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Research Article

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Factor IX Antigen by Radioimmunoassay

ABNORMAL FACTOR IX PROTEIN IN PATIENTS ON WARFARIN THERAPY AND WITH HEMOPHILIA B

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ABSTRACT Factor IX, isolated from normal human plasma, was homogenous by polyacrylamide gel electrophoresis in urea and sodium dodecyl sulfate. On the latter, it migrated as a single polypeptide chain with or without reducing agents and had an apparent mol wt of 62,000. After iodination by chloramine-T, a single peak of ^{125}I was found on gels. Immunoelectrophoresis in agarose with rabbit anti-factor IX sera gave a single arc against both isolated and partially purified factor IX preparations. The rabbit antibody was specific as it failed to inhibit the activities of prothrombin or factors VII or X in normal plasma. At an additional 20-fold dilution, factor IX activity was inhibited 50%.

In a double-antibody radioimmunoassay, excess rabbit anti-human factor IX precipitated 90–95% of the ^{125}I-human factor IX. Control without specific antibody gave 6–8%. Dilutions of a pool of normal human plasma paralleled dilutions of the isolated preparation and were used for the standard curve. Of 39 plasma samples from normal donors, the mean factor IX antigen level was 93% of that of a separate normal pool.

The radioimmunoassay detected the abnormal factor IX produced in patients on warfarin therapy. After $Al(OH)_3$ adsorption of warfarin treated patient's plasma, factor IX antigen, but not activity, was present in the supernate. Samples from 28 patients on warfarin gave a mean factor IX clotting activity of 27% with a mean antigen of 69%. The antigen level from the warfarin group was significantly lower than the antigen level of the normal group ($P < 0.001$).

The factor IX antigen level was then assessed in 36 patients from 29 pedigrees with hemophilia B. The median antigen level was 17% of normal. The distribution of the antigen level was wide with two patients around 100% of normal; only two had levels below the limits of resolution of the radioimmunoassay as currently performed (<2%). Within each of the five pedigrees in which more than one affected member was tested, activity and antigen levels were the same. The degree of neutralization of the antibody's inhibition of normal plasma by patient's plasma was highly correlated.

Additional evidence for the detection of abnormal protein was provided by immunodiffusion of plasmas concentrated by lyophilization. Reactions of complete identity occurred between normal, a warfarin treated and a hemophilia B subject's plasmas.

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INTRODUCTION

Factor IX is a coagulant protein which is required for intrinsic clotting. Congenital defects occur as the X-linked syndrome, hemophilia B (Christmas disease), and account for one-fifth the cases of hemophilia. Acquired defects are most frequently associated with the dependence of factor IX upon vitamin K for synthesis as an active protein.

Factor IX levels are usually assessed kinetically as clotting activity. For assay, the degree of correction of the prolonged intrinsic clotting of hemophilia B plasma is measured. The normal range is wide, generally 50–150% as compared to a normal plasma pool, and precision is moderate at best. On the other hand, most antigen assays of factor IX have been indirect, based upon neutralization of an antibody's inhibition of factor IX clotting activity. Antibodies used have been either human circulating anticoagulants (1) or antisera prepared in rabbits against partially purified factor IX preparations (2). However, tests with either of these preparations have lacked sensitivity, the lack of specificity has been an addi-

tional problem with rabbit antisera. A significant advance has been reported by Orstavik et al. (3) in which a quantitative immunoelectrophoresis system was developed in a highly purified, though not monospecific, system. Heretofore, a direct assay of factor IX antigen has not been achieved with specific, precipitating antibody.

Demonstration of a circulating, nonfunctional factor IX protein in patients on vitamin K antagonists could be achieved by a comparison of clotting activity and antigen level. Coumarin anticoagulation produces an acquired defect in the activities of prothrombim and factors VII, IX, and X. In bovine prothrombin, deficient carboxylation of glutamyl residues near the amino terminus occurs (4) and a nonfunctional protein circulates. The amino terminal sequence of bovine factor IX is homologous with bovine prothrombin (5), indicating an identical mechanism of action of the coumarins on these proteins. Although there has been no direct evidence for an abnormal, circulating human factor IX molecule, kinetic (6) and neutralization (7, 8) studies have suggested its presence.

Patients with hemophilia A have normal levels of factor VIII antigen (9), and circulate an immunologically cross-reactive, nonfunctional protein. In von Willebrand's disease, inherited as an autosomal dominant trait, there are low levels of factor VIII antigen (9) such that there is either rapid clearance of an abnormal factor or decreased production. There is current controversy about the nature of the factor VIII antigen, von Willebrand's factor (10), which raises questions as to the validity of the data for hemophilia A or its use as a model for other hemophilias.

In groups of patients with hemophilia B, the majority of cases have been associated with either undetectable (1, 11, 12) or normal (11) levels of factor IX antigen by neutralization tests. The presence of antigen in reduced quantities in some patients with hemophilia B was first suggested by Neal et al. (13) by using an immunosorbent technique. Indeed, of the 11 pedigrees studied by Orstavik et al. (3) 5 had significantly positive neutralization tests and of these, 3 had reduced levels on quantitative immunoelectrophoresis. It is not clear, however, if the previous factor IX antigen assays are a valid indication of the presence or incidence of factor IX protein in patients with hemophilia B.

Factor IX was isolated from plasma and was used to produce a specific rabbit anti-factor IX antibody. The antigen was then labeled for use in a radioimmunoassay with the rabbit antibody and goat anti-rabbit IgG sera. Antigen level was then compared to clotting activity in a series of normal subjects, a group of patients on warfarin therapy, and in patients from pedigrees with hemophilia B. A preliminary account of these results has been presented (14).

