (1976) has recently purified and characterized a fifth plasma protein from bovine plasma containing γ-carboxyglutamic acid. The biological role of this protein, called protein C, however, is not known (Esmon et al., 1976; Kisiel et al., 1976).

The purification of human factor IX has been reported by Østerud and Flengsrud (1975), Andersson et al. (1975), and Rosenberg et al. (1975a). It is composed of a single polypeptide chain with an amino-terminal tyrosine (Fryklund et al., 1976). Human prothrombin has been well characterized (Shapiro and Waugh, 1966; Lanchantin et al., 1968; Kisiel and Hanahan, 1973; Downing et al., 1975), and major portions of its structure have been determined (Pirkle et al., 1973; Thompson et al., 1974; Walz and Seegers, 1974; Butkowski et al., 1976). Human factor X has been isolated and partially characterized by Aronson et al. (1969) and Rosenberg et al. (1975b). No physical-chemical characterization of the final preparation was made. Properties of the corresponding bovine coagulation factors, however, are well known (Fujikawa et al., 1974a), and the amino acid sequences for bovine prothrombin and bovine factor X have been completed (Magnusson et al., 1975; Enfield et al., 1975; Titani et al., 1975).

In the present communication, we report the isolation and characterization of human factor IX, factor X, and a new plasma protein of unknown function, called protein S.[2] A comparison of the properties of these three proteins with those of human prothrombin and the corresponding proteins from bovine plasma is also presented.

Experimental Section

Materials

Heparin sodium salt (grade I, 150 USP units/mg), soybean trypsin inhibitor (type I-S), imidazole (grade I), morpholinoethanesulfonic acid (Mes),[3] morpholinopropanesulfonic acid

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

[2] The new protein was arbitrarily called protein S in reference to its isolation and characterization in Seattle. When its function is discovered, a more appropriate name can be assigned to this plasma protein.

[3] Abbreviations used are: Mes, morpholinoethanesulfonic acid; Mops, morpholinopropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Dip-F, diisopropyl fluorophosphate; $PhCH_2SO_2F$, phenylmethylsulfonyl fluoride; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

(Mops), *p*-aminobenzamidine hydrochloride, thiobarbituric acid, acetylacetone, galactosamine, galactose, *N*-acetylneuraminic acid, and poly(L-lysine) (type I-B) were obtained from Sigma Chemical Co., St. Louis, Mo. Cyclohexylmorpholinoethylcarbodiimide, diisopropyl fluorophosphate (Dip-F), *O*-methylisourea, cyclohexanone, and benzamidine hydrochloride were purchased from Aldrich Chemical Co., Milwaukee, Wis. All benzamidine stock solutions were filtered before use. Barium chloride and cyanogen bromide were obtained from Baker Chemical Co., Phillipsburg, N.J. DEAE-Sephadex A-50, Sephadex G-150, and Sepharose 4B were products of Pharmacia Fine Chemicals, Piscataway, N.J. 2-Mercaptoethanol, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Kodak Co., Rochester, N.Y. Acrylamide was a product of Bio-Rad Laboratories, Richmond, Calif. Kaolin was obtained from Fisher Scientific Co., Fair Lawn, N.J. Guanidine hydrochloride (ultrapure) was purchased from Schwarz/Mann, Van Nuys, Calif. All guanidine solutions were filtered before use. Calcium chloride was obtained from Matheson Coleman and Bell, Los Angeles, Calif. All stock solutions of calcium chloride were filtered and autoclaved before use. Fresh human cryosupernatant was obtained from the Puget Sound Blood Center. The blood (450 ml) was collected in 63 ml of citrate–phosphate–dextrose anticoagulant mixture. The anticoagulant mixture contained 26.3 g of trisodium citrate dihydrate, 3.27 g of citric acid monohydrate, 2.22 g of sodium dihydrogen phosphate monohydrate, and 25.5 g of glucose in 1 l. All other materials were of the highest quality available.

Methods

Protein concentrations of crude fractions were determined from absorbance at 280 nm assuming an $E_{280}^{1\%}$ of 10. For purified preparations, protein concentration was determined from absorbance at 280 nm employing an $E_{280}^{1\%}$ of 13.2 and 11.6 for factor IX and factor X, respectively. These extinction coefficients were determined in an analytical ultracentrifuge by the method of Babul and Stellwagen (1969) in 0.02 M Mops buffer, pH 7.2. An extinction coefficient of 13.8 was used for human prothrombin (Kisiel and Hanahan, 1973).

Amino acid analyses and preparation of samples were carried out by the methods of Moore and Stein (1963) and Spackman et al. (1958) employing a Durrum Model D500 amino acid analyzer. Samples were hydrolyzed in 6 N HCl at 110 °C for 24, 48, 72, and 96 h in evacuated tubes. The values of threonine and serine were determined by extrapolation to zero hydrolysis time. Isoleucine and valine values were calculated from the 96-h hydrolysis time. Tryptophan and tyrosine were estimated by the method of Bencze and Schmid (1957). Half-cystine was determined as cysteic acid by the method of Hirs (1967).

N-Acetylneuraminic acid was determined by the thiobarbituric acid method of Warren (1959) using *N*-acetylneuraminic acid as a standard. Neutral sugar was determined by the phenol–sulfuric acid method of Dubois et al. (1956) using a 1:1 mixture of mannose and galactose as a standard. For the determination of hexosamine, samples were hydrolyzed in 2 N HCl for 24 h at 110 °C and analyzed for galactosamine and glucosamine employing the amino acid analyzer.

Sodium dodecyl sulfate gel electrophoresis was performed by the method of Weber and Osborn (1969). Samples were run on 8.5% gels for 6 h with a current of 5 mA/tube. Gels were stained for protein with Coomassie brilliant blue R. Molecular weights were estimated by interpolation from a linear semilogarithmic plot of apparent molecular weight vs. migration distance using the following protein standards: phosphorylase *b* (95 000), bovine serum albumin (68 000), ovalbumin (45 000), bovine carbonic anhydrase (29 000), and myoglobin (17 000).

Ultracentrifugation was carried out in a Beckman Model E analytical ultracentrifuge which was equipped with an electronic speed control. Sedimentation equilibrium experiments were performed by the method of Yphantis (1964) employing a six-channel Kel-F centerpiece. Rayleigh patterns were recorded on Kodak II-G photographic plates. The plates were read on a modified Nikon microcomparator, which had been automated as described by De Rosier et al. (1972). With this procedure, the entire fringe envelope is read at 50-μm intervals and the calculated Fourier transform parameter employed to compute the actual fringe displacement using the program developed by De Rosier et al. (1972). The data were then used to calculate the various point-by-point molecular weight averages employing a computer program developed by Teller et al. (1969).

