

## **Purification and characterization of an abnormal factor IX (Christmas factor) molecule. Factor IX Chapel Hill.**

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**Research Article**

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# Purification and Characterization of an Abnormal Factor IX (Christmas Factor) Molecule

## FACTOR IX CHAPEL HILL

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**ABSTRACT** Human Factor IX (Christmas factor) was isolated from the plasma of a patient with mild hemophilia B. The patient's plasma contained 5% Factor IX clotting activity but 100% Factor IX antigenic activity as determined by immunological assays, which included inhibitor neutralization and a radioimmunoassay for Factor IX. This abnormal Factor IX is called Factor IX Chapel Hill (Factor IX<sub>CH</sub>). Both normal Factor IX and Factor IX<sub>CH</sub> have tyrosine as the NH<sub>2</sub>-terminal amino acid. The two proteins have a similar molecular weight, a similar amino acid analysis, the same number of gamma-carboxyglutamic acid residues (10  $\gamma$ -carboxyglutamic acid residues), and a similar carbohydrate content. Both exist as a single-chain glycoprotein in plasma. The major difference between normal Factor IX and Factor IX<sub>CH</sub> is that the latter exhibits delayed activation to Factor IXa in the presence of Factor XIa and Ca<sup>2+</sup>. Thus, Factor IX<sub>CH</sub> differs from other previously described abnormal Factor IX molecules.

## INTRODUCTION

Factor IX (plasma thromboplastin component, Christmas factor) is a clotting factor which participates in the middle phase of intrinsic blood coagulation. Diminished activity of this factor results in hemophilia B. Factor IX is a glycoprotein that has been isolated from both the human and bovine species and many of the characteristics of the normal factor have been published (1-8). In the circulation, Factor

IX exists as a single-chain zymogen which is converted to an active serine protease in the presence of Factor XIa (plasma thromboplastin antecedent) and Ca<sup>2+</sup> ions (9). During activation, the Factor IX zymogen undergoes proteolysis in a two-stage reaction that results in a two-chain molecule composed of a light and heavy chain linked by disulfide bonds. The first step in the proteolysis is the cleavage of a specific arginine-alanine peptide bond which results in an inactive intermediate product. The second cleavage splits an arginine-valine peptide bond with the release of a carbohydrate-rich activation peptide and an active Factor IX enzyme (10, 11). The active site serine resides on the heavy chain (11).

Activated Factor IX (Factor IXa), in the presence of phospholipids, thrombin-modified Factor VIII, and calcium ions, converts Factor X to Factor Xa (2). However, the precise molecular interactions of Factor IXa with calcium, phospholipid, Factor VIII, and Factor X are not completely understood. Elucidation of the nature of the defect in abnormal Factor IX molecules found in some patients with hemophilia B (Christmas disease) may provide insights into function of normal Factor IX in hemostasis and its interaction with other clotting factors. Roberts et al. (12), and other investigators (13-19) have demonstrated that some patients with hemophilia B possess an abnormal Factor IX molecule which has low to undetectable clotting activity, but which is present in relatively normal amounts when measured by immunological techniques (12, 20-22). Because they contain cross-reacting material (CRM)<sup>1</sup> to specific homologous and heterologous

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<sup>1</sup> *Abbreviations used in this paper:* CRM, cross-reacting material; CRM<sup>+</sup>, CRM positive variants; CRM<sup>-</sup>, CRM negative variants; CRM<sup>R</sup>, CRM reduced variants; Factor IX<sub>CH</sub>, Factor IX Chapel Hill; SDS, sodium dodecyl sulfate.

anti-Factor IX antibodies, these patients have been termed CRM<sup>+</sup> (CRM positive variants). They can be distinguished from other hemophilia B patients who have reduced, but not absent, levels of both Factor IX antigen and Factor IX clotting activity (CRM<sup>R</sup>; CRM reduced variants). The CRM<sup>+</sup> patients can also be distinguished from those hemophilia B patients in whom Factor IX antigen and Factor IX clotting activity are absent or undetectable (CRM<sup>-</sup>; CRM negative variants) (22).

The purpose of this paper is to describe the isolation and characteristics of an abnormal Factor IX molecule from a mildly affected CRM<sup>+</sup> hemophilia B patient. This abnormal Factor IX has been named Factor IX Chapel Hill (Factor IX<sub>CH</sub>) (23). Factor IX<sub>CH</sub> is compared with purified normal human Factor IX in terms of its molecular weight, NH<sub>2</sub>-terminal amino acid, content of  $\gamma$ -carboxyglutamic acid residues, carbohydrate content, and activation by Factor XIa and Ca<sup>2+</sup>. The major abnormality in Factor IX<sub>CH</sub> appears to be defective activation by Factor XIa and Ca<sup>2+</sup>, although abnormalities in interactions with other clotting factors cannot be excluded.