METHODS

Subject samples. Normal plasma samples were taken from volunteer blood donors at the Puget Sound Blood Center. The age range of the control group was 18–60 yr and 54% were women. Samples from consecutive patients on warfarin therapy were taken from specimens submitted for prothrombin time measurements to the Hematology Laboratory, U. S. Public Health Service Hospital. Patients' charts were reviewed and samples were excluded where there was any evidence of concurrent liver disease. Patients from pedigrees with hemophilia B had samples drawn for routine or diagnostic testing under the Hemophilia Care Program of the Puget Sound Blood Center. They had received no blood products for 2 wk before drawing the sample. Samples were collected into 1/10th vol 0.12 M sodium citrate (pH 6.8), rendered platelet poor by final centrifugation at 45,000 *g* for 20 min, and were either assayed immediately or flash frozen in dry ice-ethanol and stored at −80°C before assay.

Materials. Materials were obtained from the following suppliers: Ammonium sulfate, ultra pure, from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.; DEAE-A50 Sephadex (fine), G-50 Sephadex (fine), and G-25 Sephadex from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; heparin, grade 1, and rabbit brain cephalin, from Sigma Chemical Co., St. Louis, Mo.; benzamidine HCl from Aldrich Chemical Co., Inc., Milwaukee, Wis.; Freund's adjuvants from Difco Laboratories, Detroit, Mich.; $Al(OH)_3$, unflavored, from Wyeth Laboratories, Philadelphia, Pa.; Na ^{125}I, 100 mCi/ml, from New England Nuclear, Boston, Mass.; chloramine-T from Eastman Kodak Co., Rochester, N. Y.; bovine serum albumin, fraction V, from Reheis Chemical Co., Chicago, Ill.; agarose from Sea Kem, Rockland, Maine; and Coomassie brilliant Blue, R-250, from Bio-Rad Laboratories, Richland, Calif. Phosphate-buffered saline was a solution of 0.12 M NaCl, 5.5 mM Na_2HPO_4, and 3.2 mM KH_2PO_4, pH 7.2. Other chemicals were at least reagent grade. Thrombotest reagent was from Nyegaard, Oslo, Norway (distributed by BioQuest, BBL & Falcon Products, Cockeysville, Md.). Unless otherwise indicated, radioimmunoassays were performed with normal rabbit IgG and goat anti-rabbit IgG prepared as ammonium sulfate fractions from sera of these animals (15). Similar results were obtained with IgG (4 mg/ml) and goat anti-rabbit IgG (P 4, SA) from Antibodies Inc., Davis, Calif. (see legend, Fig. 3). Heparin agarose was prepared by the cyanogen bromide technique as previously described (16, 17).

Purification of factor IX. This was accomplished by a modification of the previously described method for bovine factor IX (16, 17). Normal blood was collected from volunteer donors into citrate-phosphate-dextrose and, after removal of erythrocytes, heparin (4 U/ml) was added to the plasma. For the initial purification step, $BaCl_2$ (45 gm) was added to 3 liters plasma at 4°C and stirred for 30 min. Barium citrate was collected by centrifugation and washed with 1 liter 0.15 M $BaCl_2$ then 1 liter H_2O. Proteins were eluted with 1 liter 0.15 M sodium citrate (pH 7.5) containing 1 mM benzamidine for 30 min at 23°C. Ammonium sulfate, 267 g (30% saturation) was added and stirred for 15 min at 4°C. After centrifugation, an additional 133 g ammonium sulfate was added to the supernate (60%), the pellet collected by centrifugation, and dissolved by the addition of 10 ml 1 mM benzamidine (in H_2O). The preparation was desalted on 2.5 × 20-cm columns of G-25 Sephadex equilibrated with 70 mM sodium citrate (pH 7.5) with 1 mM benzamidine. Protein was then applied to a 5 × 20-cm column of DEAE-Sephadex equilibrated with the same buffer

TABLE I
Purification of Factor IX

	Volume	Protein	Factor IX	Yield	Purification
	ml	*A.U. (280 nm)**	*clotting units*	*%*	*fold*
Plasma	3,500	245,000	2,905	100	
Barium citrate eluate	1,000	2,450‡			100*
Ammonium sulfate (30–60%)	50		2,250	77	
DEAE-Sephadex	215				
Ammonium sulfate (60%)	24	312	1,200	41	300
Heparin-agarose	20				
Ammonium sulfate (60%)	5	4.8	360	12	6,000

* A.U., absorbancy units.

‡ Approximate value; preparation is turbid at this point.

and then eluted with an exponential gradient containing 70 mM sodium citrate (pH 7.5) in a 450-ml lower chamber to 0.15 M sodium citrate (pH 7.5) in a 2-liter reservoir; both buffers contained benzamidine. The peak containing the vitamin K-dependent clotting factors was pooled, concentrated by precipitation with 390 g/liter solid ammonium sulfate at 4°C, centrifuged, taken up in 5 ml 50 mM Tris (pH 7.5) with 50 mM NaCl and 1 mM benzamidine, and then gel filtered through G-25 Sephadex in this buffer. At this point the preparation was divided in half and applied to 2.5 × 3.0-cm bed volume of heparin agarose equilibrated with the Tris-NaCl buffer with 1 mM $CaCl_2$. The gel-filtered, concentrated, DEAE eluate had calcium added (to 1 mM) before application to the heparin agarose column. After application, 20-ml washes of buffer containing 50 mM NaCl, 0.2 M NaCl, 0.3 M NaCl, and 0.5 M NaCl were applied. The breakthrough protein contained predominately prothrombin; the 0.2-M NaCl wash, prothrombin with some factor X; the second wash, factor X activity with some factor IX (up to 20% of that applied); and the 0.5-M wash, only factor IX activity. Where necessary, the effluent protein was concentrated to 1 mg/ml by ammonium sulfate (60%) after the chromatography. The entire purification procedure could be carried out in 33 h.