Sedimentation equilibrium measurements of factor IX and factor X were performed at three different concentrations (0.9, 0.7, and 0.5 mg/ml) in 6 M guanidine hydrochloride. Salt-free factor IX and factor X were dissolved in 6 M guanidine hydrochloride containing 0.1 M ammonium acetate, pH 5.6, and dialyzed against the same solvent for 48 h at room temperature prior to ultracentrifugation; the appropriate sample concentrations were obtained by dilution with the diffusate. The determinations were carried out at 20 °C with a rotor speed of 22 000 rpm. Partial specific volumes of $\bar{v}$ = 0.713 and 0.719 ml/mg were calculated for human factor IX and factor X, respectively, by the method of Lee and Timasheff (1974). These values were then corrected for carbohydrate content (Gibbons, 1966).

Automated Edman degradations were performed with a Beckman sequenator Model 890A. The mode of operation of the instrument and the methods of sequenator analysis are adaptations (Hermodson et al., 1972) of the technique of Edman and Begg (1967). For amino-terminal analysis, 7 mg of the heavy chain of *S*-pyridylethyl factor X, 3 mg of the light chain of *S*-pyridylethyl factor X, 3.2 mg of protein S, 6 mg of factor IX, and 7 mg of prothrombin were employed. Determinations were performed twice for each protein. In the quantitation of terminal residues, protein concentration was determined in an amino acid analyzer after hydrolysis of the sample. Norleucine was employed as an internal standard to calculate protein recovery.

Factor IX was routinely assayed by the kaolin partial thromboplastin time according to the method of Proctor and Rapaport (1961) as modified by Fujikawa et al. (1973). One unit of activity was defined as the amount of factor IX or factor X activity present in 1.0 ml of normal human plasma.

Factor X activity was assayed by the method of Bachmann et al. (1958) as modified by Fujikawa et al. (1972). Factor VII was assayed according to the method of Nemerson and Clyne (1974), and prothrombin was assayed in a one-stage assay by the method of Hjort et al. (1955).

Heparin-Sepharose was prepared by a modification of the method of Fujikawa et al. (1973). Fifty milliliters of wet-packed Sepharose 4B was suspended in 50 ml of distilled water. Fifteen grams of CNBr was then added and the temperature was maintained between 18 and 20 °C for 15 min and the pH was held at 11.0 with 6 M NaOH. The activated Sepharose was immediately filtered and washed with 1 l. of cold 0.1 M $NaHCO_3$ adjusted to pH 9.5 with 6 M NaOH and added to

3 g of sodium heparin in 50 ml of 0.1 M $NaHCO_3$, pH 9.5. The reaction was allowed to proceed overnight at 4 °C, and the next day the heparin-Sepharose was washed with 1 l. of 2.0 M NaCl in 0.1 M $NaHCO_3$, pH 10.5, followed by 1 l. of distilled water, and finally in 0.02 M Mes-Tris buffer, pH 5.85, containing 0.02% sodium azide.

Benzamidine-Sepharose was prepared by the following modification of the method of Schmer (1972): ϵ-aminocaproic acid–Sepharose 4B (50 ml of settled volume) was suspended in 50 ml of 0.1 M Mes buffer, pH 5.6, and incubated for 30 min with 100 mg/ml of cyclohexylmorpholinoethylcarbodiimide. Two grams of *p*-aminobenzamidine was then added and the reaction was allowed to proceed with gentle stirring for 1 day at 25 °C and then for 2 additional days at 4 °C. The resin was then filtered and washed with 2.0 M NaCl followed by distilled water and 0.02 M Mes-Tris buffer, pH 5.85, containing 0.02% sodium azide.

Poly(homoarginine)-Sepharose was prepared as follows: 100 ml of wet-packed Sepharose 4B was suspended in 100 ml of water, and 30 g of CNBr was then added to the slurry. The temperature was maintained between 18 and 20 °C with ice chips, and the pH was held at 11.0 with 6.0 M NaOH. After 15 min, the slurry was quickly filtered and washed with 2 l. of 0.1 M $NaHCO_3$, pH 9.5. The activated Sepharose was added to 2 g of poly(L-lysine) (mol wt greater than 85 000) in 100 ml of 0.1 M $NaHCO_3$, pH 9.5. The slurry was gently stirred overnight at 4 °C and then at room temperature for 6 h. The poly(L-lysine)-Sepharose was filtered, washed with 1 l. of 2 M KCl, and then washed with 2 l. of distilled water. Poly(homoarginine)-Sepharose was prepared from poly(L-lysine)-Sepharose by a guanidylation reaction as reported by Kimmel (1967). In this method, 100 ml of wet-packed poly(L-lysine)-Sepharose was added to 100 ml of 0.8 M NaOH containing 8.8 g of *O*-methylisourea, pH 10.5. The reaction was allowed to proceed for 4 days with gentle stirring at 4 °C. The poly(homoarginine)-Sepharose was filtered and washed with 2 l. of 2 M NaCl and then with 2 l. of distilled water. Finally, the poly(homoarginine)-Sepharose was washed and equilibrated in 0.02 M Mes-Tris buffer, pH 6.0, containing 0.02% sodium azide.

Purification of Human Factor IX, Factor X, and Protein S. In this procedure, all operations were performed at 4 °C and contact with glass was avoided by employing siliconized glassware and plastic containers. Heparin (5000 units), 250 mg of polybrene, and *O*-phenanthroline (1.5 mmol final concentration) were added to 5 l. of human citrated cryosupernatant and the mixture was stirred for 15 min at 4 °C. Two hundred milliliters of 1 M $BaCl_2$ was then added and the mixture was stirred at 4 °C for an additional 30 min. The barium citrate pellet was washed twice with 1 l. of 0.02 M imidazole buffer, pH 6.0, containing 0.2 M NaCl and 1 mM benzamidine. The adsorbed proteins were eluted from the barium citrate pellet by homogenizing the pellet in a siliconized glass homogenizer with 1 l. of elution buffer containing 0.2 M Mes-HCl buffer, pH 5.85, 0.15 M sodium citrate, 10 mM benzamidine, 1000 units of heparin, and 10 mg of soybean trypsin inhibitor. The mixture was subsequently stirred for 1 h at 4 °C and the precipitate was removed by centrifugation at 5000*g* for 30 min.

