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Characterization of Two Glycoprotein Variants of Bovine Factor X and Demonstration That the Factor X Zymogen Contains Two Polypeptide Chains†

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ABSTRACT: Bovine factor X can be resolved into two distinct forms, factor X_1 and factor X_2, by DEAE-Sephadex A-50 chromatography (Jackson, C. M., and Hanahan, D. J., *Biochemistry 7*, 4506 (1968)). These two species have been demonstrated to differ in carbohydrate composition, factor X_1 containing 8 mol of hexose, 8 mol of sialic acid, and 6–7 mol of hexosamine *vs.* 10 mol of hexose, 9–10 mol of sialic acid, and 7–8 mol of hexosamine for factor X_2. Both factor X_1 and X_2 are shown to be made up of two, unique polypeptide chains. The heavy chain has a molecular weight of 39,000 and contains all the carbohydrate. The light chain has a molecular weight of 15,000 and is speculated to be involved in the binding of factor X in its activated form to lipid surfaces. Evidence is cited which suggests that the oligosaccharide moiety of factor X may be important in the activation process.

Factor X[1] occupies a key position in the sequence of reactions leading to blood clot formation. Activation of the zymogen form of factor X generates the proteolytic enzyme directly responsible for converting prothrombin to thrombin (Barton *et al.*, 1967; Milstone, 1964; Seegers, 1964). This key zymogen activation is achieved by at least two separate routes involving plasma components. The intrinsic pathway of blood clotting *via* factors XII, XI, IX, and VIII results in formation of an activator of factor X which most probably directly involves factor VIII and factor IX in a complex with phospholipid (Macfarlane, *et al.*, 1964; Lundblad and Davie, 1964, 1965; Osterud *et al.*, 1971). The extrinsic path for factor X activation utilizes clotting factor VII (Straub and Duckert, 1961; Nemerson and Spaet, 1964) and is thought to be the route initiated as a consequence of tissue damage. The convergence of these separate reaction sequences at the stage of activation of factor X makes this position in the sequence an attractive point for control of the blood clotting process. Recent observations (Seegers and Marciniak, 1962; Yin and Wessler, 1968; Yin *et al.*, 1971a) have demonstrated the existence of a separate plasma protein inhibitor of activated factor X. This stage of the clotting process also appears to be the locus of action of the anticoagulant heparin (Yin *et al.*, 1971b).

Factor X and its catalytically active form have been isolated and partially characterized in a number of laboratories (Esnouf and Williams, 1962; Jackson *et al.*, 1968; Jackson and Hanahan, 1968; Seegers *et al.*, 1967; Milstone, 1964; Tishkoff *et al.*, 1968). The physical and chemical properties of factor X as isolated in these various laboratories are not generally in agreement. However, there no longer appears to be much disagreement about the basic enzymatic action of the catalytically active form of factor X and the other equivalent clotting species (Seegers, 1964; Jackson and Hanahan, 1968). Factor X's position in enzyme classification as a DFP[2]-inhibitable serine protease also appears now to be generally established (Jackson and Hanahan, 1968; Leveson and Esnouf, 1969; Seegers *et al.*, 1969).

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[1] Factor X is the designation given by the International Committee on the Nomenclature of Blood Clotting Factors to the blood component abnormal in patients first described by Graham and Hougie (1956), Telfer *et al.* (1956), and Bachmann *et al.* (1957). The biological role of activated factor X appears to be identical with that of thrombokinase (Aronson and Menache, 1968; Barton *et al.*, 1967; Milstone, 1964) and autoprothrombin C (Seegers *et al.*, 1969; Seegers, 1964). Use of the name factor X to describe the component under investigation in this laboratory is a consequence of the fact that the bioassay used to define factor X here is directly related to a procedure which is based in the deficiency state known as factor X deficiency.

[2] Abbreviations used are: DFP, diisopropyl phosphofluoridate; Tame, *p*-toluenesulfonyl-L-arginine methyl ester; $GalNH_2$, galactosamine; $GlcNH_2$, glucosamine; Nana, *N*-acetylneuraminic acid; Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl (lauryl) sulfate.

The present investigation was undertaken with the objective of determining the properties of the two forms of the zymogen factor X responsible for their separation on DEAE-Sephadex A-50 which was reported by Jackson and Hanahan (1968). This characterization was considered a necessary prerequisite to the investigation of the process of activation of factor X and the concomitant changes in the molecule which result in the conversion of a protein which does not bind to a lipid surface to one which does bind. During the course of this investigation it was discovered that the factor X molecule is made up of two disulfide-linked polypeptide chains. At essentially the same time a preliminary report from Fujikawa *et al.* (1971) appeared which in general seems to present virtually identical observations.

Materials and Methods

Common inorganic chemicals were of analytical reagent grade and were purchased from major domestic suppliers.

Barium sulfate, suitable for X-ray diagnosis, was the product of Merck and Co., Rahway, N.J., and Mallinckrodt Chemical Co., St. Louis, Mo.

DEAE-cellulose (Selectacel Type 20, Schleicher and Schuell, N. Y.) was washed as described in Jackson *et al.* (1968), except that the cellulose was not dried. The equilibrated cellulose was added to the factor X solution in just sufficient quantity to absorb 95% of the factor X activity as determined by clotting assay.

Sephadex G-100, A-50, G-25, Russell's viper venom, phenylmethanesulfonyl fluoride, dansylamino acid standards, heparin, factor VII–X deficient substrate plasma, DFP,[1] Tame, glycine ethyl ester, sodium lauryl sulfate, bovine plasma albumin, ovalbumin, aldolase, cytochrome *c*, mannose, galactose, *N*-acetylneuraminic acid, ATP, NAD, NADP, β-galactose dehydrogenase, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, hexokinase, phosphomannose isomerase, fucose, glucosamine, and galactosamine were purchased from Sigma Chemical Co., St. Louis, Mo.

Catalase and chymotrypsinogen were obtained from Worthington Biochemical Corp, Freehold, N. J.

Guanidine hydrochloride was purchased from Heico, Inc., Delaware Water Gap, Pa. Benzamidine hydrochloride was the product of Aldrich Chemical Co., Milwaukee, Wis. Acrylamide, *N,N,N',N'*-methylenebisacrylamide, and tetramethyleneethylenediamine were purchased from Eastman Organic Chemicals, Rochester, N. Y. Electrophoresis grade acrylamide was used for preparative disc electrophoresis.

Bovine factor X was prepared according to the procedure described by Jackson *et al.* (1968) with the following modifications. Bovine blood was collected into an anticoagulant solution containing 0.1 M sodium oxalate, 0.01 M benzamidine hydrochloride, and two units of heparin per ml. Blood from 20 to 50 animals was pooled during plasma preparation. Plasma was separated from the red blood cells using a Westphalia LG-205 continuous-flow separator (Centrico, Englewood, N. J.). Adsorption of the "prothrombin complex" clotting factors onto $BaSO_4$ was performed immediately after preparation of the plasma.