## METHODS

**Materials.** The patient with Factor IX<sub>CH</sub> is a 30-yr-old white male who has been followed at the University of North Carolina Hospital for 16 yr. He has a mild bleeding tendency and a Factor IX level of 5% of normal when tested in one-stage clotting assays. With a specific antibody to Factor IX, however, the patient was found to have 100% of Factor IX when measured by an antibody neutralization test and a radioimmunoassay for Factor IX (*vide infra*). All other clotting factors were normal. Plasma for purification of Factor IX<sub>CH</sub> was obtained from this patient who was previously shown to be CRM<sup>+</sup> (24). The patient was plasmapheresed every 2 wk to 1 mo, after informed consent, until sufficient plasma was obtained for the studies outlined below. The blood was handled in a manner similar to that for normal blood as described below. The resultant plasma was stored at -70°C and thawed at 37°C just before use. Normal, pooled human plasma was obtained from the Blood Bank of the North Carolina Memorial Hospital, Chapel Hill, N. C. Human blood was collected in plastic bags that contained standard citrate phosphate dextrose anticoagulant and centrifuged at 3,000 g for 30 min at 4°C to render the plasma platelet poor and free of erythrocytes. The plasma was then centrifuged under the same conditions. Plasmas used for the purification of normal Factor IX were either fresh or stored for <2 mo at -70°C.

Alumina C- $\gamma$  gel was purchased from Calbiochem, San Diego, Calif. Benzamidine hydrochloride and cyanogen bromide were purchased from Eastman Organic Chemicals, Div. Eastman Kodak Co., Rochester, N. Y. Heparin sodium salt, bovine serum albumin, dithioerythritol, dansyl chloride, dansyl amino acids, galactose, mannose, glucose, fucose, N-acetylneuraminic acid, and amino sugars were obtained from Sigma Chemical Co., St. Louis, Mo. DEAE-cellulose was Whatman DE-52 (Whatman Chemicals, Div. W & R Balston, Maidstone, Kent, England). DEAE-Sephadex (A-50), Sephadex G-150, Ribonuclease A, chymotrypsinogen A, and ovalbumin were purchased from Pharmacia Fine Chemicals, Uppsala,

Sweden. Bovine brain cephalin (Thromboplastin) was purchased from Ortho Pharmaceuticals Corporation, Raritan, N. J. Thromboplastin reagent (Simplastin) was purchased from General Diagnostics, Warner Lambert Company, Morris Plains, N. J. Agarose (Bio-Gel A-15 m, 100-200 mesh), Aminex A-4 Resin,  $\beta$ -mercaptoethanol, and materials used in gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, Calif. Celite 545 was purchased from Johns-Manville, Denver, Colo. All other chemicals were of reagent grade or better.

**Protein concentrations.** The Lowry method (25), with bovine serum albumin as a standard, was used for the estimation of protein concentration for the early stages of purification of Factor IX. Protein concentration on the final product was estimated by optical adsorption at 280 nm with experimentally determined extinction coefficients (*vide infra*).

**Coagulation assays.** 1 U of Factor VII, VIII, IX, or X clotting activity was defined as that amount in 1 ml of normal, pooled human plasma. A one-stage assay, based on the partial thromboplastin time, was used for the measurement of Factors VIII and IX (26, 27). Factors X and VII were measured by a one-stage assay based on the prothrombin time (28). Prothrombin was assayed with a two-stage method (29). Factor XI was assayed with artificially depleted human plasma (30) as well as plasma congenitally deficient in Factor XI (31). Factor IX immunological activity was assayed by an inhibitor-neutralization technique (12, 21). Factor IX was also measured by a radioimmunoassay with purified normal human Factor IX and a human anti-Factor IX antibody (32).

**Preparation of heparin-agarose.** Heparin-agarose was prepared by a modification of the method described by Porath (33) and Cuatrecasas (34).

**Electrophoresis.** Disc gel electrophoresis was performed in 0.2 M N,N-bis[2-hydroxyethyl]glycine-imidazole buffer at pH 7.8 for nondenatured protein and 0.025 M Tris-glycine buffer at pH 8.3 for sodium dodecyl sulphate-treated protein (35). 7.5 and 10% polyacrylamide gels were used for analytical separation of the proteins with 3% polyacrylamide as the stacking gel.  $\beta$ -Mercaptoethanol and dithioerythritol were used as reducing agents. Immuno-electrophoresis was performed on 1% agarose gel in 0.075 M Tris-barbital buffer at pH 8.6 which contained 2 mM of calcium lactate.