The supernatant was brought to 10% saturation with ammonium sulfate by the slow addition of 76 g of solid enzyme-grade ammonium sulfate. Prior to addition, 1.5 ml of 1.0 M Tris base was mixed with the ammonium sulfate. The precipitate was removed by centrifugation at 5000*g* for 20 min and discarded. The supernatant was raised to 40% saturation with ammonium sulfate by the slow addition of an additional 186 g of ammonium sulfate to which had been added 9 ml of 1 M Tris. The precipitate was removed by centrifugation at 5000*g* for 20 min and discarded. The supernatant was then adjusted to 70% saturation by the slow addition of 229 g of ammonium sulfate to which had been added 10 ml of 1 M Tris. The precipitate was collected by centrifugation at 5000*g* for 30 min and dissolved in 50 ml of 2×10^{-2} M Tris-H_3PO_4 buffer, pH 5.85, containing 0.1 M EDTA, 2 mM benzamidine, and 0.02% sodium azide. The protein solution was then dialyzed against 1 l. of this buffer and then twice against 1 l. of buffer A which contained 2×10^{-2} M Tris-H_3PO_4 buffer, pH 5.85, 2 mM benzamidine, and 0.02% sodium azide. A fine precipitate that appeared upon dialysis was removed by centrifugation at 9000*g* for 10 min.

The protein solution was applied to a DEAE-Sephadex A-50 column (3 × 25 cm) which had previously been equilibrated in buffer A, and the column was then washed with 250 ml of 0.15 M NaCl in buffer A. Elution of the proteins was achieved by a linear gradient formed from 250 ml of buffer A containing 0.15 M NaCl and 250 ml of buffer A containing 0.55 M NaCl. The flow rate was 1.4 ml/min, and 3.5-ml fractions were collected. The peak containing the factor IX and factor X was pooled and dialyzed against buffer B which contained 0.02 M Mes-Tris buffer, pH 5.9, 2 mM benzamidine and 0.02% sodium azide.

After dialysis, the solution was applied to a benzamidine-Sepharose column (2 × 20 cm) which had been previously equilibrated with buffer B, and the column was washed with 80 ml of buffer B containing 0.3 M NaCl. Elution of the protein was achieved by a linear gradient formed by 250 ml of buffer B containing 0.3 M NaCl and 250 ml of buffer B containing 1.3 M NaCl. The flow rate was 0.4 ml/min, and 3.5-ml fractions were collected. This column separated factor IX from factor X.

The fractions from the benzamidine-Sepharose column which contained factor X were immediately pooled and brought to 1×10^{-3} M Dip-F and applied to a poly(homoarginine)-Sepharose column (1.5 × 27 cm). This column was previously equilibrated with buffer C which contained 0.02 M Mes-Tris buffer, pH 5.9, and 0.02% Na azide. After application of the sample, the column was washed with 250 ml of buffer C containing 3.0 M NaCl, 10^{-3} M Dip-F, and 2×10^{-4} M $PhCH_2SO_2F$. The column was further washed with 250 ml of buffer C containing 2.9 M guanidine hydrochloride, 2.4 M NaCl, 10^{-3} M Dip-F, and 10^{-3} M $PhCH_2SO_2F$. The flow rate was 0.2 ml/min, and 2.5-ml fractions were collected. Protein S, factor X, and prothrombin were readily separated from each other on this column. The peaks containing protein S and factor X were pooled separately and brought to 10 mM in benzamidine. They were then dialyzed once against 500 ml of 0.02 M Mes-Tris buffer, pH 5.9, containing 2.4 M NaCl, 0.02% sodium azide, and 10 mM benzamidine. Each sample was then dialyzed two more times against 1 l. of buffer B, frozen, and stored at −70 °C. The poly(homoarginine)-Sepharose column was regenerated by washing with 250 ml of buffer C containing 3.5 M guanidine hydrochloride and 2.3 M NaCl followed by washing and equilibrating with buffer C.

The fractions from the benzamidine-Sepharose column containing factor IX were pooled, concentrated to approximately 85 ml by ultrafiltration, and dialyzed for 20 h against buffer B. The sample was then dialyzed against 1 l. of 50 mM NaCl in buffer B. The sample was brought to 2.5 mM $CaCl_2$ and 10^{-3} M Dip-F, and then applied to a heparin-Sepharose column (1 × 13 cm). The heparin-agarose column was pre-

TABLE I: Purification of Human Factor IX and Factor X.

Purification Step	Vol (ml)	Total Protein[a] (mg)	Total Act. (units)[b] IX	Total Act. (units)[b] X	Sp Act. (units/mg) IX	Sp Act. (units/mg) X	Overall Recovery (%) IX	Overall Recovery (%) X	Purification (fold) IX	Purification (fold) X
Cryosupernatant	5000	360×10^3	5000	5000	0.014	0.014	100	100		
Ba citrate eluate	1000	1.1×10^3	4750	4750	4.32	4.32	95	95	310	310
Ammonium sulfate (40–70%)	70	460	3200	2950	7.0	6.4	64	59	500	460
DEAE-Sephadex column	110	270	2850	2400	10.6	8.9	57	48	750	630
Benzamidine-Sepharose column (IX)	85	76	2350	150	30.9		47		2210	
(X)	60	177	50	1950		11		39		790
Poly(homoarginine)-Sepharose column (X)	14	14.3[c]	0	1750	0	125	0	35	0	8930
Heparin-Sepharose (IX)	6	6.2[d]	2100	0	325	0	42	0	25100	0

[a] Protein concentration was determined by absorbance at 280 nm assuming $E_{280}^{1\%} = 10$. [b] One unit is defined as the amount of factor IX or factor X activity in 1 ml of normal human plasma. [c] Protein concentration was determined employing an extinction coefficient of $E_{280}^{1\%} = 13.3$. [d] Protein concentration was determined employing an extinction coefficient of $E_{280}^{1\%} = 11.6$.

viously layered with Sepharose 4B beads to a thickness of 3 cm and equilibrated with buffer B containing 50 mM NaCl and 2.5 mM $CaCl_2$. After application of the sample, the column was washed with 40 ml of buffer B containing 50 mM NaCl and 2.5 mM $CaCl_2$. The protein was eluted with a linear gradient consisting of 100 ml of buffer B containing 50 mM NaCl, 2.5 mM $CaCl_2$, and 10^{-3} M Dip-F, and 100 ml of buffer B containing 1.50 M NaCl, 2.5 mM $CaCl_2$, and 10^{-3} M Dip-F. Fractions (2.5 ml) were collected at a flow rate of 0.3 ml/min.