Preparative disc electrophoresis was used with two batches of factor X in addition to the procedures described earlier (Jackson *et al.*, 1968). One of these batches was used in determining the amino acid compositions of the two glycoprotein species which are obtained from DEAE-Sephadex A-50 chromatography and are described in this manuscript. The other batch was used in one of the composition studies of the two isolated polypeptide chains which are shown to form the zymogen factor X. In both cases the preparative electrophoresis was carried out using the Canalco Prep disk apparatus. The conditions were those described by Canalco for a steady-state stacking run, but using the formulations for the sample gel and stacking gel ordinarily used with the pH 9.5 preparative separating gel system.

The factor X clotting specific activity of preparations used in this study varied from 60 to 140 units per mg. In all these preparations, the activity after total activation was 58–64 μequiv/min per mg per ml when 0.1 M Tame was used as substrate in the pH-Stat at 37° and pH 7.8. All preparations showed a single band by analytical disc electrophoresis at pH 9.5. All clotting factor activities were assayed by the procedure described previously (Jackson *et al.*, 1968).

Molecular weight determinations were made using the analytical ultracentrifuge and the high-speed, meniscus depletion technique of Yphantis (1964). Both the Rayleigh interference and the Beckman uv scanner were employed in various ultracentrifuge runs. The protein and polypeptide chain partial specific volumes were calculated from the amino acid composition by the procedure of McMeekin *et al.* (1949). Specific volumes for the sugar residues of the glycoprotein were obtained from Bezkorovainy and Doherty (1962).

Disc electrophoresis at pH 9.5 was done by the technique of Ornstein (1964) using the Tris–glycinate buffer system devised by Davis (1964) for plasma proteins. Acrylamide gel electrophoresis in SDS was carried out using both the system described by Weber and Osborn (1969) and the procedure of Laemmli (1970) which stacks the protein–SDS complexes into thin starting zones.

DFP was used as a 1 M solution in 2-propanol.

Carbohydrate analyses were performed using the procedures of Roe (1955) and Dubois *et al.* (1956) for hexoses, the procedure of Warren for sialic acids (1959), and the procedure described by Fanger and Smyth (1970) using the amino acid analyzer for hexosamines. Fucose was assayed using the cysteine–sulfuric procedure of Dische and Shettles (1948).

Amino acid compositions were determined on a Beckman 120C amino acid analyzer using the single-column technique and DC-1a resin from Durrum Chemical Corp., Palo Alto, Calif.

Amino-terminal amino acids were determined quantitatively by the procedure of Stark and Smyth (Stark, 1967) and qualitatively by the dansyl chloride procedure of Gray (1967). Dansylamino acids were identified using the polyamide sheets and procedure of Woods and Wang (1967).

Free carboxyl groups of aspartate and glutamate residues were determined using glycine ethyl ester and the carbodiimide coupling procedure developed by Hoare and Koshland (1967).

Ninhydrin analyses were done using the procedure of Troll and Cannan (1953) after hydrolysis of the protein in 13.5 N NaOH at 100° for 1.5 hr.

Factor X disulfide bridges were reduced using β-mercaptoethanol. The resulting sulfhydryl groups were carboxymethylated at pH 8.0 in 0.5 M Tris-HCl–8 M urea–0.25% EDTA. The procedure is a minor modification of that described by Hirs (1967).

Mannose present in the hexoses isolated from sulfuric acid hydrolysates (Spiro, 1966) was determined using ATP, hexokinase, phosphomannose isomerase, phosphoglucose isomerase, glucose 6-phosphate dehydrogenase, and NADP as described by Kaplan (1967) and Doudoroff (1962). Galactose was determined using galactose dehydrogenase and NAD as described by Doudoroff (1962).

TABLE I: Carbohydrate Composition of Bovine Factor X.

Sample Description	Hexose (Mol/Mol)[a] C_6H_5OH–H_2SO_4	Hexose (Mol/Mol)[a] Anthrone	Sialic Acid (Mol/Mol)[a] Thiobarbituric Acid	Hexosamines (Mol/Mol)[a] $GlcNH_2$	Hexosamines (Mol/Mol)[a] $GalNH_2$
DEAE-cellulose	7.0 (1)	10.0 (1)	6.0 (3)		
Sephadex G-100	7.6 (2)	10.5 (2)	5.7 (2)	4.9 (1)	2.7 (1)
DEAE-Sephadex A-50	6.8 (2)	7.7 (4)	8.3 (3)	4.0[b]	2.5[b]
X_1		(7.0–8.3)	(6.3–10.0)	5.8[c]	2.4[c]
DEAE-Sephadex A-50	8.0 (2)	10.1 (4)	9.4 (3)	4.5[b]	2.0[b]
X_2		(9.5–10.6)	(7.7–11.0)	6.3[c]	2.5[c]
Factor X polypeptide Chains (X_1 and X_1)					
Heavy Chain	7.3[d]			4–4.5[e]	Detectable[e]
Light Chain	Not detectable			Not detectable[e]	Not detectable[e]

[a] Moles of sugar residue per mole of glycoprotein of mol wt 54,000. The number in parentheses adjacent to the sugar value is the number of separate preparations included in the average. In the hexose and Nana demonstration the number in parentheses below the sugar value represents the range for these separate preparations. For ease of comparison to other glycoproteins the percentage compositions for the carbohydrates of factors X_1 and X_2 are: for hexose, X_1, 2.3%; X_2, 3.0%; for sialic acid as Nana, X_1, 4.4%; X_2, 5.0%; and for hexosamines as N-acetylated amino sugars, X_1, 2.6%; X_2, 2.9%. [b] Determined from a time course hydrolysis in 6 N HCl and represents zero time extrapolated values. [c] Determined after hydrolysis for 4 hours in 4 N HCl with peptide removal carried out using the procedure of Fanger and Smyth (1970). [d] For ease of comparison the number of residues are calculated for a glycoprotein of mol wt 54,000. [e] Hexosamines have been determined on chromatograms from the amino acid analyses of the reduced, carboxymethylated polypeptide chains after 24-hr hydrolysis in 6 N HCl at 110°. Glucosamine is present in precisely the amount to be expected in a 24-hr hydrolysate. The galactosamine peaks cannot be accurately integrated. No hexosamine can be detected in the light chain.

Trypsin was treated with tosyl-L-phenylalanine chloromethyl ketone as described by Carpenter (1967). Digestion of the polypeptide chains of factor X was carried out in 0.1 M NH_4HCO_3 and 0.01 M $CaCl_2$ overnight at 37° using a polypeptide: trypsin ratio of approximately 100:1.

Peptide mapping was carried out on Whatman No. 3MM chromatography paper with chromatography in the first dimension using 1-butanol–HOAc–H_2O (4:1:5, v/v) and electrophoresis at pH 5.2 using pyridine–HOAc–H_2O (2:1:97, v/v).

Results

Ion-exchange chromatography on DEAE-Sephadex A-50 was reported by us earlier (Jackson and Hanahan, 1968) to result in separation of factor X into two distinguishable species. These species will be designated X_1 and X_2 with the subscript referring to the order of elution from the DEAE-Sephadex A-50 column. These forms of factor X were not, however, demonstrably different in their behavior on acrylamide gel electrophoresis at pH 9.5 or in their clotting or esterase specific activity. The enzymatic activity of the two species was determined in both the single stage factor X clotting assay and after activation of the zymogen by the rate of Tame hydrolysis. Such separation of the zymogem of factor X into these two forms has been observed repeatedly with factor X_1 accounting for 60 ± 5% and factor X_2, 40 ± 5% of the total protein. A detailed discussion of the chromatographic characteristics of these two factor X variants, X_1 and X_2, can be found in Jackson and Hanahan (1968); Figure 1 of that article shows the separation of factors X_1 and X_2 on DEAE-Sephadex A-50. More complete separation than shown in Figure 1 of Jackson and Hanahan (1968) is obtained by using columns of greater length.