**Purification of Factor IX<sub>CH</sub>.** 60 ml of cold alumina gel was stirred into 600 ml of cold platelet-poor plasma, obtained from the patient with Factor IX<sub>CH</sub>, for 5 min at 25°C. The plasma was centrifuged and the supernate decanted. The adsorbate was washed twice with cold, normal saline and then eluted with 30 ml 0.3 M (NH<sub>4</sub>)<sub>2</sub> HPO<sub>4</sub> buffer at pH 8.5. The elution was repeated at least once. Pooled eluate was dialyzed against 4 liters of 0.01 M sodium phosphate buffer at pH 7.0. The dialysate was then applied to a DEAE-cellulose (DE-52, Whatman Chemicals) column (2.5  $\times$  25 cm) which was washed extensively with sodium phosphate buffer containing 0.13 M NaCl until the OD (280 nm) of the effluent was <0.01. The column was eluted with a linear gradient of NaCl (from 0.13 M to 0.30 M) with an automatic level sensor. Factor IX<sub>CH</sub>-rich fractions were pooled and dialyzed against 4 liters of 0.05 M sodium citrate at pH 7.0. The dialysate was applied to a DEAE-Sephadex column (2.3  $\times$  12 cm) (Pharmacia Fine Chemicals) which was washed with 0.08 M sodium citrate (pH 7.0) until the OD (280 nm) was <0.01. A linear gradient of sodium citrate from 0.08 to 0.30 M (pH 7.0) was then used to elute the column. Factor IX<sub>CH</sub>-rich fractions were pooled and dialyzed against 4 liters of 0.05 M imidazole-HCl buffer (pH 6.0) overnight, and the dialysate was brought to 2.5 mM CaCl<sub>2</sub> concentration with a 1-M CaCl<sub>2</sub> stock solution. A heparin-agarose column (1.1  $\times$  4.5 cm) was used for the last step of purification. After the Factor IX solution was applied, the column was washed with 250 ml of imidazole-HCl buffer which contained

0.25 M NaCl and 2.5 mM CaCl<sub>2</sub>. Factor IX was eluted with 0.4 M NaCl and 2.5 mM CaCl<sub>2</sub> in the same buffer. The procedures were carried out at 4°C except when stated otherwise. All solutions used in the purification procedure contained 1 mM benzamidine hydrochloride. Purification was monitored by a two-stage Factor IX neutralization assay as well as clotting assays for Factors II, VII, VIII, IX, and X.

**Purification of normal human Factor IX.** Purification of normal Factor IX was similar to that described for Factor IX<sub>CH</sub>.

**Preparation of Factor XIa.** 10 ml of normal, pooled plasma was adsorbed twice with alumina C-γ gel for 30 min at 25°C. After centrifugation, the supernatant plasma was incubated with 200 mg of prewashed Celite 545 (Johns-Manville) in a plastic tube at 37°C for 10 min. The tube was inverted every 1–2 min. The Celite suspension was collected by centrifugation and then washed 3 times with 10 ml of distilled water. Factor XIa was eluted from the Celite with 10 ml of 10% saline, and dialyzed against buffered citrated saline (pH 7.2). Factor XIa was also purified by the method of Lundblad and Kingdon (36) and used for the activation of purified Factor IX.

**Molecular weight.** The molecular weight was estimated by the electrophoretic mobility method with sodium dodecyl sulfate (SDS) disc gel electrophoresis as described above (35).

**Inhibitor neutralization assay and radioimmunoassay for Factor IX.** The inhibitor neutralization assay was performed according to the method of Roberts et al. (12). A radioimmunoassay was carried out according to the method described by Reisner et al. (32).

**Activation of Factor IX by Factor XIa and Ca<sup>2+</sup>.** 3 mg of purified, normal human Factor IX were incubated with 100 μg of partially purified Factor XIa at 37°C in 0.05 M imidazole buffer (pH 6.0) in a final volume of 3 ml which contained 5 mM of CaCl<sub>2</sub>. At selected time intervals an aliquot of the incubation mixture was added to platelet-poor, unactivated Factor IX-deficient substrate plasma obtained from a severely affected CRM<sup>-</sup> hemophilia B patient. A nonactivated, partial thromboplastin time and a Factor IX assay were performed on the final mixture. At the same time intervals, aliquots from the first incubation mixture were evaluated on SDS gel electrophoresis to determine the time of appearance of lower molecular weight components. Factor IX<sub>CH</sub> was also activated in the same manner, and monitored in a similar fashion. Appropriate controls were run by substituting imidazole buffer for Factor XIa, Factor IX, and Ca<sup>2+</sup> in the incubation mixture.

**NH<sub>2</sub>-terminal amino acid analysis.** The NH<sub>2</sub>-terminal amino acid was determined by Edman degradation (37) with a Beckman model 890-C Sequencer (Beckman Instruments, Inc., Fullerton, Calif.). Phenyl isothiocyanate was coupled to the free amino terminal group of the protein by incubation at 55°C for 15 min. Anhydrous heptafluorobutyric acid was used to release the phenyl isothiocyanate derivative of the NH<sub>2</sub>-terminal amino acid which was identified by gas chromatography. The NH<sub>2</sub>-terminal amino acid was also determined by thin layer chromatography with dansyl chloride (38).