After the peak of the prothrombin was eluted from the column, the gradient was interrupted and the column was washed with 100 ml of buffer B containing 0.3 M NaCl, 0.05 M NaCl, 10^{-3} M Dip-F, and 2.5 mM $CaCl_2$. The gradient was then reattached and continued, and factor IX was subsequently eluted. Factor IX was made 5.0 mM in EDTA and concentrated to approximately 6 ml by ultrafiltration using an Amicon Diaflo concentrator with a PM10 filter and a pressure of 30 lb/in.^{2}. The sample was dialyzed for 5 h against buffer B containing 0.05 M EDTA and an additional 5 h against buffer B containing 0.1 M NaCl. Factor IX was frozen and stored at −70 °C. The heparin-Sepharose column was regenerated by washing with 200 ml of 2.0 M NaCl in 0.02 M $NaHCO_3$, pH 10.5, followed by washing and equilibration with buffer B containing 0.05 M NaCl and 2.5 mM $CaCl_2$.

Preparation of the Heavy and Light Chains of Human Factor X. Factor X (11 mg) was reduced and pyridylethylated according to the procedure of Friedman et al. (1970), and the heavy and light chains were separated by gel filtration on a Sephadex G-150 column (1.5 × 84 cm) in the presence of 10% formic acid. The elution profile was essentially identical with that obtained by Fujikawa et al. (1972) for bovine factor X.

Results

Isolation of Human Factor IX, Factor X, and Protein S. A typical purification of factor IX and factor X is shown in Table I starting with 5 l. of human cryosupernatant. Factor IX, factor X, prothrombin, and protein S were purified from human plasma by steps that included barium-citrate adsorption and elution, ammonium sulfate fractionation, DEAE-Sephadex column chromatography, and benzamidine-Sepharose column chromatography. Factor IX was further purified by heparin-Sepharose column chromatography, and factor X was further purified by poly(homoarginine)-Sepharose column chromatography. The overall purification of factor IX was 25 000-fold with a recovery of about 40%. Factor X was purified 9000-fold with a recovery of about 35%.

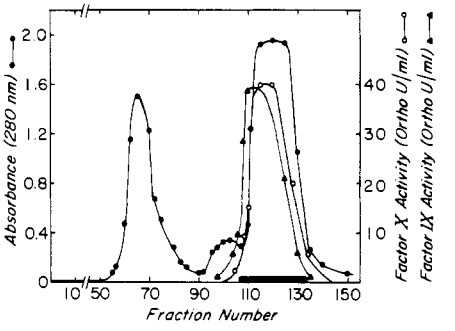

FIGURE 1: Elution pattern of human factor IX and factor X from DEAE-Sephadex. Protein was eluted from the column (3 × 25 cm) with a linear gradient of 250 ml of 0.02 M Tris-phosphate buffer, pH 5.9, containing 0.15 M NaCl and 250 ml of Tris-phosphate buffer, pH 5.9, containing 0.55 M NaCl. Fractions (3.5 ml) were collected at a flow rate of 1.4 ml/min. Factor IX, factor X, prothrombin, and factor VII were determined as described under Methods. Fractions that were pooled are indicated by the bar. (●—●) Absorbance at 280 nm; (▲—▲) factor IX activity; (○—○) factor X activity.

In this purification procedure, factor VII was separated from prothrombin, factor IX, factor X, and protein S on DEAE-Sephadex (Figure 1). On this column, factor VII appeared mainly in fractions 72 through 92. Subsequently, factor IX was separated from factor X, prothrombin, and protein S on benzamidine-Sepharose (Figure 2). The ascending portion of the protein peak contained factor X, prothrombin, and protein S, while the descending portion contained factor IX and prothrombin. Prothrombin, factor X, and protein S were then separated from each other on a poly(homoarginine)-Sepharose column (Figure 3). Protein S appeared as a small peak eluting just prior to factor X. The yield of this protein was 0.5 mg from 5 l. of plasma. Factor X was present in the second peak, while the third peak contained essentially pure prothrombin. Factor IX was separated from prothrombin by heparin-Sepharose column chromatography (Figure 4). The first peak contained essentially pure prothrombin, while the second peak contained factor IX.

Factor IX isolated by this procedure was free of detectable levels of factor X, prothrombin, and factor VII. Similarly, factor X was free of prothrombin, factor IX, and factor VII, and prothrombin was free of factor X, factor IX, and factor

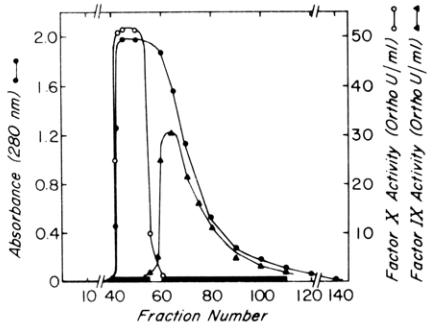

FIGURE 2: Elution pattern of human factor IX and factor X from benzamidine-Sepharose. Protein was eluted from the column (2 × 20 cm) with a linear gradient of 250 ml of buffer B containing 0.3 M NaCl and 250 ml of buffer B containing 1.3 M NaCl as described in Methods. Fractions (3.5 ml) were collected at a flow rate of 0.4 ml/min. Factor IX and factor X were determined as described under Methods. Fractions that were pooled are indicated by the bar. (●—●) Absorbance at 280 nm; (▲—▲) factor IX activity; (○—○) factor X activity.

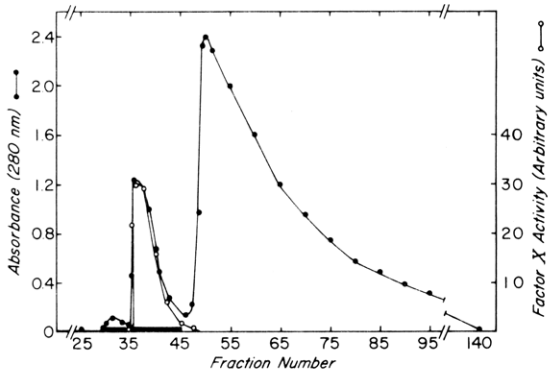

FIGURE 3: Elution pattern of human protein S, factor X, and prothrombin from poly(homoarginine)-Sepharose. Protein was eluted from the column (1.5 × 27 cm) with 0.02 M Mes-Tris buffer, pH 5.9, containing 2.9 M guanidine hydrochloride, 2.4 M NaCl, 0.02% Na azide, 10^{-3} M Dip-F, and 10^{-3} M $PhCH_2SO_2F$. Fractions (2.5 ml) were collected at a flow rate of 0.2 ml/min. Factor X and prothrombin were determined as described under Methods. Fractions that were pooled for protein S and factor X are indicated by the bars. (●—●) Absorbance at 280 nm; (○—○) factor X activity.