Carbohydrate Composition. Examination of the carbohydrate compositions for the two forms of factor X has repeatedly demonstrated a difference between the oligosaccharide moieties of factor X_1 and factor X_2. The carbohydrate composition data are shown in Table I. In order to be certain that the carbohydrate differences between factor X_1 and X_2 were not the result of glycosidase action on factor X during isolation, the carbohydrate composition was determined for the first product which from previous data (Jackson *et al.*, 1968) is at least 90% factor X. The necessary condition that the carbohydrate content of factor X preparations before resolution into factor X_1 and X_2 be at least as great as the average of X_1 and X_2 is seen to be met from the data of Table I. In an early factor X preparation of this series complete desialylation occurred between the Sephadex G-100 gel filtration step and DEAE-Sephadex A-50 chromatography. For this reason, the above investigation was considered necessary for the differences which have been found to be truly conclusive. The ranges shown for sialic acid residues in factor X_1 and X_2 can be seen to overlap. However, in each individual batch of factor X, factor X_1 always contained at least one fewer residue of Nana than factor X_2. Determination of the carbohydrate content of each factor X variant was always performed on at least three aliquots of the glycoprotein sample and have been done at the same time.

Hexoses have been determined by both the phenol–H_2SO_4 and anthrone reactions. In the latter procedure the extinction coefficients of the chromophores derived from galactose and mannose are nearly identical (Spiro, 1966). This procedure consequently determines total nonspecific hexose. The phenol–H_2SO_4 reaction, however, yields products with different extinction coefficients for these two monosaccharides and thus by combining phenol–H_2SO_4 estimates and an independent determination of the ratio of galactose to mannose,

TABLE II: Amino Acid Compositions of the Factor X Variants X_1 and X_2.[a]

Residue	X_1 (μmol)[a]	Residues/ Mol[b]	X_2 (μmol)	Residues/ Mol[b]
Asp	0.1269	40.1	0.1265	40.0
Thr	0.0943	29.8	0.0954	30.1
Ser	0.0927	29.3	0.0927	29.3
Glu	0.1944	61.4	0.1915	60.5
Pro	0.0641	20.2	0.0634	20.0
Gly	0.1290	40.7	0.1308	41.3
Ala	0.0966	30.5	0.0978	30.9
Cys	0.0696	22.0 (24)	0.0719	22.7 (24)
Val	0.0744	23.5	0.0787	24.9
Met	0.0173	5.5	0.0179	5.7
Ile	0.0351	11.1	0.0331	10.5
Leu	0.1003	31.7	0.0957	30.2
Tyr	0.0296	9.3	0.0309	9.8
Phe	0.0673	21.2	0.0650	20.5
Lys	0.0666	21.0	0.0650	20.5
His	0.0329	10.4	0.0347	11.0
Arg	0.0784	24.8	0.0798	25.2
Trp[c]		10–11		10–11

[a] Data are from 3 separate preparations of bovine factor X_1 and 4 preparations of X_2 with duplicate hydrolyses in each case at 24, 48, 72, and 96 hr. The numbers are the averages for all the samples examined. Performic acid oxidation was used to determine the half-cystine content. The performic acid oxidation values are given in parentheses. [b] 1 mol of polypeptide has a molecular weight of 49,000. [c] Data from Jackson and Hanahan (1968).

the number of residues of each of the two monosaccharides can be found. The ratio of galactose to mannose was found to be 1.8 ± 0.2 using the specific enzymatic assays described in the Methods section. From this ratio and the estimate of "total hexose" by the phenol-H_2SO_4 reaction, factor X_1 can be shown to contain approximately 3 mannose and 6 galactose residues and factor X_2, 4 mannose, and 6–7 galactose residues. The total number of hexose residues determined in this way agrees well with the total found by the anthrone reaction.

Hexosamines have been determined using two different conditions for hydrolysis. In the first entry of Table I for X_1 and X_2, hydrolysis was carried out in 6 N HCl at 110° for 12, 16, and 20 hr. The hexosamines were determined directly on the hydrolysates for each interval and extrapolation to zero time was used to determine the amino sugar content of the glycoprotein. In the second entry and for the Sephadex G-100, factor X hydrolysis was for 4 hr in 4 N HCl and the peptides which interfere with the quantitative estimation of the hexosamines removed using a Dowex 2 column as described by Fanger and Smyth (1970).

No fucose could be found in either factor X_1 or X_2 under conditions which would easily have detected less than 0.1 residue/mole.

Factor X is made up of two polypeptide chains (*vide infra*). All the sugar is found on the heavy polypeptide chain. This is demonstrated by the data of the last entry in Table I.

An examination of the total number of residues of sialic acid per mole of factor X in the preparations handled to date suggests a correlation between sialic acid and the one stage clotting assay specific activity. However, in view of the observed desialylation with one batch of glycoprotein and the demonstrated difference between the sugars of factor X_1 and X_2, a much more detailed investigation will be required to demonstrate such a relationship if it exists. Such an investigation is now under way.

Amino Acid Compositions. Preliminary data from single hydrolysate amino acid analyses of factor X_1 and X_2 did not demonstrate significant differences in amino acid composition between factor X_1 and factor X_2 (Jackson and Hanahan, 1968). However, to be certain that this conclusion did not simply reflect the ambiguity inherent in such single hydrolysate data sets, an additional investigation of the composition of factor X_1 and X_2 was undertaken. Composition data from time course hydrolyses of three separate batches of factor X_1 and four batches of X_2 are given in Table II. This extensive investigation of the glycoprotein amino acid composition was undertaken as part of an attempt to determine if the carbohydrate composition differences given in Table I might actually reflect a loss of glycopeptide as a consequence of limited partial proteolysis. Absolutely no significant differences between factor X_1 and X_2 could be found in any of the batches of factor X examined, including the data from the batch of factor X which had been subjected to preparative disc electrophoresis in addition to the usual steps. The number of residues of each amino acid is calculated for a polypeptide of 49,000 molecular weight; this corresponds to a glycoprotein of 54,000 (*vide infra*).

Amino-Terminal Amino Acids. The second approach taken to determine if the carbohydrate differences might be the consequence of an obvious proteolysis was by examination of N-terminal amino acid residues of both factor X_1 and X_2.

Amino-terminal Ala (0.85–0.97 mol/mol) has been reproducibly found in all factor X preparations and in equal amounts in both factor X_1 and X_2. In one preparation, a decrease in the amount of Ala was compensated by the appearance of an equivalent amount of Gly. Glutamate also appears in the N-terminal samples of both factor X_1 and X_2 in amounts ranging from 0.8 to 1.2 residues per mol and from its reproducibility probably represents a second N-terminal amino acid. However, a nearly equivalent amount of Glu/mol also appears in the carbamyl protein blank. Hydrolysis in 3 N HCl and chromatography on 0.4 × 5 cm Dowex 50 column were performed to remove "spurious Glu" derived from pyrrolidonecarboxylic acid peptides in both the hydantoin sample and in the blank determination.