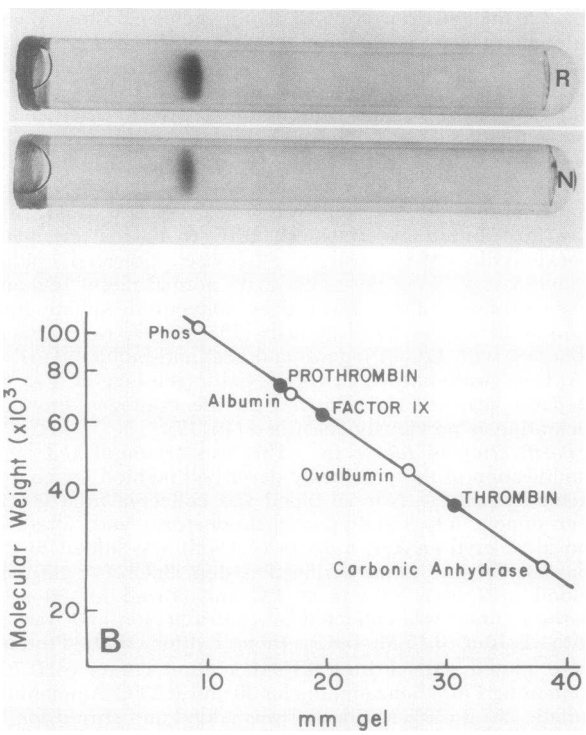

FIGURE 1 Gel electrophoresis in sodium dodecyl sulfate and 7.5% polyacrylamide (18). (A) Gel R represents dithiothreitol reduced and N depicts non-reduced samples of 30 μg factor IX protein. Anode is to the right; gels measured 95 mm. (B) Relative migration of factor IX vs. standard proteins (see 19; Phos is phosphorylase), prothrombin and thrombin. Mol wt estimate is 62,000 for factor IX.

The purification is summarized in Table I, where the final preparation contained 75 factor IX U/mg. Assays of 1:10 dilutions of the factor IX preparation (1 mg/ml) for prothrombin, factor VII, and factor X were equal to buffer control values. Gel electrophoresis of purified factor IX showed a single band in sodium dodecyl sulfate (Fig. 1A). It was also homogenous by disc-gel electrophoresis in urea (20) (not shown). Factor IX is more anodal than factor X in the latter gels but they migrate the same in sodium dodecyl sulfate.

Radioiodination. Factor IX was labeled with ^{125}I by the chloramine-T method of Hunter (21). To 100 μl factor IX (50–100 μg) in 25 μl 0.5 M sodium phosphate buffer (pH 7.5) and 10 μl Na ^{125}I (1,000 μCi), 10 μl chloramine-T (3 μg) was added and reacted for 10 s. The reaction was stopped by the addition of 10 μl of a fresh solution of sodium metabisulfite (containing 8 μg) and 10 μl KI (2 mg) in the phosphate buffer. The sample was applied to a 0.5 × 20-cm column of G-50 Sephadex through which 200 mg bovine serum albumin had been run to prevent adsorption. The reaction tube was washed with an additional 100 μl of the phosphate buffer (containing 2 mg KI) which was then applied to the column and the column begun. Equilibration and elution of this column was performed with phosphate-buffered saline. In eight preparations, the initial elution peak contained from 5 to 30% of the total counts per minute. For the 10-s reaction as described above, factor IX clotting activity was unaltered by iodination. Gel electrophoresis of the radioiodinated factor IX gave a single sharp peak of radioactivity (Fig. 2).

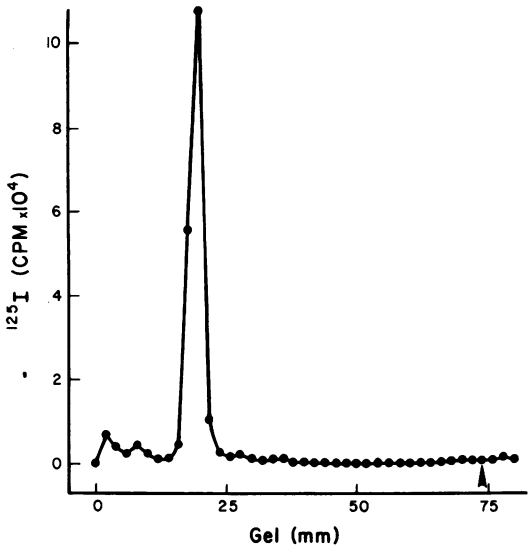

FIGURE 2 Gel electrophoresis in 10% polyacrylamide of ^{125}I-factor IX revealing the preservation of a single molecular species after iodination. Arrow (73 mm) indicates anodal migration of marker dye. Gels were fractionated in a Gilson Aliquogel Fractionator (model GMA/GCB, Gilson Medical Electronics, Inc., Middleton, Wis.).

Radioimmunoassay. The double antibody system was adapted from the radioimmunoassay of Buchanan et al. (22), employing final precipitation by goat anti-rabbit IgG. Rabbit anti-factor IX antibody was prepared by intradermal injections of Freund's adjuvants, emulsified with 0.5–1 mg aqueous antigen, and given 2–4 wk apart. Blood was collected at 7–14 days after the final boost and sera used for clotting assays was adsorbed with 1/10th vol $Al(OH)_3$, which was removed by centrifugation (45,000 *g*, 20 min).

For the assay, anti-factor IX (10 μl, 1:400–1:5000 dilution in 1:5 normal rabbit sera or IgG) was added to 250-μl tubes (Beckman Instruments, Inc., Fullerton, Calif.) containing dilutions of plasma samples (20 μl), and ^{125}I-factor IX (20 μl, 1:4–1:100 dilution in phosphate-buffered saline with 1% albumin) and incubated overnight. Goat anti-rabbit IgG (50 μl containing 25–40 μl) was added and incubated 1 h. The precipitate was then centrifuged 5 min in a Beckman model 152 microfuge, the supernate aspirated, and the pellet counted. No wash was necessary; incubations were performed at 4°C. To enhance sensitivity in samples with low antigen levels, the diluted plasma was incubated 1 or 2 h with rabbit anti-factor IX before adding the labeled antigen. Four or more dilutions (e.g. 1:2, 1:4, 1:10, 1:20, 1:40, 1:80, in phosphate-buffered saline with albumin) of patient's plasma were compared to dilutions of pooled plasma from 30 normal human subjects and the percent of normal determined from a plot of percent bound vs. log dilution (Fig. 3). Dilution curves of isolated factor IX paralleled those of normal plasma. Using $E_{1\,cm}^{1\%} = 13.2$ for human factor IX,[1] the level of factor IX protein in the normal plasma pool was 4.2 μg/ml. The radioimmunoassay was sensitive to 2% of normal levels;[2] for duplicate determinations, counts per minute agreed to within ±5%. Data relating to experimental error is presented in results.