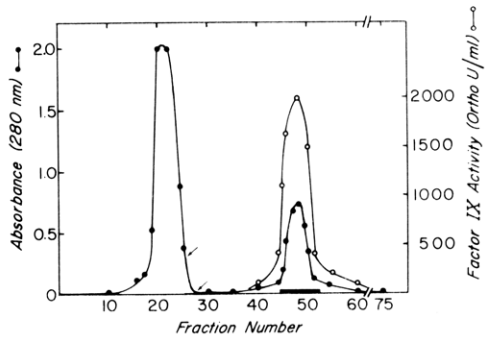

FIGURE 4: Elution pattern of human factor IX from heparin-Sepharose. Protein was eluted from the column (1 × 13 cm) with a linear gradient formed by 100 ml of 0.02 M Mes-Tris buffer, pH 5.9, containing 0.05 M NaCl, 2×10^{-3} M benzamidine, 10^{-3} M Dip-F, and 2.5×10^{-3} M $CaCl_2$, and 100 ml of 0.02 M Mes-Tris buffer, pH 5.9, containing 1.45 M NaCl, 2×10^{-3} M benzamidine, 10^{-3} M Dip-F, and 2.5×10^{-3} M $CaCl_2$. Fractions (2.5 ml) were collected at a flow rate of 0.3 ml/min. After the bulk of the prothrombin was eluted, the gradient was interrupted (shown by the first arrow) and the column was washed with 100 ml of 0.02 M Mes-Tris buffer, pH 5.9, containing 0.35 M NaCl, 2×10^{-3} M benzamidine, 10^{-3} M Dip-F, and 2.5×10^{-3} M $CaCl_2$. The gradient was then reengaged (shown by the second arrow) and factor IX was eluted. Fractions that were pooled are indicated by the bar. Factor IX was determined as described under Methods. (●—●) Absorbance at 280 nm; (○—○) factor IX activity.

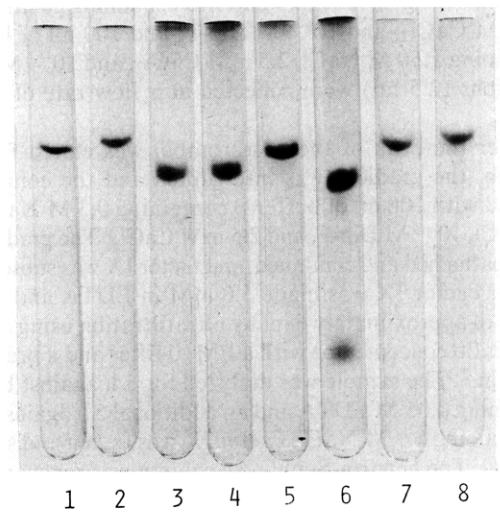

FIGURE 5: Sodium dodecyl sulfate gel electrophoresis of human prothrombin, factor IX, factor X, and protein S. Gel electrophoresis was carried out in 8.5% polyacrylamide gels for 6 h, and the gels were stained with 2% Coomassie brilliant blue R as described in Methods. Gel 1 is 8 μg of unreduced prothrombin; gel 2 is 8 μg of reduced prothrombin; gel 3 is 10 μg of unreduced factor IX; gel 4 is 10 μg of reduced factor IX; gel 5 is 7 μg of unreduced factor X; gel 6 is 12 μg of reduced factor X; gel 7 is 8 μg of unreduced protein S; gel 8 is 8 μg of reduced protein S. The anode was at the bottom of the gel.

VII. Protein S contained no coagulant activity when measured for prothrombin, factor VII, factor IX, or factor X.

Inhibitors of proteolytic enzymes and blood coagulation were routinely added during the isolation of factor IX, factor X, prothrombin, and protein S to restrict degradation of these proteins during the isolation procedure. This resulted in some variation, however, in the assay of factor IX during the early stages of purification.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Single, sharp protein bands were obtained for human prothrombin, factor IX, factor X, and protein S when these proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 5). Prothrombin is shown in samples 1 and 2 before and after reduction with 2-mercaptoethanol. It has an apparent molecular weight of 70 000 by this technique. Factor IX is shown in samples 3 and 4 before and after reduction. It migrates faster than prothrombin with an apparent molecular weight of 58 000. Factor X is composed of two chains held together by a disulfide bond(s). The intact molecule migrates with an apparent molecular weight of 67 000 (sample 5). Upon reduction, a heavy chain (mol wt 49 000) and a light chain (mol wt 17 000) are observed (sample 6). Protein S migrates as a single protein band before and after reduction with an apparent molecular weight of 69 000 (samples 7 and 8).

The migration of human prothrombin, factor IX, and factor X is very similar to the corresponding bovine preparation (Kisiel and Davie, 1975). The heavy chain of human factor X (mol wt 49 000) migrated slightly slower, however, than the heavy chain of bovine factor X (mol wt 46 000).

Sedimentation Equilibrium Studies. Sedimentation equilibrium studies on human factor IX and factor X in the presence of 6 M guanidine hydrochloride indicated that these two proteins were heterogeneous at all concentrations tested (Table II). The observed heterogeneity of factor IX and factor X was

TABLE II: Molecular Weights of Human Factor IX and Factor X by Sedimentation Equilibrium.

	M_1[a]	M_n	M_w	M_z
Factor IX	57 100 ± 620	59 800 ± 680	62 500 ± 1900	69 300 ± 3000
Factor X	58 900 ± 900	63 300 ± 1750	67 700 ± 3300	74 500 ± 3200

[a] M_1 refers to the smallest molecular weight species calculated by the methods of Teller et al. (1969). M_n, M_w, and M_z refer to the number average molecular weight, the weight average molecular weight, and the z-average molecular weight, respectively, as defined by Kraemer (1940).

TABLE III: Amino Acid and Carbohydrate Compositions of Human and Bovine Factor IX, Factor X, and Prothrombin, and Human Protein S.[a]