**Amino acid composition of human Factor IX.** Amino acid analysis was performed by the method of Spackman et al. (39). Samples in 6 N HCl were sealed in Pyrex (Corning Glass Works, Corning, N. Y.) tubes (Research & Development Products, Berkeley, Calif.) at reduced pressure and hydrolyzed at 110°C for 24 and 72 h. γ-Carboxyglutamic acid was quantitated from alkaline hydrolysates of protein with a modification of the method of Hauschka et al. (40). 1 mg of each of these samples was dissolved in 2 ml of 2 N KOH and sealed at reduced pressure in glass tubes lined with polypropylene. Hydrolysis was carried out at 110°C for 24 h. After alkaline hydrolysis, samples were brought to pH 7.0 by drop-wise addition of 35% HClO<sub>4</sub>. The solutions were evaporated and the

amino acids were eluted from the precipitate cake with aliquots of 0.2 N sodium citrate buffer, pH 2.2. Amino acid analyses were performed on either a modified Phoenix model VG6000-B (Phoenix Precision Instrument, Div. Virtis Co., Inc., Gardiner, N. Y.) or a Beckman model 116 analyzer (Beckman Instruments, Inc.). The determination of cystine as cysteic acid was performed after Moore (41) with bovine prothrombin as a standard to determine the yield of the reaction. The relative amount of tryptophan to tyrosine in Factor IX was determined by a spectrophotometric method (42) in 0.1 N NaOH with a Cary 118 spectrophotometer (Cary Instruments, Fairfield, N. J.). Tryptophan residues per molecule of Factor IX were calculated from the tyrosine residues obtained in the acid hydrolysis.

**Carbohydrate composition.** Neutral sugars were determined by quantitative gas liquid chromatography (43) after being released from the protein by hydrolysis in 2 N H<sub>2</sub>SO<sub>4</sub> at 100°C for 5 h and converted to alditol acetates (44). A colorimetric method with the phenolsulfuric acid reaction and a 1:1 mixture of galactose and mannose as standard was also employed to confirm the result of total hexoses (45). Total hexosamine was determined according to Gardell (46). Sialic acid was assayed with 2-thiobarbituric acid (47).

**Extinction coefficient.** Purified normal Factor IX and Factor IX<sub>CH</sub> were rendered salt-free and lyophilized. The lyophilized proteins were then dissolved in 0.05 M imidazole-HCl buffer (pH 6.0) and the optical absorption at 280 nm was measured and extinction coefficients were calculated.

## RESULTS

Summaries of the purification results of Factor IX<sub>CH</sub> and normal Factor IX are shown in Tables I and II, respectively. Both normal Factor IX and Factor IX<sub>CH</sub> were purified from 17,000- to 18,000-fold with a yield ranging from 47 to 53%.

When the final products were electrophoresed on SDS gels only one band was seen for normal Factor IX and Factor IX<sub>CH</sub> as shown in Fig. 1. The apparent molecular weights according to this technique were 70,000 for both the normal product and Factor IX<sub>CH</sub>. When both normal Factor IX and IX<sub>CH</sub> were run in SDS gels in the presence of β-mercaptoethanol or dithioerythritol, only one band was seen, which in-

TABLE I  
Purification of Factor IX<sub>CH</sub>

Stage	Vol- ume	Total protein	Clotting activity	Yield	Purification
	ml	mg	U	%	-fold
Plasma	600	3.1 × 10 <sup>4</sup>	28.2	100	1
Al (OH) <sub>3</sub>	60	155	26.8	95	190
DE-52	150	5.89	19.5	69	3,640
DEAE- Sephadex	60	2.65	17.7	63	7,340
Heparin- agarose	35	0.96	16.3	58 (54)*	18,700 (17,300)*

\* Antigenic determination in parentheses.

TABLE II  
Purification of Normal Human Factor IX

Stage	Volume	Total protein	Clotting activity	Yield	Purification
	ml	mg	U	%	-fold
Plasma	2,000	$15.3 \times 10^4$	$1.7 \times 10^3$	100	1
Al(OH) <sub>3</sub>	203	$7.3 \times 10^2$	$1.66 \times 10^3$	97.2	204
DEAE-Sephadex	90	$1.1 \times 10^2$	$1.6 \times 10^3$	94.2	1,301
DE-52	180	10.3	$1.0 \times 10^3$	59.0	8,769
Heparin-agarose	45	4.2	$0.8 \times 10^3$	47.0	17,163

indicates that the zymogen form of both molecules is composed of a single polypeptide chain (Fig. 1). To show that the bands on SDS gels were indeed Factor IX, the purified normal Factor IX and Factor IX<sub>CH</sub> were electrophoresed on non-denatured polyacrylamide gels. Two gels for each product were used. One gel was stained with Coomassie Blue (Bio-Rad Laboratories, Richmond, Calif.) to identify the position of the protein band while the other gel was sliced into 1–2 mm lengths and eluted with buffered citrated saline as shown in Figs. 2 and 3. As can be seen in Fig. 2, normal Factor IX clotting activity was eluted in the same position as the protein band. In Fig. 3 both clotting and immunologic assays were used to detect Factor IX<sub>CH</sub>. On immunoelectrophoresis and immunodif-