Clotting assays. Factor IX clotting activity was determined by a modification of the one-stage, kaolin-activated partial thromboplastin time (23) in which substrate plasma was from patients with severe congential hemophilia B who lacked neutralizing activity. For the assay, 0.1 ml deficient plasma, 0.1 ml 1:20–1:40 dilution of rabbit brain cephalin, and 0.1 ml kaolin (2% in 0.1 M NaCl) were preincubated with 0.1-ml sample (diluted in veronal buffer, 0.1 ionic strength, pH 7.5) for 2 min at 37°C, and then 0.1 ml 35 mM $CaCl_2$ was added. The siliconized tubes were then tipped and timed until a visible clot was formed. Four dilutions of sample were assayed and compared to the log dilution vs. activity of a normal human plasma pool (30 donors).

The prothrombin time, partial thromboplastin time, and specific assays of prothrombin, factor VII, and factor X were performed as previously described (24). The thrombotest was run according to Owren (25) with fibrometers (Bio-Quest, BBL & Falcon Products). For samples from the preparation before DEAE-Sephadex chromatography, heparin was adsorbed by ECTEOLA cellulose columns (24) before assay.

Neutralization of anti-factor IX inhibition was determined by using the factor IX assay in a method similar to that of Roberts et al. (1): 0.1 ml patient sample or buffer (0.1 M NaCl, 50 mM Tris, pH 7.5) was incubated at 37°C for 5 min with 1 μl of a 1:5 or 1:10 dilution of $Al(OH)_3$-adsorbed rabbit anti-human factor IX. Then 0.1 ml normal human plasma was added for an additional 25 min incubation before dilution and assay. The buffer controls (buffer, normal plasma, and antibody) gave 50 and 88% inhibition for the rabbit antibody for 1:200 and 1:100 final dilution, respectively. Values reported are the percent of this inhibition that was neutralized by the patient's sample. For 1:200 antibody, for example, 50% neutralization of inhibition would then correspond to a patient sample with normal antigen but no activity, allowing full expression of the normal plasma's activity.

Statistical methods were as described in Bruning and Kintz (26).

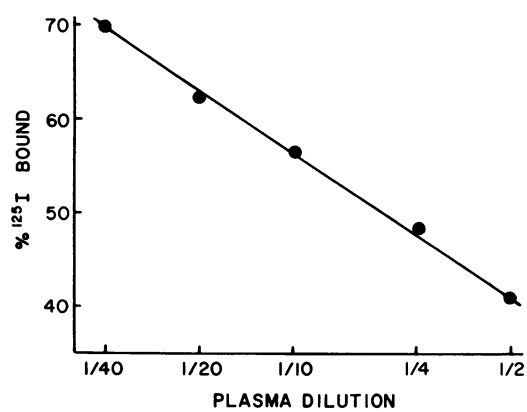

FIGURE 3 Standard curve of normal plasma. For this assay, rabbit anti-factor IX was diluted 1:400 in a 1:5 dilution of rabbit IgG; added goat anti-rabbit IgG contained 25 μg. When commercial rabbit IgG and goat anti-rabbit IgG were employed, a range of 25–50% inhibition was used. Pellets were counted in a Packard Auto Gamma Scintillation Spectrometer (model 5220, Packard Instrument Co., Inc., Downers Grove, Ill.).

[1] Desipio, R., and E. W. Davie. Unpublished results.

[2] 2% was the lowest level at which three or more dilution points could be compared with the dilution curve of normal plasma.

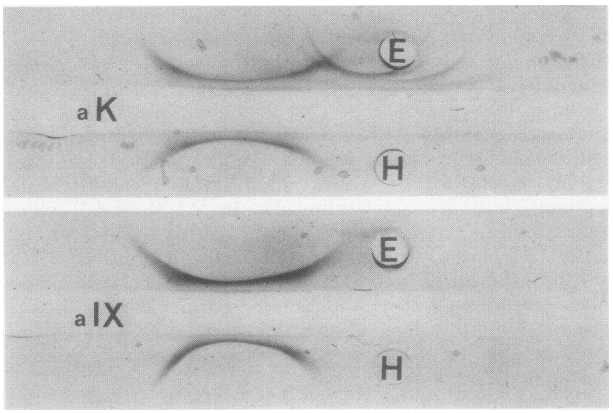

FIGURE 4 Immunoelectrophoresis of factor IX in agarose. Center trough contains rabbit antisera where aK represents antibody which neutralized prothrombin and factors IX and X, and aIX was the specific anti-factor IX antibody. H represents 20 μg heparin-agarose purified factor IX; E represents 100 μg barium citrate eluate. After drying, gels were stained with Coomassie brilliant blue. With either antibody preparation, a single precipitin arc was formed with purified factor IX (H). In addition, crude or purified factor IX gave single arcs to the specific antibody (a-IX), with or without $Al(OH)_3$ adsorption. A faint streaking to the left of well E in the lower slide was present when eluate was run against normal rabbit sera and thus represents an artifact. It was not seen in eight preparations of purified antigen. 1% agarose was used in barbital, 0.04 ionic strength, pH 8.6 (27).