The demonstration of two polypeptide chains in factor X (*vide infra*) requires a second N-terminal residue; however, an independent method will be required for its unequivocal demonstration.[3]

Free Carboxyl Groups of Factor X_1 and X_2. As the primary basis for separation on an anion exchange Sephadex is expected to be electrostatic charge, the numbers of free carboxyl groups on factors X_1 and X_2 were determined by the technique of Hoare and Koshland (1967). The results of these determinations are given in Table III. No significant differences between factor X_1 and X_2 could be found. Although these data show no differences, conservatively one can only say that out of a total of 74 free carboxyl groups, any difference must be extremely small and very unlikely to be responsible

[3] As factor X is currently being sequenced by Davie, Neurath, and their colleagues at the University of Washington, no further effort to resolve the N-terminal ambiguity has been expended in this laboratory.

TABLE III: Comparison of Factor X_1 and X_2 Free Carboxyl Groups.

Residue	X_1		X_2	
	μmol[a]	Residues/ Mol[b]	μmol[a]	Residues/ Mol
Asp	0.0931	40	0.0928	40
Glu	0.1369	60	0.1375	60
Gly	0.2664	115	0.2676	115
Protein[b]	0.00231		0.00232	
Calculated glycine[c]	0.0947	41	0.0951	41
Free carboxyl excess Gly	0.1717	74	0.1725	74
Carboxamide	0.0583	26	0.0578	26

[a] Average of three 24-hr hydrolysates. [b] Calculated for a glycoprotein of mol wt 54,000 and are from the data of Table II. The number of μmoles of protein is calculated from the μmoles of Asp, Glu, Ala, Phe, Lys, His, and Arg in the hydrolysates and the residues per mole. [c] Calculated using Gly content given in Table II for 0.00231 μmol of protein.

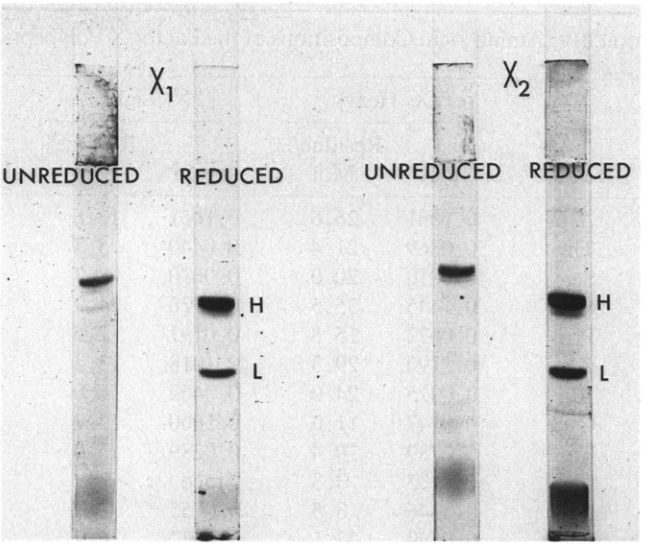

FIGURE 1: Sodium dodecyl sulfate gel electrophoresis of bovine factor X. Sodium dodecyl sulfate system of Laemmli (1970). Approximately 50 μg of protein is present in each gel.

for the separation of factor X into X_1 and X_2 during chromatography on DEAE-Sephadex. This conclusion is in agreement with the lack of resolution of X_1 and X_2 by disc acrylamide gel electrophoresis reported earlier (Jackson and Hanahan, 1968).

Polypeptide Chains of Factor X. Factor X_1 and X_2 have been examined by SDS acrylamide gel electrophoresis both before and after reduction of the disulfide bridges. With intact disulfide bridges a single band is seen in the SDS gel electrophoretogram; however, after disulfide reduction two bands are found. Photographs of such SDS gels for both factor X_1 and X_2 are seen in Figure 1. No significant differences between variants X_1 and X_2 have ever been seen by this technique, both in reduced and unreduced samples. *A priori*, two alternative structural explanations for the SDS gel results obtained after reduction of the disulfides of factor X must be considered. First, and subsequently shown to be the case, the factor X zymogen may be made up of two unique polypeptide chains. Alternatively, however, the SDS gel electrophoresis data might only imply that some fraction of the factor X preparation has undergone a proteolytic cleavage to yield two fragments of approximately equal molecular weight. The molecular weight estimates from SDS gel electrophoresis seen in Table V indicate the necessity for this consideration. This "bisected" factor X would be represented by the fast migrating band of the SDS gels. Consideration of this second explanation is necessary in view of the number of artifactual forms of factor X which have been described earlier (Jackson and Hanahan, 1968) and the marked disparity in the physical and chemical data for factor X (Esnouf and Williams, 1962; Jackson and Hanahan, 1968) or its activated form (Tishkoff *et al.*, 1968) and autoprothrombin C (Seegers *et al.*, 1967).

Although molecular weight estimates from SDS gel electrophoresis might permit one to distinguish between these alternatives, the SDS molecular weight estimtes are complicated by the fact that bovine factor X is a glycoprotein and the relationship between glycopolypeptide molecular weitht and SDS gel electrophoretic mobility is not unequivocally known (Pitt-Rivers and Impiombato, 1968; Reynolds and Tanford, 1970; Trayer *et al.*, 1971). Using SDS gel electrophoresis, the faster migrating species actually appears to possess approximately one-half the molecular weight of the large polypeptide. In view of the foregoing considerations, an extensive investigation of the two polypeptide chain products obtained after reduction of the disulfide bonds of factor X was undertaken.

Factor X_1 and factor X_2 after being isolated by DEAE-Sephadex A-50 chromatography were separately reduced using β-mercaptoethanol and S-carboxymethylated as described in the Methods section. The reduced, carboxymethylated polypeptide chains in a volume of approximately 5 ml were separated from urea and the slight excess of mercaptoethanol on a 1.5 × 45 cm column of Sephadex G-25 fine in 50% acetic acid. Lower concentrations of acetic acid were not adequate to keep the S-carboxymethylated chains in solution. The desalted polypeptide chain mixtures were dried on a rotary evaporator and then dissolved in 1 ml of 50% acetic acid for application to a Sephadex G-100 column. The large and small polypeptide materials were readily separated by gel filtration in 50% acetic acid on a Sephadex G-100 column (1.5 × 90 cm). An elution profile from the separation of the chains of factor X_2 is shown in Figure 2. Indistinguishable profiles were obtained for factor X_1 and for factor X from the Sephadex G-100 chromatography step which precedes the separation of factor X into the two variants. In all cases 70 ± 2% of the total ninhydrin reacting material was found associated with the first peak of polypeptide material and 30% ± 2% for the second peak of material. Greater than 95% of the ninhydrin reacting and 280-nm-absorbing material applied to the column was recovered in the two peaks.

The amino acid compositions of the separated polypeptide chains were determined and are reported in Table IV. The number of residues per mol in each of the chains is based on the partitioning of the ninhydrin reacting material from the column and a hologlycoprotein polypeptide chain molecular weight of 49,000. From this composition data it is extremely difficult to see how the smaller polypeptide material could

TABLE IV: Amino Acid Compositions of the Factor X Polypeptide Chains.