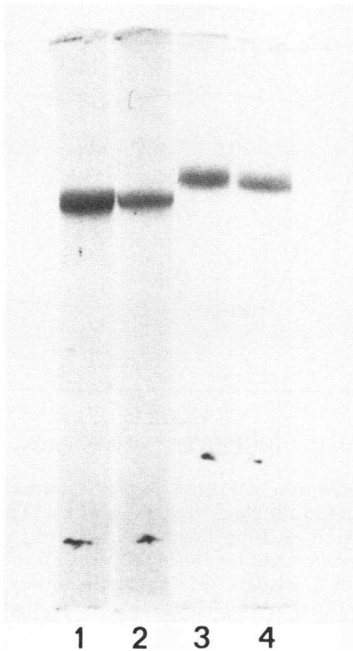


FIGURE 1 SDS gel electrophoresis of: (1) nonreduced Factor IX<sub>CH</sub>; (2) nonreduced normal Factor IX; (3) reduced Factor IX<sub>CH</sub>; (4) reduced normal Factor IX.

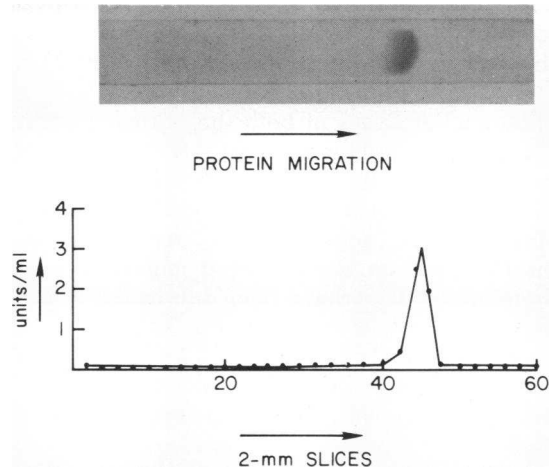


FIGURE 2 Disc gel electrophoresis of normal human Factor IX. 15  $\mu$ g of purified normal human Factor IX was electrophoresed in 7.5% polyacrylamide as shown on the top panel. Protein was identified by staining with Coomassie Blue. The migration was toward the anode. A duplicate gel was sliced into 2-mm segments, as shown in the lower panel, and each segment was crushed and incubated with 0.2 ml 0.15 M NaCl in 0.05 M imidazole-HCl buffer (pH 6.0) at 4°C overnight. The sample was centrifuged and the supernate assayed for Factor IX.

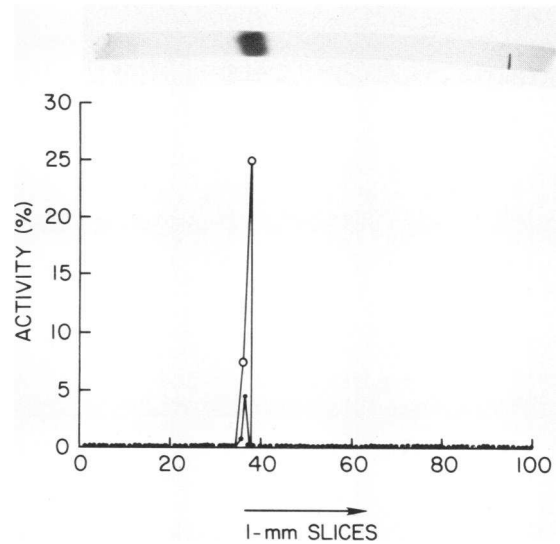


FIGURE 3 Disc gel electrophoresis of Factor IX<sub>CH</sub>. 15  $\mu$ g of purified Factor IX<sub>CH</sub> was electrophoresed on 7.5% polyacrylamide as shown in the top panel. The protein was identified by staining with Coomassie Blue. The migration was toward the anode. A duplicate gel was sliced into 1-mm segments and each segment was crushed and incubated with 0.1 ml 0.15 M NaCl in 0.05 M imidazole buffer (pH 6.0) at 4°C overnight. The Factor IX clotting activity was assayed with the one-stage method and the antigenic activity was assayed by the techniques described in Methods: (O) antigenic activity; (●) clotting activity.

fusion, purified normal Factor IX and IX<sub>CH</sub> were not recognized by antibodies to whole human serum, albumin, prothrombin, and Factor X but were reactive with anti-Factor IX antibodies.