RESULTS

The factor IX preparation was homogenous by polyacrylamide gel electrophoresis in urea or sodium dodecyl sulfate. With the latter, there was no change on reduction (Fig. 1A) indicating a single polypeptide chain, and the mol wt estimate was 62,000 (Fig. 1B). When co-electrophoresed with bovine factor IX (17), the migration was identical (not shown). Factor IX was also homogenous by immunoelectrophoresis. As shown in Fig. 4, rabbit antibody to partially purified or isolated factor IX gave single precipitin arcs when run against the purified factor IX antigen. Likewise, antibody to a partially purified factor IX gave a single arc against purified factor IX (Fig. 4). The resulting factor IX preparation contained no detectable clotting activity of other vitamin K-dependent factors. A 1:100 initial dilution of the factor IX preparation (0.5 mg/ml) gave a clotting time of 58 s, but in the absence of kaolin, a 1:10 dilution had not clotted in 300 s. Thus, activated forms of factors IX and X, if present, could account for less than 1% of the total activity.

Specificity of the antibody. The rabbit antibody prepared against isolated factor IX was further tested for inhibitory activity against prothrombin and factors VII, IX, and X. For inhibition, 0.4 ml normal plasma was incubated 30 min, 37°C, with 40 μl $Al(OH)_3$-adsorbed rabbit anti-factor IX. Specific factor assays were performed with inhibition representing the decrease in activity vs. an incubated control sample of plasma without antibody. Undiluted there was no inhibition of prothrombin or factors VII or X; factor IX activity was inhibited to 5%. A 1:200 final dilution was necessary to achieve 50% inhibition of factor IX clotting activity. Immunodiffusion experiments with this antibody failed to detect factor IX in normal plasma without concentration. When plasmas were concentrated by lyophilization, immunodiffusion in agarose gave single precipitin lines. A reaction of complete identity was seen between the lines of normal, warfarin-treated, and hemophilia B plasmas (Fig. 5).

Radioimmunoassay. With excess rabbit anti-factor IX (e.g. 1:30, 1:100), the second antibody precipitated 90–96% (e.g. Fig. 6) of counts in eight preparations of labeled antigen. When rabbit anti-factor IX was omitted from the assay, the pellet gave 6–8% of the initial antigen counts (without washing). For antigen level determinations, the absolute values were somewhat dependent upon experimental conditions (e.g. preincubation, concentration, and preparation of iodinated antigen or rabbit or goat anti-sera). Day to day variation of the standard curve was $< \pm 10\%$ for a given set of conditions.

To assess experimental error, three separate dilutions were performed on plasma samples containing either near normal, half normal, or low antigen levels. The assay results were 118, 113, and 105% for a normal subject (whose levels had been 105 and 112 on previous assays); 52, 53, and 58% for a warfarin-

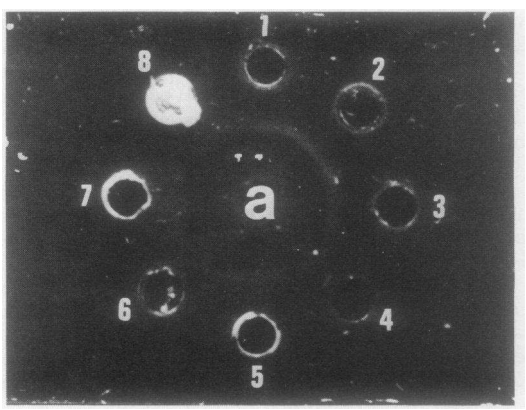

FIGURE 5 Immunodiffusion in 0.5% agarose (27). Center well contains rabbit antifactor IX serum (a) and plasma samples were concentrated by lyophilization and reconstitution with water to one-fifth starting volume. Plasma sample wells are numbered as follows: 1, 3, 5, and 7 are normal; 2 and 4 are hemophilia B (patients 12 and 1, respectively, from Table IV); and 6 and 8 are warfarin treated (patients 27 and 28, respectively, from Table III).

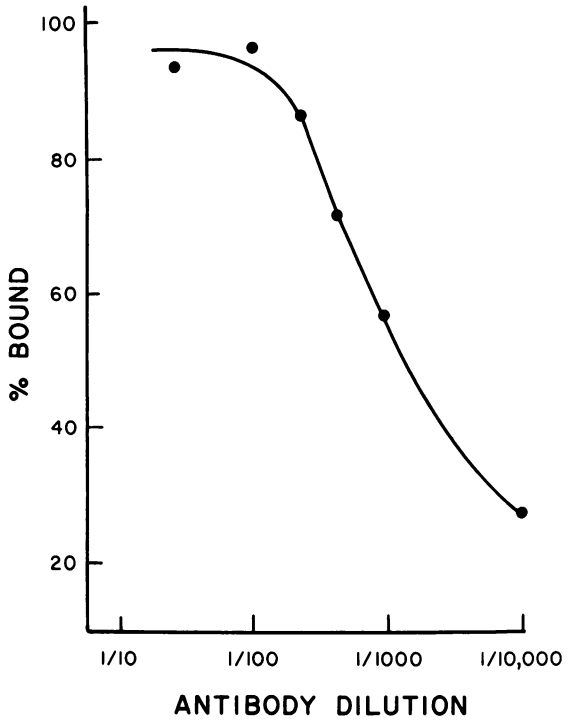

FIGURE 6 Antibody dilution curve of ^{125}I-factor IX and specific rabbit anti-factor IX, demonstrating greater than 90% precipitation of labeled antigen at high antibody concentrations.

treated patient (previously 53); and 14, 14, and 15% for a patient with hemophilia B (previously 15%), such that the reproducibility of the assay was $< \pm 10\%$ of the given value throughout a wide antigen range.

Normal controls. Of the 39 normal control subjects, the mean factor IX clotting activity was 93% and the mean antigen level was also 93% (Table II). The correlation coefficient (r) for the activity vs. antigen was 0.39 ($P < 0.02$); with log transformed data, r was calculated as 0.52 ($P < 0.001$). Of note, when the data in Table II are rank ordered, Spearman's rho = 0.85. The distribution of log clotting activity vs. log antigen level is presented in Fig. 7.