Residue	X_1 Heavy		X_1 Light		X_1[a]	X_2 Heavy		X_2 Light		X_2[a]
	μmol[b]	Residue/Mol	μmol[b]	Residue/Mol	H + L	Mol	Residue/Mol	μmol[b]	Residue/Mol	H + L
Asp	0.1081	26.6	0.1051	13.6	40.2	0.1151	27.4	0.1113	14.5	41.9
Thr	0.0869	21.4	0.0440	5.7	27.1	0.0922	22.0	0.0434	5.7	27.7
Ser	0.0810	20.0	0.0670	8.7	28.7	0.0730	17.4	0.0820	10.7	28.1
Glu	0.1445	35.5	0.1926	24.9	60.4	0.1416	33.7	0.1927	25.1	58.8
Pro	0.0622	15.3	0.0191	2.5	17.8	0.0714	17.0	0.0228	3.0	20.0
Gly	0.1190	29.3	0.1018	13.2	42.5	0.1176	28.0	0.1025	13.4	41.4
Ala	0.0975	24.0	0.0462	6.0	30.0	0.1049	25.0	0.0467	6.1	31.1
Cys	0.0447	11.0	0.1060	13.7	24.7	0.0418	10.0	0.0958	12.5	22.5
Val	0.0789	19.4	0.0389	5.0	24.4	0.0798	19.0	0.0360	4.7	23.7
Met	0.0135	3.3	Trace		3.3	0.0071	1.7	0.0041	0.5	2.2?
Ile	0.0346	8.5	0.0155	2.0	10.5	0.0366	8.7	0.0157	2.0	10.7
Leu	0.0899	22.1	0.0532	6.9	29.0	0.0972	23.1	0.0524	6.8	29.9
Tyr	0.0272	6.7	0.0187	2.4	9.1	0.0307	7.3	0.0188	2.5	9.8
Phe	0.0536	13.2	0.0545	7.0	20.2	0.0530	12.6	0.0573	7.5	20.1
Lys	0.0635	15.6	0.0515	6.7	22.3	0.0568	13.5	0.0470	6.1	19.6
His	0.0321	7.9	0.0213	2.8	9.7	0.0329	7.8	0.0205	2.7	10.5
Arg	0.0746	18.4	0.0565	7.3	25.7	0.0780	18.6	0.0556	7.2	25.8

[a] H + L is the sum of the residues in the two chains. [b] Average number of μmoles from the duplicate samples from 24, 48, 72, and 96 hr of hydrolysis. Ser and Thr were obtained from the zero-time extrapolation values by assuming pseudo-first-order destruction. Val, Ile, and Leu were the averages of the values obtained after 72 and 96 hr of hydrolysis. Cys was determined as *S*-carboxymethylcysteine.

be derived from the larger. Amino acid analysis of a trace amount of ninhydrin-reacting material which was found in the internal volume peak of the Sephadex G-100 (50% HOAc) column showed a composition which did not differ significantly from the composition of the native factor X. Thus it appears on both the basis of mass conservation and the amino acid composition data that the factor X is made up of two polypeptide chains with no significant other peptide material detectable as very small peptides.

In an attempt to obtain further evidence for this conclusion, samples of the heavy and light chains were digested with trypsin and the peptide maps obtained by paper chromatography–high-voltage electrophoresis were examined. The heavy chain showed 31–34 well-separated peptides. This number of peptides agrees with the number expected from the residues of lysine and arginine as determined by amino acid analysis. The light-chain digests have not been satisfactory and have shown extensive streaking toward the anode in all electrophoretic separations attempted. Of the approximately eight peptides which can be clearly distinguished on the light-chain maps, no more than one is found to be even near a peptide spot from any of the heavy-chain peptides. The observations of the requisite number of peptides in the peptide map of the heavy polypeptide chain and the absence of overlapping peptides in the maps of the two separated chains, coupled with the amino acid composition data for the separated chains, conclusively exclude the possibility that smaller molecular weight polypeptide material is derived from the larger polypeptide. It can therefore be concluded that factor X is made up of two unique polypeptide chains.

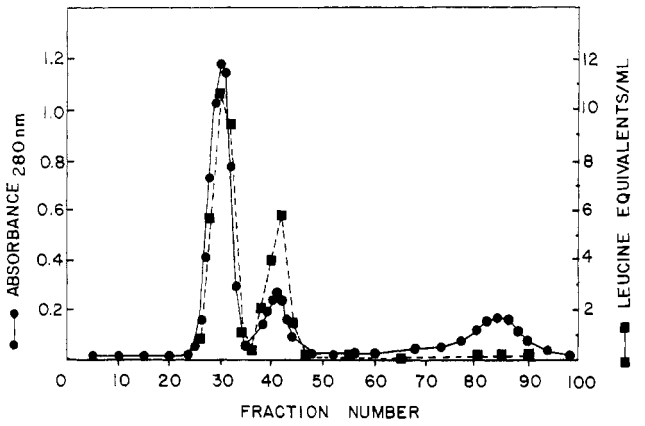

FIGURE 2: Separation of the reduced, carboxymethylated polypeptide chains of bovine factor X. Column: 1.5 × 90 cm Sephadex G-100 in 50% HOAc. Flow rate: 8 ml/hr. Sample: 10 mg of X_2 in 1 ml of 50% HOAc.

An examination of the hexose distribution between the isolated polypeptide chains has demonstrated that all hexose is attached to the heavy chain and none to the light chain. Using the single column technique for amino acid analysis, both glucosamine and galactosamine were found in the 24-hr hydrolysates of the heavy polypeptide chain; no hexosamines were detectable in hydrolysates of the light chain. These data are included in Table I. No attempt was made to look for residual sialic acid in view of the conditions required for polypeptide chain separation.

The molecular weights for the factor X hologlycoprotein, the glycoprotein polypeptide moiety, and the separated polypeptide chains have been determined by a variety of procedures. The resulting molecular weight values for the various factor X constituents are given in Table V. Molecular

weights of the separated polypeptide chains were determined by sedimentation equilibrium in 6 M guanidine hydrochloride. Values are given using the partial specific volumes calculated from the amino acid and carbohydrate composition both without and with correction for the effects of guanidinium ion binding on the partial specific volume (Hade and Tanford, 1967). In the case of the light chain, the maximum change in $\bar{v}$ from guanidine binding produces the best agreement between the chemical estimate of the molecular weight from the partition of ninhydrin reacting material and the value from sedimentation equilibrium. The molecular weight determined for the heavy glycopeptide chain by SDS gel electrophoresis agrees well with the other two estimates for this species. However, the agreement is with the glycopeptide chain and would not be in agreement if the carbohydrate were not included as contributing to the SDS gel molecular weight. No difference in this molecular weight estimate has been found using either the 0.1% SDS containing gels described by Weber and Osborn (1969) or the 1% SDS system of Laemmli (1970).

The light-chain molecular weight determined by SDS electrophoresis has been consistently larger than expected on the basis of the chemical data. Such anomalous behavior of some polypeptides with a large number of negative charges has been discussed in detail by Tung and Knight (1972). In addition, the data from analytical ultracentrifugation agree with the chemical data only when a reduction in partial specific volume of 0.02 cm^3/g is used. This is the maximum which has been previously reported (Hade and Tanford, 1967). The structural basis for this behavior of the factor X light chain is currently under further investigation.