Amino acid analyses of both the normal Factor IX and Factor IX<sub>CH</sub> are shown in Table III. The amino acid compositions of both Factor IX proteins are similar. Both normal Factor IX and Factor IX<sub>CH</sub> contain 10  $\gamma$ -carboxyglutamic acid residues per molecule of Factor IX based on our observed molecular weight for the peptide. They have been subtracted from the glutamic acid content as reported in Table III.

The NH<sub>2</sub>-terminal amino acid of both the normal and abnormal molecule is tyrosine, as determined by both automatic and manual methods on several determinations.

The carbohydrate content of normal Factor IX and IX<sub>CH</sub> is also shown in Table III. The carbohydrate content of each protein is nearly identical except for a

TABLE III  
Amino Acid and Carbohydrate Compositions of Normal Factor IX and Factor IX<sub>CH</sub>

Components	Factor IX	Factor IX <sub>CH</sub>
		<i>no. of residues</i>
Amino acid		
Asp	51.9	50.4
Thr	32.8	30.9
Ser	32.6	33.7
Glu	53.8	56.1
Pro	17.6	20.4
Gly	43.3	43.1
Ala	28.6	30.1
Cys/2	24.0	26.5
Val	32.1	31.5
Met	6.3	3.9
Ile	19.9	17.9
Leu	32.2	32.2
Tyr	18.3	18.1
Phe	23.0	19.8
Lys	29.7	29.1
His	10.0	10.7
Arg	22.1	20.5
Trp	10.0	10.1
Gla*	9.8	10.2
Total residues	498.0	495.2
Mol wt	56,600	56,600
Carbohydrates		%
Galactose	1.81	1.93
Mannose	1.72	1.65
Glucose	0.31	0.41
Fucose	0.39	0.51
Hexosamines	7.30	7.00
Sialic Acid	6.40	7.60
Total	17.93	19.10

\*  $\gamma$ -Carboxyglutamic acid.

slightly higher sialic acid content of Factor IX<sub>CH</sub>, but this is within the limits of experimental error.

The results of activation of both normal Factor IX and Factor IX<sub>CH</sub> are shown in Fig. 4. In this experiment, when normal Factor IX is activated by Factor XIa and Ca<sup>2+</sup>, the clotting times are progressively shortened. When purified Factor IX<sub>CH</sub> is activated by Factor XI and Ca<sup>2+</sup> under the same conditions, very little shortening of the unactivated partial thromboplastin time is observed. If Factor XIa, Factor IX, or Ca<sup>2+</sup> is omitted from the incubation mixture as a control, there is again very little shortening of the clotting time.

When the activation of normal Factor IX is followed by SDS gel electrophoresis, as shown in Fig. 5, the 70,000-mol wt band is converted to a lower molecular weight component (estimated mol wt 50,000) within 30 min. When Factor IX<sub>CH</sub> is activated under the same conditions, no change in molecular weight is observed. Even after incubation for 20 h, only very little lower molecular weight component was formed. These findings were consistent on several determinations.

The extinction coefficients ( $E_{280}^{1\%}$ ) were similar, 11.8 for Factor IX and 12.5 for Factor IX<sub>CH</sub>.

## DISCUSSION

An abnormal Factor IX molecule obtained from a patient with mild hemophilia B has been isolated and

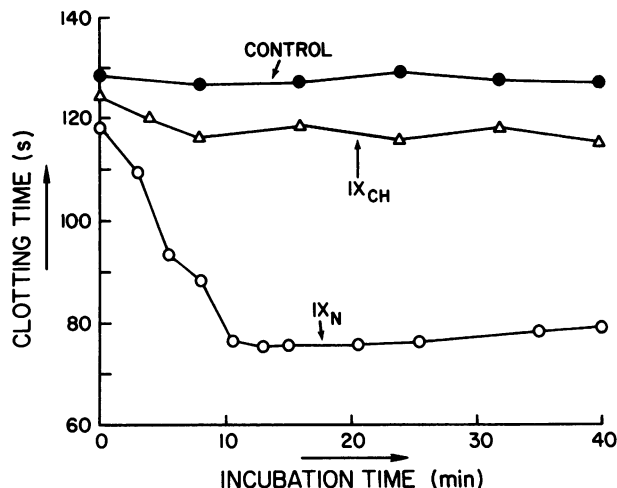


FIGURE 4 Activation of Factor IX<sub>CH</sub> and normal human Factor IX by activated human Factor XI and Ca. The incubation mixture consisted of 3 mg Factor IX protein (or buffer control); 100  $\mu$ g Factor XIa (or buffer control); 5 mM CaCl<sub>2</sub> in a final volume of 3 ml (0.05 M imidazole buffer, pH 6.0). At the indicated time intervals, 0.1 ml of the incubation mixture was diluted with imidazole buffer (1:200 for Factor IX and 1:20 for Factor IX<sub>CH</sub>) and was added to 0.1 ml unactivated Factor IX-deficient substrate. Clotting was achieved by the addition of 0.2 ml of a 1:1 mixture of 32 mM CaCl<sub>2</sub> and 10% Thrombafax in normal saline.