Components	Human Factor IX (57 100)[i]	Bovine Factor IX[b] (55 400)[i]	Human Factor X (58 900)[i]	Bovine Factor X[c] (55 070)[i]	Human Prothrombin[d] (72 000)[i]	Bovine Prothrombin[e] (71 552)[i]	Human Protein S (69 000)[i]
Amino acid							
Lys	26.6	27.6	31.1	23	29.2	31	46.7
His	8.8	8.1	8.9	12	10.0	9	12.1
Arg	17.3	17.0	22.1	25	38.5	45	23.0
Asp[f]	45.9	36.3	45.8	27	61.9	35	74.4
Thr	29.4	20.1	35.3	31	35.0	28	32.2
Ser	26.6	29.0	26.7	31	36.1	36	47.5
Glu[g]	47.0	46.6	61.5	45	73.7	44	68.0
Pro	14.9	13.1	19.6	18	31.8	35	29.0
Gly	34.1	29.7	38.8	41	48.5	48	42.7
Ala	22.3	18.8	26.2	31	38.1	35	42.8
½-Cystine	19.6	17.2	20.0	24	17.7	24	23.1
Val	33.0	24.9	21.1	26	33.1	35	39.0
Met	2.4	2.6	6.3	5	7.1	6	6.0
Ile	22.3	19.3	15.6	12	21.0	20	35.1
Leu	25.4	18.7	29.4	30	41.6	45	56.0
Tyr	14.0	16.9	12.8	10	19.0	19	13.2
Phe	21.5	15.2	20.9	21	24.8	20	20.9
Trp	11.7	11.4	6.5	7	12.7	14	10.3
Asn				14		25	
Gln				14		18	
Total residues[h]				447		582	
Protein mol wt	47 540	42 391	50 355	49 801	65 762	64 397	68 685
Carbohydrate	17.1%	25.5%	15%	9.6%	8.2%	10%	
Hexose	16.2 (5.1%)	32.6 (10.6%)	15.0 (4.6%)	10 (3.2%)	11.2 (2.8%)	15 (3.9%)	
N-Acetylhexosamine	18.1 (6.6%)	16.4 (6.2%)	12.5 (4.1%)	8 (3.0%)	10.7 (3.1%)	7 (2.1%)	Present
N-Acetylneuraminic acid	10.0 (5.4%)	15.6 (8.7%)	12.0 (6.3%)	6 (3.4%)	5.3 (2.3%)	9 (4.0%)	

[a] Expressed in residues per molecule of glycoprotein. [b] From the data of Fujikawa et al. (1973, 1974b). [c] From the data of Fujikawa et al. (1975). [d] Modified from the data of Kisiel and Hanahan (1973). [e] Calculated from the data of Magnusson et al. (1975). [f] The aspartic acid includes asparagine except for bovine factor X and bovine prothrombin; the Asx in bovine prothrombin reported by Magnusson et al. (1975) is included with aspartic acid. [g] The glutamic acid residues include glutamine except for bovine factor X and bovine prothrombin; the 44 glutamic acid residues shown for bovine prothrombin include 10 residues of γ-carboxyglutamic acid; the glutamic acid shown for the remaining proteins probably includes about 10 residues of γ-carboxyglutamic acid, but this has not been determined. [h] Calculated from amino acid sequence data. [i] Molecular weight in parentheses.

attributed, however, to reversible monomer aggregation rather than a contaminating protein species. The minimum molecular weight of human factor IX was calculated to be 57 100 ± 620, and the minimum molecular weight of human factor X was calculated to be 58 900 ± 900 employing the method of Teller et al. (1969). Protein S was not included in these studies because of the small amounts available.

Amino Acid and Carbohydrate Compositions. The amino acid and carbohydrate compositions for human factor IX and factor X are shown in Table III. This table also includes the amino acid composition for human protein S, as well as the corresponding data for prothrombin taken from the literature.

Human factor IX and factor X are glycoproteins containing 17 and 15% carbohydrate, respectively. Protein S is also a glycoprotein since it contains hexosamine, as shown in the amino acid analyzer. A complete carbohydrate analysis of this protein was not carried out, however, due to the small amounts available. Accordingly, the amino acid analysis of protein S was calculated on the basis of a molecular weight of ~68 000 for the carbohydrate-free protein. This value was employed in these calculations since the migration of protein S on sodium dodecyl sulfate-polyacrylamide gels was nearly identical with human prothrombin which also has a molecular weight of about 65 800 for the carbohydrate-free protein. Thus, the amino acid composition for protein S will require some minor readjustment when the carbohydrate and molecular weight studies are completed.

It is clear from these data that human factor IX, factor X, protein S, and prothrombin have different amino acid com-

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Prothrombin	Human	Ala	Asn	Thr	-	Phe	Leu	Gla	Gla	-	Val	Arg	Lys	Gly	Asn	Leu	
Prothrombin	Bovine	Ala	Asn	Lys	Gly	Phe	Leu	Gla	Gla	-	Val	Arg	Lys	Gly	Asn	Leu	
Factor IX	Human	Tyr	Asn	Ser	Gly	Lys	Leu	(Gla)	(Gla)	Phe	Val	Gln	-	Gly	Asn	Leu	
Factor IX	Bovine	Tyr	Asn	Ser	Gly	Lys	Leu	Gla	Gla	Phe	Val	Arg	-	Gly	Asn	Leu	
Factor X*	Human	Ala	Asn	Ser	-	Phe	Leu	(Gla)	(Gla)	-	Met	Lys	?	Gly	?	Leu	
Factor X*	Bovine	Ala	Asn	Ser	-	Phe	Leu	Gla	Gla	-	Val	Lys	Gln	Gly	Asn	Leu	
Factor VII	Bovine	Ala	Asn	-	Gly	Phe	Leu	(Gla)	(Gla)	Leu	Leu	-	Pro	Gly	Ser	Leu	
Protein C*	Bovine	Ala	Asn	Ser	-	Phe	Leu	Gla	Gla	-	Leu	Arg	Pro	Gly	Asn	Leu	
Protein S	Human	Ala	Asn	Ser	?	-	Leu	(Gla)	(Gla)								

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Factor X†	Human	Ser	Val	Ala	Gln	Ala	Thr	Ser	?	Ser	Gly	Glu	Ala	Pro	Asp	Thr	Ile
Factor X†	Bovine	Trp	Ala	Ile	His	Thr	Ser	Glu	Asp	Ala	Leu	Asp	Ala	Ser	Glu	Leu	Glu
Protein C†	Bovine	Asp	Thr	Asn	Gln	Val	Asp	Gln	Lys	Asp	Gln	Leu	Asp	Phe	Arg	Ile	Val

FIGURE 6: Amino-terminal sequences of prothrombin, factor IX, factor X, factor VII, protein C, and protein S. Amino acids that are identical are shown in blocks. Dashes refer to spaces that have been inserted to bring the six proteins into alignment for better homology. Gla, ?, *, and † refer to γ-carboxyglutamic acid, amino acids that are not known, light chain, and heavy chain, respectively. Taken in part from Fujikawa et al. (1974a), Titani et al. (1975), Kisiel and Davie (1975), Kisiel et al. (1976), and Stenflo (1976).

positions. Human factor IX, factor X, and prothrombin also show some differences with the corresponding proteins from bovine plasma.

Amino-Terminal Sequences. The amino-terminal sequences of the *S*-pyridylethyl derivatives of human prothrombin, factor IX, factor X (heavy and light chains), and protein S are shown in Figure 6. Similar sequences of bovine prothrombin, factor VII, factor IX, factor X, and protein C are also included for comparison. In these experiments, the repetitive yield for each of the degradation steps was about 95%. The yield of the phenylthiohydantoins was 60% or more based on the weight of each of the proteins analyzed. Amino-terminal analysis of human factor X was carried out on the heavy and light chains after reduction, pyridylethylation, and separation by gel filtration as described in Methods.