The amino-terminal amino acid of the light chain was identified as Ala by the dansyl chloride technique of Gray (1967). The Dns-Ala was identified by chromatography on polyamide sheets as described by Woods and Wang (1967).

TABLE V: Molecular Weights of Bovine Factor X Components.

	Component	$\bar{v}$ (cm^3/g)	Mol Wt
	Glycoprotein (Jackson and Hanahan, 1968)		
X_1 plus X_2	0.1 M NaCl–0.001 M Tris-HCl (pH 7.4) (corrected for carbohydrate)[a]	0.718 0.707	55,092 53,084
X_2	This investigation polypeptide chain[b]	0.707	54,415 48,900–49,700
	Heavy Chain		
X_2	6 M Gdn·HCl (corrected for Gdn·HCl binding)[c]	0.707[a] 0.697	40,700 38,400
X_1 and X_2	Acrylamide gel electrophoresis in SDS[d]		38,000–42,000
X_1 and X_2	Fraction of total ninhydrin-reacting material after separation of the two polypeptide chains[e] (Figure 2)		39,100
	Light Chain		
X_2	6 M Gdn·HCl, c = 0.2 mg/ml (corrected for Gdn·HCl binding)[c] (extrapolated to c = 0)[f]	0.708 0.698 0.688 0.708	17,000 16,000 15,200 14,200 ± 1,000
X_1 and X_2	Acrylamide gel electrophoresis in SDS[d]		22,000–24,000
X_1 and X_2	Fraction of the total ninhydrin-reacting material after separation[e]		15,200

[a] Calculated using $\bar{v}$ which includes the contribution of the carbohydrate. [b] Polypeptide chain molecular weight is the difference between the glycoprotein molecular weight determined by ultracentrifugation and the molecular weight of the sugar residues taken from Table I. [c] The partial specific volume has been decreased by 0.010 cm^3/g to compensate for guanidine hydrochloride binding as determined and discussed by Hade and Tanford (1967). [d] SDS gel electrophoresis yields identical values and range of values in both the 0.1% SDS system of Weber and Osborn (1969) and the 1% stacking system of Laemmli (1970). [e] This mass conservation estimate for the polypeptide chain molecular weights was determined from the fraction of the total ninhydrin reacting material in each peak of the separated polypeptide chains (Figure 2). [f] Extrapolation was molecular weight *vs.* concentration, and the limits of error were determined from the range at c = 0 using different pairs of the three experimental points.

Discussion

Factor X_1 and X_2 have been shown to differ in carbohydrate composition with factor X_2 containing 2 more hexose residues and 1–2 more sialic acid residues than factor X_1. No simple relationship between the factor X variants and either the number or identity of the hexosamine residues has been found. The presence of galactosamine in factor X appears to distinguish it from prothrombin which has been reported to contain glucosamine only (Magnusson, 1965; Hartley, 1970). Prothrombin isolated in this laboratory has not been found to contain galactosamine in 24-hr 6 N HCl hydrolysates in agreement with the data of Magnusson (1970) (W. G. Owen and C. M. Jackson, unpublished observations). On a residue per mole basis, carbohydrate compositions of prothrombin and factor X appear very similar, although these proteins differ in molecular weight by at least 14,000. If total hexosamine is considered, factor X contains 6.5–8.8 residues/mol whereas prothrombin is reported to contain 6.8 residues/mol (Hartley, 1970). Prothrombin and factor X contain nearly identical amounts of sialic acid/mol, 9.5 residues for prothrombin and 8.8 and 9.4 for factors X_1 and X_2, respectively. Hexose compositions are both qualitatively and quantitatively similar; prothrombin contains 6 mol of galactose against 6 or 7 mol for factors X_1 and X_2 and for prothrombin 5.3 mol of mannose *vs.* 3–4 for the factor X variants. Nothing is yet known about the number of structures of the oligosaccharide chains of these proteins; however, both are under investigation in this laboratory. The preliminary report by Fujikawa *et al.* (1971) that factor X_1 is activated 70 times more slowly by factor IX[a] than factor X_2 and the recent reports of the involvement of vitamin K in glycosylation of the "K-dependent" clotting factors (Pereira and Couri, 1971; Johnson *et al.*, 1971) suggest that the carbohydrate differences reported here may be of considerable biological significance. The failure to see such a relationship in this study very likely is a result of the nonphysiological activator, the coagulant protein of Russell's viper venom, which is used to activate factor X (Williams and Esnouf, 1962; Jackson *et al.*, 1971). In this same

TABLE VI: Thrombin and the Factor X Heavy Polypeptide Chain: a Comparison between the Amino Acid Compositions.

Residue	X_H	Thrombin: Magnusson A + B Chains[a] (Bovine)	Thrombin: Seegers *et al.*[b] (Bovine)	Thrombin: Fenton *et al.*[c] (Human)	Thrombin: Batt *et al.*[d] (Bovine)
Asp	27	27	28	27	32
Thr	22	13	13	11	16
Ser	17	17	14	13	18
Glu	34	34	29	28	35
Pro	17	16	14	17	18
Gly	28	25	21	22	30
Ala	25	16	12	12	18
Cys	10	8	6	6	8
Val	19	22	16	15	19
Met	3–4	5	4	5	4
Ile	9	16	11	13	13
Leu	23	27	24	21	32
Tyr	7	12	10	10	11
Phe	13	11	10	10	14
Lys	14	25	18	18	26
His	8	7	5	5	8
Arg	19	22	17	17	24
Trp	<10[e]	10		6	10
Total residues	303	313	252	253	336

[a] Calculated from the provisional amino acid sequence of the A and B chains (Magnusson, 1969). [b] Seegers *et al.* (1967). [c] Fenton *et al.* (1971). [d] Batt *et al.* (1970). [e] This value is from Jackson and Hanahan (1968) and represents the maximum possible in the heavy chain.

context, Denson (1970) has reported that clinical factor X deficiency states can be distinguished on the basis of the activator used to assay the factor X. It is interesting, therefore, to ask if the oligosaccharide moiety of the factor X molecule might not be important in determining the biological activity of the factor X, activity in this instance varying as a consequence of differences in the rate of factor X activation. Hemker *et al.* (1970) have reported that patients on dicoumarol exhibit a slower rate of activation of prothrombin than do normal individuals, an observation which might also be explicable on the basis of the structure of the glycoprotein oligosaccharide chains. Morell *et al.* (1971) have reported that removal of sialic acid residues from plasma glycoproteins can serve as a signal for removal of the glycoprotein from the circulation. It would be extremely interesting to know if this mechanism functions for the blood clotting components as well as the plasma glycoproteins investigated by Ashwell's group.

Additional support for the proposed role of the oligosaccharide chains in the activation of factor X is obtained from the recent data of Radcliffe and Barton (1972) on activated factor X. These workers have reported that no carbohydrate could be detected in activated factor X. In addition, the difference in molecular weight between the zymogen form of factor X (Jackson and Hanahan, 1968) and Table V, and activated factor X, mol wt 48,500 (Radcliffe and Barton, 1972), can be accounted for almost completely by the carbohydrate of factor X. (Unpublished data from this laboratory are in agreement with Radcliffe and Barton.)