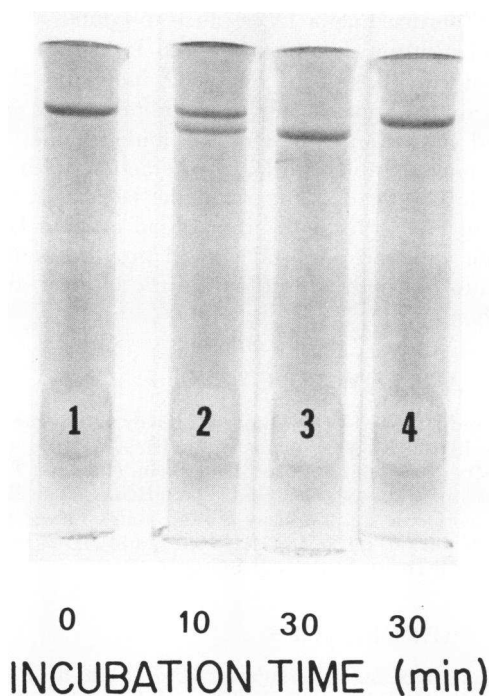


FIGURE 5 Activation of Factor IX<sub>CH</sub> and normal human Factor IX by activated human Factor XI and Ca. The activation conditions were the same as those described in Fig. 4 except that at the indicated time interval, an aliquot of the incubation mixture which contained  $\approx 15 \mu\text{g}$  protein was subjected to SDS gel electrophoresis. Gels 1–3 show normal Factor IX which was converted to a lower molecular weight component. Gel 4 shows Factor IX<sub>CH</sub> which showed no change in molecular weight.

characterized. The patient's plasma contains 5% Factor IX clotting activity but 100% Factor IX antigen as determined by radioimmunoassay and by antibody neutralization experiments as previously described. This variant Factor IX (Factor IX<sub>CH</sub>) was first described by Aronson (23) who noted that addition of SrCl<sub>2</sub> to the patient's plasma did not result in activation of the patient's Factor IX, while addition of SrCl<sub>2</sub> to normal plasma caused rapid activation of normal Factor IX. After activation promoted by SrCl<sub>2</sub>, the apparent molecular weight of the patient's Factor IX did not change, while that of normal Factor IX, as estimated on a Sephadex column (Pharmacia Fine Chemicals, Inc.), had a smaller molecular weight (23). However, Aronson used a plasma preparation, not a purified Factor IX.

We have purified Factor IX<sub>CH</sub> 18,000-fold. The product is homogeneous by several criteria. In the purification procedure, the DE-52 ion exchange and the heparin-agarose affinity chromatography are critical. In the former, the flow rate must be maintained at least at 60 ml/h. On heparin-agarose chromatography, calcium ions were used to enhance the binding of both normal Factor IX and Factor IX<sub>CH</sub> to the column. In

the presence of 2.5 mM CaCl<sub>2</sub>, prothrombin does not bind to the column and can be collected in relatively pure form after the sample application. The column is washed extensively with imidazole buffer, which contains 0.25 M NaCl and 2.5 mM CaCl<sub>2</sub>, to remove Factor X and prothrombin remaining in the column. Factor IX<sub>CH</sub> can then be eluted with 0.4 M NaCl in the same buffer.

Factor IX<sub>CH</sub> resembles normal human Factor IX in several respects. Although several other values of molecular weight for normal human Factor IX have been reported (2–6, 8) including one as low as 57,000 as determined by sedimentation equilibrium (8), higher molecular weights are found with the Laemmli SDS gel method (35). Nevertheless, it is apparent that both Factor IX moleculars have a similar molecular weight. In addition, both normal Factor IX and Factor IX<sub>CH</sub> have tyrosine as the NH<sub>2</sub>-terminal amino acid. Amino acid analyses of both proteins are very similar. Compared to analyses for normal human Factor IX published by other investigators (2, 3, 6, 8), by normalizing molecular weights to 50,000, our analyses were found to be in good agreement with some reports in terms of most of the individual amino acid (3, 8) as well as the total residues (8). This type of analysis, however, obviously does not exclude a single amino acid substitution in Factor IX<sub>CH</sub>. Furthermore, the carbohydrate content of the normal and abnormal molecules is similar and is in agreement with that reported by others for the normal molecule (3, 6, 8). Moreover, Factor IX<sub>CH</sub> contains the same number of  $\gamma$ -carboxyglutamic acid residues as normal Factor IX. Thus, there is no evidence that the abnormality in Factor IX<sub>CH</sub> is the result of defective  $\gamma$ -carboxylation of the molecule during postribosomal modification in the hepatic parenchymal cells.