To assess the effect of increasing the number of data points on precision, samples from 10 subjects (see asterisks, Table II) were chosen for repeat measurements. Two dilution sets and replicates of each dilution were performed such that four times as many assays were determined for both clotting activity and antigen level. Compared to a normal plasma pool (which differed from that in Table II), clotting activities increased and decreased in four subjects each. Antigen levels were increased in eight and unchanged in two subjects. The correlation coefficient increased from $r = 0.16$ (data in Table II) to $r = 0.56$ for the repeat data on these subject's samples.

Patients on warfarin. Table III presents the assay data for samples from 28 patients on warfarin therapy in the order of their factor IX clotting activity. The mean prolongation of the prothrombin time was 23 s or 1.9 times normal; the thrombotest prolongation was strongly correlated ($r = 0.76$). Correlation of decreased factor IX activity with the prolongation of each of these extrinsic tests (for prothrombin time, $r = -0.73$; for thrombotest, $r = -0.53$) was varied but highly significant, such that the degree of anticoagulation is reflected by the factor IX clotting activity in this group. The mean clotting activity was 28%. The mean factor IX antigen level was 69% and dilution curves of each patient's plasma paralleled those of normal control plasma. The mean warfarin antigen level was significantly lower than that (93%) of the normal controls ($t = 4.39$, $P < 0.001$). In the warfarin group the correlation coefficient between factor IX clotting activity and antigen was $r = 0.39$ ($P < 0.05$). The log-transformed data comparing clotting activity to antigen levels are presented in Fig. 7.

To demonstrate that the radioimmunoassay indeed detects abnormal factor IX protein in warfarin pa-

TABLE II
Factor IX Results on Normal Subjects

Subject number	Factor IX Clotting activity	Factor IX Antigen level	Subject number	Factor IX Clotting activity	Factor IX Antigen level
	%			%	
1	125	62	21*	96	80
2*	122	100	22	95	94
3	120	105	23	93	93
4	115	100	24	92	125
5	114	100	25*	92	82
6	113	70	26*	91	90
7*	112	84	27	84	84
8	110	100	28	83	110
9	108	108	29	83	83
10	105	113	30	81	105
11	105	100	31	79	105
12	103	108	32*	77	96
13	103	104	33*	76	88
14	102	108	34*	73	111
15	101	140	35	72	105
16	101	100	36*	61	66
17	100	95	37	56	56
18	99	75	38	55	55
19	98	88	39	47	47
20*	97	82			
Mean				93	93
SD				19	19

* Subjects whose plasmas were re-assayed with more duplicate and replicate determinations to assess precision as described in the text.

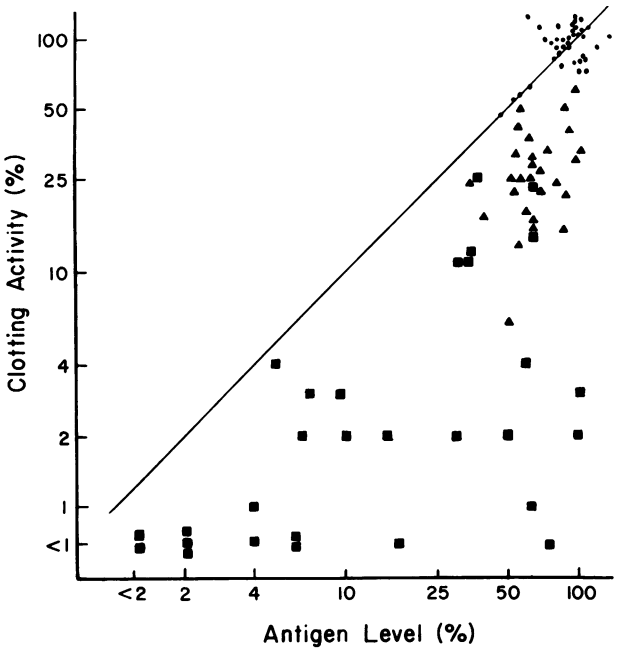

FIGURE 7 Factor IX clotting activity vs. antigen level in normal subjects (●), patients on warfarin (▲), and patient pedigrees with hemophilia B (■). The straight line represents a clotting activity:antigen level ratio of 1.0.

tients, two samples (from patients 12 and 22, Table III), were treated with 1/10th vol of $Al(OH)_3$, which primarily adsorbs active vitamin K-dependent factors, and, after centrifugation, <1% factor IX clotting activity remained in the supernate. Factor IX antigen was detected at 10% in each dialyzed supernate from the warfarin-treated patients whereas the antigen level was undetectable (<2%) in the supernate of adsorbed, dialyzed normal plasma. Due to the partial absorption of abnormal vitamin K-dependent proteins and interference of Al salts in the radioimmunoassay, it was not possible to quantitate the level of abnormal factor IX antigen in samples from these patients.

Hemophilia B patients. Assay results from members of 29 pedigrees are presented in Table IV, in the order of their clotting activity level. The median factor IX clotting activity was 2% (range <1–25%) whereas the median antigen level was 17% (range <2–103%). Five pedigrees had more than one affected member tested and the activity and antigen levels were the same within each family (Table V). Neutralization results correlated highly with the antigen level (Spearman's rho = 0.93, $P < 0.001$). For the 16 pedigrees with ≤17% antigen, 12 were negative in the neutralization test with a lower concentration of rabbit anti-factor IX (1:200, final dilution) and 4 were weakly positive. When the neutralization test was repeated in these patients with a higher antibody concentration (1:100), only six pedigrees with the lowest antigen levels (≤4%) were consistently negative by neutralization.

As shown in Fig. 7, affected members of 11 pedigrees had the severe form of the disease defined by the clinical presence of spontaneous bleeding and factor IX activity levels of <1% of normal. Of these, 8 had antigen levels below 10%, 1 had 17%, and the 10th averaged 73%. The members of 13 pedigrees had a moderately severe form of the disease (clotting activity, 1–4%). Their antigen levels range from 4 to 103%. Six pedigrees had mild defects (clotting activity, 11–25%) and their antigen levels were 30–66%.