The origin of the two species of factor X, X_1 and X_2, is unknown, but the data of Table I for the carbohydrate composition at the earliest stage at which it may be determined make it very unlikely that these two forms of factor X arise during the isolation process. The reproducibility of the separation into X_1 and X_2 and the constancy of the relative amounts of the two variants further suggest that these differences are inherent in the native glycoprotein, although glycosidase action in the blood or plasma cannot be eliminated at this time. The presence of a variety of glycosidases in plasma and associated with blood platelets is well known (Price and Dance, 1972; Bossman, 1972).

Unfortunately the bovine blood obtained at commerical packing houses may not be entirely satisfactory for further investigation of these relationships. This qualification is necessary in view of the widespread practice of administering diethylstilbestrol to feedlot cattle (Oltjen and Lehmann, 1968), and the potential effects of this estrogenic compound on both blood clotting factor activity and glycoprotein metabolism. A decrease in serum protein sialic acid in man has been demonstrated to occur after diethylstilbestrol administration (Doe *et al.*, 1967).

No differences between factor X_1 and X_2 other than the carbohydrate differences described above have been found. The amino acid compositions, the number of free carboxyl groups and the amino-terminal amino acids are identical within the error of these determinations. In spite of the ambiguity in identifying the amino terminus of the heavy chain of the factor X molecule, the results for the N-terminal determination on two separate batches of factor X variants showed no difference between factor X_1 and X_2. The amino acid composition differences seen in Table IV between the heavy polypeptide chains of factor X_1 and X_2 and the light chains of these two species cannot be concluded to be significant. If real differences exist between these corresponding chains, the completion of the amino acid sequence (E. W. Davie, personal communication) will surely demonstrate them conclusively.

The most interesting features of the amino acid composition data of factor X can be seen in Table VI in which the heavy chain of factor X is compared to bovine thrombin. The composition similarities are striking. Only 3 amino acids are markedly different, threonine and alanine, which may be trivial or at least do not immediately suggest functionally significant differences, and lysine. The decrease in net negative charge, or the increase in positively charged residues, may be important in determining the specificity of these two obviously closely related serine proteases toward their protein substrates.

Radcliffe and Barton (1972) have shown that the active serine is found on the heavy chain in activated factor X and earlier work by Leveson and Esnouf (1969) has demonstrated that the amino acid sequence in the vicinity of the active serine is the now classic, Gly-Asp-Ser-Gly. Smith (1971) has demonstrated that activated factor X contains one active site per 50,000.

The light chain of factor X appears to be potentially very acidic, the number of Glu plus Asp residues exceeding the Lys plus Arg by approximately 25. As the active site for activated factor X is located on the heavy chain (Radcliffe and Barton, 1972), it is interesting to speculate on a possible func-

tion for the light chain or some portion of it. Papahadjopoulos and Hanahan (1964) and Esnouf and Jobin (1967) have demonstrated that activated factor X binds to phospholipid dispersions in the presence of calcium ions. On the basis of these two observations, if a lipid binding site or region exists, it might well be expected to be found on this light chain. This possible role for the light chain is under investigation.

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References

Aronson, D. L., and Menache, D. (1968), *Biochim. Biophys. Acta 167*, 378.
Bachmann, F., Duckert, F., Fluckiger, P., and Hitzig, W. H. (1957), *Schweiz. Med. Wochenschr. 87*, 1221.
Barton, P. G., Jackson, C. M., and Hanahan, D. J. (1967), *Nature (London) 214*, 923.
Batt, C. W., Mikulka, T. W., Mann, K. G., Guarracino, C. L., Altiere, R. J., Graham, R. G., Quigley, J. P., Wolf, J. W., and Zafonte, C. W. (1970), *J. Biol. Chem. 245*, 4857.
Bezkorovainy, A., and Doherty, D. G. (1962), *Arch. Biochem. Biophys. 96*, 491.
Bossman, H. B. (1972), *Biochim. Biophys. Acta 258*, 265.
Carpenter, F. H. (1967), *Methods Enzymol. 11*, 237.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Denson, K. W. E. (1970), *Zool. Soc. London Symp. 27*, 151.
Dische, Z., and Shettles, L. B. (1948), *J. Biol. Chem. 175*, 595.
Doe, R. P., Mellinger, G. T., Swaim, W. R., and Seal, U. S. (1967), *J. Clin. Endocrinol. Metab. 27*, 1081.
Doudoroff, M. (1962), *Methods Enzymol. 5*, 335, 339.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.
Esnouf, M. P., and Williams, W. J. (1962), *Biochem. J. 84*, 62.
Fanger, M. W., and Smyth, D. (1970), *Anal. Biochem. 34*, 494.
Fenton, II, J. W., Campbell, W. P., Harrington, J. C., and Miller, K. D. (1971), *Biochim. Biophys. Acta 229*, 26.
Fujikawa, K., Thompson, A. R., Legaz, M. E., and Davie, E. W. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1075.
Graham, J. B., and Hougie, C. (1956), *VI Congr. Int. Soc. Haematol. (Boston)*, 327.
Gray, W. (1967), *Methods Enzymol. 11*, 139.
Hade, E. P. K., and Tanford, C. (1967), *J. Amer. Chem. Soc. 89*, 5034.
Hartley, B. S. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 77.
Hemker, H. C., Muller, A. D., and Loeliger, F. A. (1970), *Thromb. Diath. Haemorrh. 23*, 633.
Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 197.
Hoare, D. G., and Koshland, D. E. (1967), *J. Biol. Chem. 242*, 2447.
Jackson, C. M., Gordon, J. G., and Hanahan, D. J. (1971), *Biochim. Biophys. Acta 252*, 255.
Jackson, C. M., and Hanahan, D. J. (1968), *Biochemistry 7*, 4506.
Jackson, C. M., Johnson, T. F., and Hanahan, D. J. (1968), *Biochemistry 7*, 4492.
Johnson, H. V., Martinovic, J., and Johnson, B. C. (1971), *Biochem. Biophys. Res. Comm. 43*, 1040.
Kaplan, N. O. (1957), *Methods Enzymol. 3*, 107.
Laemmli, U. K. (1970), *Nature (London) 227*, 680.
Leveson, J., and Esnouf, M. P. (1969), *Brit. J. Haematol. 17*, 173.
Lundblad, R. L., and Davie, E. W. (1964), *Biochemistry 3*, 1720.
Lundblad, R. L., and Davie, E. W. (1965), *Biochemistry 4*, 113.
Macfarlane, R. G., Biggs, R., Ash, B. J., and Denson, K. W. E. (1964), *Brit. J. Haematol. 10*, 530.
Magnusson, S. (1965), *Arkiv Kemi 23*, 285.
Magnusson, S. (1969), *Thromb. Diath. Haemorrh., Suppl. XII*, 97.
McMeekin, T. L., Groves, M. L., and Hipp, N. J. (1949), *J. Amer. Chem. Soc. 71*, 3298.
Milstone, J. H. (1964), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 23*, 742.
Morell, A. G., Gregoriadis, G., Scheinberg, I. H., Hickman, T., and Ashwell, G. (1971), *J. Biol. Chem. 246*, 1461.
Nemerson, Y., and Spaet, T. H. (1964), *Blood 23*, 657.
Oltjen, R. R., and Lehmann, R. P. (1968), *J. Nutr. 95*, 399.
Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Osterud, B., Rapaport, S. I., Schiffman, S., and Chong, M. M. Y. (1971), *Brit. J. Haematol. 21*, 643.
Papahadjopoulos, D., and Hanahan, D. J. (1964), *Biochim. Biophys. Acta 90*, 436.
Pereira, M., and Couri, D. (1971), *Biochim. Biophys. Acta 237*, 348.
Pitt-Rivers, T., and Impiombato, F. S. A. (1968), *Biochem. J. 109*, 825.
Price, R. G., and Dance, N. (1972), *Biochim. Biophys. Acta 271*, 145.
Radcliffe, R. D., and Barton, P. G. (1972), *J. Biol. Chem.* (in press).
Reynolds, J. A., and Tanford, C. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 1002.
Roe, J. (1955), *J. Biol. Chem. 212*, 335.
Seegers, W. H. (1964), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 23*, 749.
Seegers, W. H., and Marciniak, E. (1962), *Nature (London) 193*, 1188.
Seegers, W. H., Marciniak, E., Kipfer, R. K., and Yasunaga, K. (1967), *Arch. Biochem. Biophys. 121*, 372.
Seegers, W. H., Marciniak, E., and McCoy, L. (1969), *Thromb. Diath. Haemorrh. 22*, 32.
Smith, R. L. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1076.
Spiro, R. G. (1966), *Methods Enzymol. 8*, 3.
Stark, G. R. (1967), *Methods Enzymol. 11*, 125.
Straub, W., and Duckert, F. (1961), *Thromb. Diath. Haemorrh. 5*, 402.
Telfer, T. P., Denson, K. W., and Wright, D. R. (1956), *Brit. J. Haematol. 2*, 303.
Tishkoff, G. H., Williams, L. C., and Brown, D. M. (1968), *J. Biol. Chem. 243*, 4151.
Trayer, H. R., Nozaki, Y., Reynolds, J. A., and Tanford, C. (1971), *J. Biol. Chem. 246*, 4485.
Troll, W., and Cannan, R. K. (1953), *J. Biol. Chem. 200*, 803.
Tung, J., and Knight, C. A. (1972), *Anal. Biochem. 48*, 153.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Williams, W. J., and Esnouf, M. P. (1962), *Biochem. J. 84*, 52.
Woods, K. R., and Wang, K. T. (1967), *Biochim. Biophys. Acta 133*, 369.
Yin, E. T., and Wessler, S. (1968), *Thromb. Diath. Haemorrh. 12*, 465.
Yin, E. T., Wessler, S., and Stoll, P. J. (1971a), *J. Biol. Chem. 246*, 3703.
Yin, E. T., Wessler, S., and Stoll, P. J. (1971b), *J. Biol. Chem. 246*, 3712.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Bovine Factors X_1 and X_2 (Stuart Factor). Isolation and Characterization†