Of particular interest are the studies on the activation of Factor IX<sub>CH</sub>. Activation of this abnormal Factor IX by Factor XIa and Ca<sup>2+</sup>, when followed by both coagulation assays and SDS gel electrophoreses, strongly suggests that the major abnormality in Factor IX<sub>CH</sub> is defective activation. These findings are in agreement with those reported by Aronson (23), except that activation of the purified protein is even slower than in a plasma system. Thus, it is possible that the major defect of Factor IX<sub>CH</sub> is delayed activation under physiological conditions. Factor IX<sub>CH</sub> may be the result of a single amino acid substitution governed by gene(s) on the X chromosome. Such a substitution could retard the cleavage of bonds that result in the two-chain Factor IXa molecule. Prolonged incubation of Factor IX<sub>CH</sub> with Factor XIa and Ca<sup>2+</sup> for up to 20 h does result in the formation of a lower molecular weight component, but this process is very slow and most of the Factor IX<sub>CH</sub> remains in the zymogen form. Activated Factor IX<sub>CH</sub> has not yet been isolated in amounts sufficient to com-

pare specific activity of Factor IX<sub>CH</sub> with that of normal Factor IX<sub>a</sub>. Fujikawa et al. (10) and Lindquist et al. (48) have shown, with purified bovine Factor IX, that an Arg-Ala bond is selectively cleaved by Factor XIa resulting in an inactive intermediate; a second proteolytic step cleaves an Arg-Val bond, releases a carbohydrate-rich activation peptide, and results in activated Factor IX. Whether the amino acid substitution in Factor IX<sub>CH</sub> is close to sites of cleavage by Factor XIa and Ca<sup>2+</sup>, or whether the substitution is remote and exerts its effect through tertiary conformational changes that hinder the proteolytic action of Factor XIa is not yet elucidated.

While it seems clear that Factor IX<sub>CH</sub> is not activated normally, it is difficult to determine whether this is the only defect. If Factor IX<sub>CH</sub> can be activated by substances such as Russell's viper venom or kallikrein, it would be possible to study the interaction of Factor IX<sub>a</sub> with calcium, lipid, and Factors VIII and X. These studies are currently in progress in our laboratory.

It could be postulated that the patient with Factor IX<sub>CH</sub> is synthesizing both a normal protein (that is contributing the 5% coagulant activity) and a nonfunctioning protein that retains antigenic activity but not clotting activity. This explanation seems unlikely on a genetic basis because hemophilia B is inherited as an X-linked recessive characteristic and it would be unlikely to have a gene coding for normal Factor IX and another for abnormal Factor IX. Moreover, in the activation experiment with clotting assays, one would expect 5% normal Factor IX to activate normally which was not the case. The Factor IX protein purified from the affected patient is probably composed entirely of a Factor IX protein that does not function normally in coagulation reactions.

Other abnormal Factor IX molecules almost certainly exist as initially postulated by Fantl et al. (49), Roberts et al. (12), and Hougie and Twomey (50). The latter authors described a hemophilia B<sub>M</sub> kindred which was shown to be CRM<sup>+</sup> by Brown et al. (16) and which has recently been purified and characterized by Østerud et al. (51). Factor IX<sub>BM</sub> differs from Factor IX<sub>CH</sub> in that the former is associated with a prolonged ox-brain prothrombin time and can be activated by Factor XIa in the presence of Ca<sup>2+</sup> (51). Furthermore, it should be noted that Factor IX<sub>CH</sub> is different from the Factor IX abnormality in hemophilia B Leyden (52). The Factor IX level in hemophilia B Leyden increases as the patient gets older. This is not the case with the patient with Factor IX<sub>CH</sub> who has been followed since childhood. Factor IX<sub>CH</sub> is also different from CRM<sup>R</sup> and CRM<sup>-</sup> kindred previously reported by us (53). Furthermore, Factor VII levels are normal in the patient with Factor IX<sub>CH</sub> who is thus different from the Factor IX variants reported by Kasper et al. (14) and Girolami et al. (15).

Studies of abnormal Factor IX molecules should help

in resolving some of the structure function relationships of normal Factor IX because the putative amino acid substitutions in the abnormal forms may affect different functions of the Factor IX molecule. For example, some abnormal Factor IX proteins may exhibit delayed activation by Factor XIa and calcium, while others may exhibit defective interaction with other blood clotting factors. The molecular basis of the interaction of Factor IX with other blood clotting factors, calcium, and phospholipid should provide better insight into the pathophysiology of the clinical disease state, hemophilia B.

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