TABLE III
Warfarin-Treated Patient Assays

Patient	Factor IX		Prothrombin time*	Thrombotest time*
	Clotting activity	Antigen level		
	%			s
1	60	100	17.6	82
2	50	90	18.0	86
3	50	58	20.6	95
4	42	57	19.6	78
5	40	94	18.4	95
6	37	62	24.6	112
7	33	105	21.9	109
8	33	76	21.2	128
9	32	55	18.4	73
10	30	100	20.4	82
11	30	66	26.9	124
12	30	66	22.9	92
13	27	70	23.5	116
14	25	60	26.8	163
15	25	58	23.4	134
16	25	56	27.4	126
17	24	83	25.5	89
18	24	35	20.9	87
19	22	70	27.1	146
20	22	54	25.1	92
21	21	91	25.0	117
22	18	62	24.4	127
23	17	40	25.2	104
24	16	66	24.2	104
25	16	66	23.1	120
26	15	88	26.0	113
27	13	57	30.7	174
28	6	52	25.1	133
Mean	28	69	23	111
SD	12	18	3	25

* Controls were: prothrombin time, 12.4–12.9 s and thrombotest, 40 s.

The thrombotest, employing commercial ox-brain thromboplastin, was performed on patients from 28 pedigrees to determine if any represented the B_m variant described by Hougie (28). B_m is probably associated with high levels of a defective factor IX antigen, and is characterized by prolonged ox brain, but not human brain, extrinsic clotting. Partial correction of the thrombotest by 1:1 mixture with normal plasma has been given as evidence for inhibition by the abnormal antigen. As shown in Table IV, seven of our patients had ≥50 s (longest normal was 53 s). Four of these seven were corrected to 42 s or less after 1:1 mixture with normal plasma. As expected, samples which corrected also had significant prolongation of the prothrombin times with human brain thromboplastin. In the other three, patients 3, 9, and 12, the correction was to 45, 45, and 46 s, respectively. Thus, none of our patients had hemophilia B_m although the longest thrombotest times after dilution with normal plasma (while still normal) were in three patients with normal antigen levels.

TABLE IV
Hemophilia B Pedigree Assay Results

Pedigree number	Age	Factor IX		Neutralization		Thrombotest*
		Clotting activity	Antigen level	1:200	1:100	
	yr	%		%		*s*
1	22	25	37	40		36
2	‡	23	56	44		46
3	18	14	66	50		59
4	19	12	35	30		44
5	10	11	34	23	35	39
6	6	11	32	43		44
7	48	4	60	48		47
8	57	4	5	0	8	48
9	44	3	103	50		57
10	‡	3	10	0	11	40
11	26	3	7	0	6	39
12	58	2	100	50		64
13	22	2	50	23		34
14	22	2	30	28	27	38
15	‡	2	15	0	2	46
16	22	2	10	16	5	40
17	‡	2	7	0	9	47
18	8	1	63	48		46
19	46	1	4	13	4	38
20	‡	<1	73	51		43
21	22	<1	17	4	6	47
22	27	<1	6	4	7	46
23	34	<1	6	0	14	
24	34	<1	4	0	0	48
25	7	<1	2	0	0	38
26	30	<1	2	0	0	59
27	14	<1	2	0	0	41
28	28	<1	<2	0	0	39
29	62	<1	<2	0	0	56

* Normal control = 40 s.
‡ See Table V.

TABLE V
Hemophilia B Assays in Multiple Patient Pedigrees

Pedigree	Age	Factor IX	
		Clotting activity	Antigen level
	yr		%
2a	67	25	60
2b	19	20	52
10a	20	3	10
10b	3 wk	3	9
15a	16	2	14
15b	15	2	16
15c	13	2	15
15d	11	2	15
17a	18	2	7
17b	7	2	6
20a	60	<1	78
20b	13	<1	68

DISCUSSION

Immunoassays of clotting factors. Protein concentrations of clotting factors have generally been determined by three types of antigenic tests: (*a*) neutralization of inhibition of clotting activity; (*b*) quantitative immunoelectrophoresis; and (*c*) radioimmunoassay. These techniques are all dependent upon the specificity of the antibody which, in turn, is reflected by the purity of the antigen used or the validity of subsequent immunoadsorption steps.

Neutralization tests are limited first by sensitivity and specificity of the antibody. Secondly, their accuracy is compromised by dependence upon clotting assays, which are themselves the net effects of multiple enzymatic steps. Quantitative immunoelectrophoresis, as employed for factor VIII by Zimmerman et al. (9), can be made reasonably precise by testing dilutions of plasma standards and measuring relative "rocket" heights. However, this type of test is usually insensitive at low levels of antigen and requires large amounts of antibody. Radioimmunoassays as developed for factors VIII (29–31) and XI (32), offer greater sensitivity and precision and, particularly on a micro-scale, use extremely small amounts of sample and antibody.

The radioimmunoassay used in the present study employs a highly purified antigen that was homog-

enous by clotting activities, by gel electrophoresis in urea and sodium dodecyl sulfate, and by immunoelectrophoresis; it appeared unaltered by iodination. Properties of our factor IX preparation were quite similar to those of Rosenberg et al. (33) and Andersson et al. (34) who also employed heparin agarose chromatography. The preparation of Osterud and Flengsrud (35) utilized an immunoadsorption technique to remove factor X and, although homogenous electrophoretically, subsequent immunoelectrophoresis (3) detected two protein species. An additional problem with their preparation is that the amino terminus was reported as glycine whereas tyrosine has been found by Andersson et al. (34) and in another preparation similar to ours.[1] The apparent molecular weight by gel electrophoresis was somewhat higher in the three previous studies although our preparation had a migration that was clearly more anodal than prothrombin. When coelectrophoresed with bovine factor IX, the migrations were identical. Assuming the carbohydrate content of human factor IX to be similar to the bovine form, a somewhat lower molecular weight would be expected by sedimentation than the 62,000 estimated on gel electrophoresis (e.g. 55,400 [17]).