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ABSTRACT: Bovine factors X_1 and X_2 (Stuart factor) were purified by $BaSO_4$ adsorption and elution, DEAE-Sephadex batch adsorption and elution, and DEAE-Sephadex column chromatography. Each protein was homogeneous by immunoelectrophoresis, gel electrophoresis, and zone electrophoresis. Factors X_1 and X_2 were heterogeneous by sedimentation equilibrium centrifugation, but this was attributed to reversible association of the protein. The monomer molecular weight calculated for each protein was about 55,000. The amino acid and carbohydrate compositions of the two proteins are essentially identical and neither protein contains free sulfhydryl groups. The two proteins also appear to have the same immunological properties when tested against antibodies to either factor X_1 or X_2. Each protein contains 10% carbohydrate which includes 3.8% neuraminic acid (*N*-acetylneuraminic acid and *N*-glycolylneuraminic acid), 2.9% hexose (galactose and mannose), and 3.6% hexosamine (galactosamine and glucosamine). Following reduction with 2-mercaptoethanol, a heavy chain with a molecular weight of about 38,000 and a light chain with a molecular weight of about 17,000 were isolated from each protein. The heavy chain from either factors X_1 or X_2 has an amino-terminal sequence of Trp-Ala-Ile-His- and contains nearly all of the carbohydrate. The amino acid composition and carbohydrate content appear to be the same for both heavy chains. The light chain from either factor X_1 or X_2 has an amino-terminal sequence of Ala-Asn-Ser-Phe-, and the total amino acid composition also appears to be essentially the same for these two chains. It was concluded that factors X_1 and X_2 are made up of one heavy and one light chain held together by one or more disulfide bonds. Furthermore, the difference between the two proteins appears to be very minor.

Factor X (Stuart factor)[1] is a plasma protein which participates in intrinsic and extrinsic blood coagulation. It plays a central role in blood coagulation since it can be activated independently by either the intrinsic or extrinsic coagulation system. Once factor X is converted to an activated form (factor X_a), it forms a macromolecular complex in the presence of factor V (proaccelerin), phospholipid, and calcium ions, and this complex is capable of converting prothrombin to thrombin (Papahadjopoulos and Hanahan, 1964; Milstone, 1964; Denson, 1967; Barton *et al.*, 1967; Esnouf and Jobin, 1967; Jobin and Esnouf, 1967; Ferguson *et al.*, 1967).

In order to study in detail the mechanism by which factor X participates in blood coagulation, highly purified preparations with well defined physical–chemical characteristics are required. Accordingly, a number of methods have been developed in recent years for the isolation of factor X from both bovine and human plasma (Hougie and Bunting, 1960; Duckert *et al.*, 1960; Esnouf and Williams, 1962; Papahadjopoulos *et al.*, 1964; Jackson *et al.*, 1968; Aronson *et al.*, 1969). Bovine factor X can also be partially separated into two protein peaks by chromatography on DEAE-Sephadex A-50, and both of these fractions can be converted to factor X_a in the presence of Russell's viper venom (Jackson and Hanahan, 1968). Factor X is presumably identical with prothrombokinase, the precursor of thrombokinase (Milstone, 1964), and autoprothrombin III, the precursor of autoprothrombin C (Seegers *et al.*, 1967; Seegers, 1969).

In the present experiments, bovine factor X was isolated in the presence of several protease inhibitors and separated into factors X_1 and X_2 by chromatography on DEAE-Sephadex A-50. The two proteins were then studied in detail in regard to their size, electrophoretic and immunological properties, amino acid, carbohydrate and subunit compositions, and amino-terminal sequences. The following papers describe some of the molecular events which occur when this protein is activated to factor X_a by a protein present in Russell's viper venom (Fujikawa *et al.*, 1972) and the amino acid sequence of the cyanogen bromide peptide which contains the serine present in the active site (Titani *et al.*, 1972).

Materials

Benzamidine hydrochloride and cyclohexanone were purchased from Aldrich Chemical Co., Milwaukee, Wis. Heparin sodium salt (Grade I), soybean trypsin inhibitor (types I and II), cephalin (rabbit brain extract), neuraminidase (*Clostridium perfringens*), bovine albumin, mannose, galactose,

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