Five of the proteins including human prothrombin, protein S, and the light chain of factor X contain an amino-terminal alanine. Factor IX, however, contains an amino-terminal tyrosine. No amino acids were detected in turns 7 and 8 for human prothrombin, factor IX, protein S, and the light chain of factor X following the numbering system shown in Figure 6. It is highly probable, however, that these positions contain γ-carboxyglutamic acid. Accordingly, these residues, which are tentative, are shown in parentheses until further identification of γ-carboxyglutamic acid is made.

It is clear that the six different proteins from human and bovine plasma are homologous in the amino-terminal region of their molecule. Some minor differences in the sequences of these proteins, however, are evident. For instance, prothrombin from human and bovine plasma differs in position 3 where a lysine residue in bovine prothrombin has been replaced by threonine in human prothrombin. Also, position 4 contains a glycine in the bovine molecule, while in human prothrombin it is necessary to omit a residue in position 4 in order to bring this protein into alignment for greater homology with the other vitamin-K-dependent proteins. Human factor IX is identical with the bovine protein in the first 14 residues except for position 11. In this position, an arginine residue in the bovine molecule has been replaced by a glutamine residue in human factor IX.

The light chain of human factor X differs from the bovine protein in residue 10 where a valine has been replaced by a methionine residue in human factor X. An amino acid was present in position 12 and position 14 of the light chain of human factor X, but these residues were not identified.

The amino-terminal sequence of the heavy chain of human factor X was completely different from bovine factor X (bottom of Figure 6). Furthermore, this sequence is not homologous with other portions of the heavy chain of the bovine molecule. Thus, it appears probable that there are a number of additional residues on the amino-terminal end of the human protein. This is consistent with the fact that the heavy chain of human factor X is larger than the corresponding bovine preparation as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The heavy chain of human factor X also shows no homology with the heavy chain of protein C.

Protein S is a single-chain protein with an amino-terminal alanine. The amino acid present in position 4 of this protein was not identified. It was not, however, occupied by glycine or phenylalanine.

Discussion

The present work describes a procedure which is suitable for the isolation of prothrombin, factor IX, and factor X from human plasma. The yield and purity of each of these three coagulation factors are high, and sufficient quantities can be readily prepared for physical-chemical characterization. The principal step in the separation of prothrombin, factor X, and protein S was column chromatography on poly(homoarginine)-Sepharose in the presence of 2.9 M guanidine hydrochloride. The specific activity of the human factor X was essentially identical with that of bovine factor X previously prepared in our laboratory (Fujikawa et al., 1972). Occasionally, a factor X preparation was obtained with a very minor band which migrated slightly faster than the major band on sodium dodecyl sulfate–polyacrylamide gels. This band, which was less than 10% of the total protein, was probably factor X_β, a molecule which has undergone minor degradation at the carboxyl end of the heavy chain (Fujikawa et al., 1974c). The degradation of human factor X was substantially reduced by rapidly carrying out the various purification steps in the presence of protease inhibitors.

Human factor X, like the bovine preparation, is composed of two polypeptide chains. These chains have an apparent molecular weight of 49 000 and 17 000 as measured by sodium

dodecyl sulfate–polyacrylamide gel electrophoresis. The summation of the two chains is in good agreement with that observed for the intact molecule (67 000) but much higher than the minimal molecular weight of 58 900 determined by sedimentation equilibrium. The value of 58 900 is probably more reliable, however, since the molecular weight of glycoproteins tends to be high when measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis due to the decreased binding of the detergent to the glycoprotein (Segrest and Jackson, 1972). In the present experiments, the heavy and light chains were readily isolated from human factor X following reduction and gel filtration, and their amino-terminal sequences were determined.

In the present studies, no evidence was obtained for the presence of a single-chain factor X from human plasma. Rosenberg et al. (1975b) found only a single protein band with an apparent molecular weight of 54 000 when their human factor X was examined by sodium dodecyl sulfate gel electrophoresis in the presence of reducing agents. This preparation differs from the two-chain molecule described in the present investigation.

The final step in the purification of human factor IX employed heparin-Sepharose column chromatography. This procedure was originally described in our laboratory for the isolation of bovine factor IX and has also been successfully employed by others in the isolation of human factor IX (Andersson et al., 1975; Rosenberg et al., 1975a). Human factor IX was found to be a single-chain molecule with an amino-terminal tyrosine residue confirming the earlier experiments of Andersson et al. (1975). Furthermore, the amino acid and carbohydrate compositions agree reasonably well with those of Andersson et al. (1975) when their data are adjusted to a molecular weight of 57 000. The present results differ, however, from the more recent data of Fryklund et al. (1976) on the amino-terminal sequence of human factor IX. These investigators reported an amino-terminal sequence of Tyr-Asn-Ile-Gly-Lys-Asn-Gla-Gla-Phe-Val-Arg-Gly-Asn-Leu- for their preparation. This differs from the present results in positions 3, 6, and 11. Neither Ile, Asn, nor Arg were detected in positions 3, 6, and 11, respectively, in the present studies which were carried out on two different preparations of human factor IX. The reasons for the difference in the amino-terminal sequences are not known.

The biological function of protein S is not known. It migrates on sodium dodecyl sulfate–polyacrylamide gel electrophoresis at a rate nearly identical with prothrombin (Figure 5). It differs from prothrombin, however, in its elution pattern from poly(homoarginine)-Sepharose (Figure 3), its amino acid composition (Table III), and its amino-terminal sequence (Figure 6). Furthermore, it has no coagulant activity when tested for either prothrombin, factor VII, factor IX, or factor X. It appears highly probably that protein S is a vitamin-K-dependent protein when its homology with the other vitamin-K-dependent proteins is taken into consideration. Furthermore, it is likely that it is a precursor of a serine protease like the other vitamin-K-dependent proteins. Experiments are now in progress to test this possibility. Whether protein S is related to the bovine plasma protein described by Mattock and Esnouf (1973) is not known.

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References

Andersson, L.-O., Borg, H., and Miller-Andersson, M. (1975), *Thromb. Res. 7,* 451.

Aronson, D. L., Mustafa, A. J., and Mushinski, J. F. (1969), *Biochim. Biophys. Acta 188,* 25.

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

Bachmann, F., Duckert, F., and Koller, F. (1958), *Thromb. Diath. Haemorrh. 2,* 24.

Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29,* 1193.

Butkowski, R. J., Elion, J., Downing, M. R., and Mann, K. G. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1765.