The specificity of the antibody raised to factor IX was demonstrated by its failure to inhibit activities of other vitamin K-dependent clotting factors. Also, precipitation of over 90% of the iodinated antigen by antibody excess with only 6–8% binding in the controls without anti-factor IX (without washing the pellet), assure a high degree of specificity in the radioimmunoassay. Below 2% antigen, the parallel nature of dilution points could not be assessed adequately in the assay as it is presently performed. For greater sensitivity, a higher dilution of rabbit anti-factor IX or a longer preincubation could be employed (both of which would decrease the precision [21]). Alternatively, a rabbit antibody preparation with greater avidity might be obtained.

Although the factor IX activity and antigen levels were significantly correlated in normal subjects, the extent to which experimental error vs. biologic variation account for the variance from a linear relationship remains unclear. Additional dilutions and replicates improve precision (36) and did increase the correlation on the 10 normal subjects we studied further. Multiple samples from each individual might further enhance precision. A computer program, similar to that described by Williams et al. (37) for clotting assays, would facilitate more accurate, objective interpretation of the results and provide a measurement of reliability. With the present inherent error of clotting activity assays, it is doubtful that even such a rigorous approach would be definitive.

Vitamin K defects. Treatment with coumarins block the vitamin K-dependent carboxylation of bovine prothrombin (4) so that an inactive protein circulates. Evidence for an inactive bovine factor IX was found by crossed immunoelectrophoresis with nonspecific rabbit antibody (38). In subjects on coumarins, kinetic studies suggested the presence of an inactive form of circulating human factor IX (6). Denson (7) and Larrieu and Meyer (8) found some neutralization of inhibition by their partially specific rabbit antibodies in plasmas of coumarin-treated patients. The present data offer the first direct evidence for a circulating, inactive human factor IX molecule in patients on vitamin K antagonists. Abnormal factor IX was detected in the supernate after $Al(OH)_3$ adsorption of warfarin patients but not normal plasmas. Such adsorption removed the active vitamin K-dependent clotting factors from plasma but did not allow quantitation of the abnormal antigen as binding was not quantitative and even traces of $Al(OH)_3$ decreased the apparent factor IX antigen level of normal plasma. Despite the uncontrolled differences in the patient population (e.g. dosage, metabolic rates of drug, competing drugs, level, and duration of anticoagulation), all of the warfarin-treated patients had a factor IX antigen level well above their factor IX clotting activity level (Table III). Furthermore, as illustrated in Fig. 7, patients on warfarin with lower factor IX clotting activities, and thus higher degrees of anticoagulation, had increasing amounts of antigen as compared to clotting activity. In the extreme, patient 28 in Table III, there was more than eight times as much antigen as clotting activity and a reaction of complete identity with normal plasma was observed on immunodiffusion. Thus, the radioimmunoassay does detect the abnormal factor IX protein in these patients.

The fact that the factor IX antigen level was significantly lower in the warfarin-treated group than in normals may reflect increased catabolism, as has been recently illustrated for human prothrombin by both crossed and quantitative immunoelectrophoresis (39). Alternatively, decreased binding of abnormal factor IX to the rabbit anti-factor IX could explain the lower level. Concerning the latter, there is no direct evidence to exclude a quantitative difference, although the parallel nature of dilution curves in the radioimmunoassay rules against a qualitative or major quantitative difference in binding.

One would predict that patients with deficient vitamin K absorption would have a similar distribution of factor IX antigen due to decreased carboxylation. In severe liver disease, even lower levels of antigen would be found, due to the additive effect of decreased protein synthesis.

Hemophilia B. Previous studies have suggested that most cases of hemophilia B have factor IX antigen levels that are either undetectable or below

normal. In similar-sized series, patients in about two-thirds of the pedigrees have had no factor IX antigen as measured by neutralization of a human anti-factor IX (40, 11). If the human factor IX antibodies are comparable to the circulating anticoagulants in hemophilia A (41), one would expect decreased sensitivity of human vs. rabbit antibody as the former is directed against fewer antigenic sites on the clotting factor molecule. Decreased sensitivity also accounts for the lack of neutralization in all but one factor IX pedigree studied by Elodi and Puskás (12). A lack of sensitivity in their rabbit antibody, moreover, can be expected from its lower titer. In contrast, Meyer et al. (11) found 21 and 22 pedigrees positive for antigenic material with their rabbit antibody. Their antibody preparation was nonspecific and detected at least four different antigens such that their positive results can be equally well explained by neutralization of other clotting factor activities in the final stage of their assay. The neutralization and immunoelectrophoretic data of Orstavik et al. (3) suggest that 6 of their 11 pedigree had levels below 7%. Although their system was a highly purified one, their antibody was not monospecific, the titer of their anti-factor IX antibody was not given, and they ran their neutralization test in antibody excess (95% inhibition).

One-third of our pedigrees had a severe defect and one would predict that a series with predominately severe defects may have a higher percentage negative by neutralization tests, even with a specific antibody in high titer. The radioimmunoassay of factor IX represents a more sensitive and quantitative technique than those previously described, in addition to improved specificity. 6 of our pedigrees were entirely negative by the neutralization test and 10 more were weakly positive. However, only 2 of our 36 patients had antigen levels with dilution curves below the resolution of the radioimmunoassay as it is presently performed.

The correlation between neutralization and radioimmunoassay (0.93 in our series), the parallel nature of dilution curves, the presence of a wide distribution of antigen level itself, and the reaction of identity or immunodiffusion confirm that the radioimmunoassay detects abnormal factor IX antigen in patients with hemophilia B. Although a mutant protein could conceivably circulate without the same antigenic sites, rabbit antibodies are generally directed against several sites (specific sequences) in a protein of this size. Thus a mutation would at best lead to a relatively small quantitative difference in the binding of these antibodies. A mutation could alter the conformation of the proteins sufficiently to change all antigenic sites without allowing increased catabolism, but this mechanism, while theoretically possible, is unlikely to occur in plasma without altering the in vivo stability as well. The antigen levels found in our pedigrees with hemophilia B are most probably a close approximation of the circulating level of an abnormal factor IX protein.

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