Cuatrecasas, P. (1970), *J. Biol. Chem. 245,* 3059.

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

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

Downing, M. R., Butkowski, R. J., Clark, M. M., and Mann, K. G. (1975), *J. Biol. Chem. 250,* 8897.

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

Edman, P., and Begg, G. (1967), *Eur. J. Biochem. 1,* 80.

Enfield, D. L., Ericsson, L. H., Walsh, K. A., Neurath, H., and Titani, K. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 16.

Esmon, C. T., Stenflo, J., Suttie, J. W., and Jackson, C. M. (1976), *J. Biol. Chem. 251,* 3052.

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

Fryklund, L., Borg, H., and Andersson, L. O. (1976), *FEBS Lett. 65,* 187.

Fujikawa, K., Coan, M. H., Enfield, D. L., Titani, K., Ericsson, L. H., and Davie, E. W. (1974a), *Proc. Natl. Acad. Sci. U.S.A. 71,* 427.

Fujikawa, K., Coan, M. H., Legaz, M. E., and Davie, E. W. (1974c), *Biochemistry 13,* 5290.

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

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

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

Fujikawa, K., Titani, K., and Davie, E. W. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3359.

Gibbons, R. A. (1966), in Glycoproteins, Vol. 5, Gottschalk, A., Ed., New York, N.Y., Elsevier, p 61.

Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., and Walsh, K. A. (1972), *Biochemistry 11,* 4493.

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

Hjort, P., Rapaport, S. I., and Owren, P. A. (1955), *J. Lab. Clin. Med. 46,* 89.

Kimmel, J. (1967), *Methods Enzymol. 11,* 584.

Kisiel, W., and Davie, E. W. (1975), *Biochemistry 14,* 4928.

Kisiel, W., Ericsson, L. H., and Davie, E. W. (1976), *Biochemistry 15,* 4893.

Kisiel, W., and Hanahan, D. J. (1973), *Biochim. Biophys. Acta 304,* 103.

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

p 345.
Lanchantin, G. F., Friedmann, J. A., and Hart, D. W. (1968), *J. Biol. Chem. 243,* 476.
Lee, J. C., and Timasheff, S. N. (1974), *Arch. Biochem. Biophys. 165,* 268.
Magnusson, S., Petersen, T. E., Sottrup-Jensen, L., and Claeys, H. (1975), in Proteases and Biological Control, Vol. 2, Reich, E., Rifkin, D. B., and Shaw, E., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory, p 123.
Magnusson, S., Sottrup-Jensen, L., Petersen, T. E., Morris, H. R., and Dell, A. (1974), *FEBS Lett. 44,* 189.
Mattock, P., and Esnouf, M. P. (1973), *Nature (London), New Biol. 242,* 90.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.
Nelsestuen, G. L., Zytokoviez, T. H., and Howard, J. B. (1974), *J. Biol. Chem. 249,* 6347.
Nemerson, Y., and Clyne, L. P. (1974), *J. Lab. Clin. Med. 83,* 301.
Østerud, B., and Flengsrud, R. (1975), *Biochem. J.* 145, 469.
Pirkle, H., McIntosh, M., Theodor, I., and Vernon, S. (1973), *Thromb. Res. 2,* 461.
Proctor, R. R., and Rapaport, S. I. (1961), *Am. J. Clin. Pathol. 36,* 212.
Rosenberg, J. S., Beeler, D. L., and Rosenberg, R. D. (1975b), *J. Biol. Chem. 250,* 1607.
Rosenberg, J. S., McKenna, P. W., and Rosenberg, R. D. (1975a), *J. Biol. Chem. 250,* 8883.
Schmer, G. (1972), *Hoppe-Seyler's Z. Physiol. Chem. 353,* 810.
Segrest, J. P., and Jackson, R. L. (1972), *Methods Enzymol. 28,* 54.
Shapiro, S. S., and Waugh, D. F. (1966), *Thromb. Diath. Haemorrh. 16,* 469.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Stenflo, J. (1976), *J. Biol. Chem. 251,* 355.
Stenflo, J., Fernlund, P., Egan, W., and Roepstorff, P. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2730.
Teller, D. C., Horbett, J. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N.Y. Acad. Sci. 164,* 66.
Thompson, A. R., Ericsson, L. H., and Enfield, D. L. (1974), *Circulation, Suppl. III, 49–50,* 292.
Titani, K., Fujikawa, K., Enfield, D. L., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3082.
Walz, D. A., and Seegers, W. H. (1974), *Biochem. Biophys. Res. Commun. 60,* 717.
Warren, L. (1959), *J. Biol. Chem. 234,* 1971.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Wright, I. (1959), *J. Am. Med. Assoc. 170,* 325.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.

The Complete Amino Acid Sequence of the Major Component Myoglobin from the Arctic Minke Whale, *Balaenoptera acutorostrata*†

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ABSTRACT: The complete primary structure of the major component myoglobin from the Arctic minke whale, *Balaenoptera acutorostrata,* was determined by specific cleavage of the protein to obtain large peptides which are readily degraded by the automatic sequencer. Over 80% of the amino acid sequence was established from the three peptides resulting from the cleavage of the apomyoglobin at the two methionine residues with cyanogen bromide along with the four peptides resulting from the cleavage of the methyl acetimidated apomyoglobin at the three arginine residues with trypsin. The further digestion of the central cyanogen bromide peptide with trypsin and *S. aureus* strain V8 protease enabled the determination of the remainder of the covalent structure. This myoglobin differs from that of the dwarf sperm whale, *Kogia simus,* at 16 positions, and the common dolphin, *Delphinus delphis,* at 14 positions, from that of the common porpoise, *Phocaena phocaena,* and the bottlenosed dolphin, *Tursiops truncatus,* at 13 positions, from that of the Amazon River dolphin, *Inia geoffrensis,* at 10 positions, and from that of California gray whale, *Eschrichtius gibbosus,* at 3 positions. All of the substitutions observed in this sequence fit easily into the three-dimensional structure of the sperm whale myoglobin.

In preceding papers, the complete amino acid sequence of the myoglobin from the Amazon River dolphin (Dwulet et al., 1975), California gray whale (Bogardt et al., 1976), and the Atlantic bottlenosed dolphin (Jones et al., 1976) were reported. These sequences of Cetacean myoglobins were determined by automatic Edman degradation. This paper reports the application of the peptide fragmentation and analytical procedures that were used in these papers in determining the complete amino acid sequence of the major component myoglobin from the Arctic minke whale. Completion of this sequence extends the number of known Cetacean myoglobin sequences to seven, including the above mentioned Amazon River dolphin, California gray whale, and Atlantic bottlenosed dolphin, Black Sea

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