Synthesis of Intrinsic Factor X Activator. Inhibition of the Function of Formed Activator by Antibodies to Factor VIII and to Factor IX*

Bjarne Østerud and Samuel I. Rapaport

ABSTRACT: The generation of an activator for clotting factor X was studied utilizing purified human reagents free of significant contamination with native factor X. When a subsample from an incubation mixture of activated factor IX, lipid, and calcium ions was added to a subsample from an incubation mixture of factor VIII and weak thrombin, a more potent clotting activity (factor X activator) was generated within seconds. This did not occur if activated factor IX, factor VIII, or lipid was left out of the incubation mixture.

Activator of factor X that formed in this reaction mixture still required both activated factor IX and factor VIII for its clotting activity. Thus, incubation of the factor X activator with either an antibody to factor IX or an antibody to factor VIII markedly inhibited its clotting activity. These data mean that a former hypothesis that activated factor IX enzymatically converts factor VIII into the factor X activator must be abandoned. They are compatible with the hypothesis that the factor X activator is a complex of lipid, activated factor IX, thrombin-altered factor VIII, and calcium.

In the "cascade" or "waterfall" theory of intrinsic blood clotting (Macfarlane, 1964; Ratnoff and Davie, 1962; Davie and Ratnoff, 1964) the clotting mechanism was viewed as a series of proenzyme–enzyme transformations in which factor IX_a[1] activates factor VIII and factor $VIII_a$ activates factor X. However, after the discovery that lipid is required to generate an intrinsic factor X activator (Lundblad and Davie, 1964; Schiffman *et al.*, 1966), several investigators suggested that the intrinsic factor X activator consists of a complex of factor IX_a, factor VIII, lipid, and calcium (Hougie *et al.*, 1967; Hemker and Kahn, 1967; Barton, 1967).

The experiments reported herein were undertaken to examine these alternative hypotheses further by answering two questions: (1) In reaction mixtures with purified human reagents free of significant contamination with factor X, can one demonstrate a time-consuming reaction between factor IX_a, factor $VIII_t$, lipid, and calcium to form an intrinsic factor X activator? (2) Does the product of this interaction retain its ability to activate factor X following exposure to an antibody against factor IX and following exposure to an antibody against factor VIII?

Materials and Methods

Barbital buffer was a solution containing 0.028 M sodium chloride and 0.125 M sodium barbital adjusted to pH 7.25 with 0.1 N HCl. Citrate–saline was a solution of 0.017 M trisodium citrate and 0.125 M sodium chloride. Cephalin, an acetone-insoluble, ether-soluble extract of human brain which supplies an activity equivalent to platelet factor 3, was prepared and stored as a stock solution as described earlier (Hjort *et al.*, 1955). The stock solution was diluted in barbital buffer prior to use. Thromboplastin was a saline extract of human brain (Owren, 1949). Standard plasma was pooled plasma prepared from five normal donors and stored at −20°. It was used to express clotting activities as per cent of normal plasma activity.

Prothrombin Reagent. Normal human-citrated plasma (380 ml) was absorbed with 20 mg/ml of bentonite powder (Prolabo, Rhone-Poulenc, France) in a plastic cylinder which was slowly tilted back and forth for 10 min at room temperature. The bentonite was removed by centrifugation at 10,000 rpm for 10 min, and the supernatant was absorbed a second time in the same manner with 10 mg/ml of bentonite for 2 min. The supernatant of this second absorption was stored at −20°. It contained 9% prothrombin activity but no measurable factor V, factor VIII, factor IX, or factor X activity (see Table I).

Factor V–Fibrinogen Reagent. Citrated hereditary factor VIII deficiency (hemophilia A) plasma (39 parts) was absorbed twice with one part of undiluted $Al(OH)_3$ gel (Alhydrox, Cutter Laboratories, Berkeley, Calif.) for 10 min at room temperature with constant stirring. The resultant absorbed plasma contained 32% factor V activity but no measurable prothrombin, factor VII, factor VIII, factor IX, or factor X activity (see Table I). It was stored at −20°.

Factor VIII Reagent. Plasma (9 parts) from a patient with hereditary factor V deficiency (parahemophilia) was absorbed with one part of $Al(OH)_3$ gel that had been diluted 1:4 with distilled water. The mixture was stirred for 5 min at 37°, and the $Al(OH)_3$ was removed by centrifugation. This absorbed plasma was then centrifuged in an ultracentrifuge

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[1] Clotting factors are designated by Roman numerals according to the convention adopted by the International Committee on Hemostasis and Thrombosis. An activated factor is designated by the subscript a, *e.g.*, factor IX_a. The increased factor VIII reactivity that results from exposure of factor VIII to weak thrombin is referred to as factor $VIII_t$, (Rapaport *et al.*, 1965).

TABLE I: Summary of the Clotting Factor Activities in the Reagents Used for Most of the Studies.

Reagent	Clotting Activity (sec) in Specific Factor Assays					
	Factor					
	Prothrombin	V	VII	VIII	IX	X
Citrate–saline	>300	65	116	133	145	130
Prothrombin	38 (9)[a]	70	132	123	133	115
Factor V	>300	37 (32)	110	119	131	121
Factor VIII	>300	66	119	76 (21)	135	126
Factor IX_a	>300	65	45 (23)	119	31[b]	97[c] (<0.1)
Factor X[d]	>300	56	41 (38)	81	85	20 (50)
Citrate–saline	>300	57	112	91	89	104

[a] Values in parentheses are per cent standard plasma activity. [b] Represents the clotting time of undiluted factor IX_a reagent in the contact, factor IX assay. Since the slope of the dilution curve for factor IX_a is steeper than the slope of the dilution curve for factor IX, this clotting time cannot be converted into per cent standard plasma activity. [c] Represents the clotting time of a 1:10 dilution. Although expressed as per cent factor X, the slight shortening when compared with blank time probably represented primarily an effect of a trace of factor X_a (see text). [d] The factor X reagent was tested at a different time and its clotting times should be compared with the blank times given in the last line.

at 25,000 rpm for 120 min. The middle, lipid-poor layer was separated and frozen at −20°.

A 130-ml aliquot was thawed in a 37° water bath with constant stirring to assure that its temperature did not rise above 6°. The protein remaining insoluble at 6° (cryoprecipitate) was spun down in the cold at 10,000 rpm for 10 min. This precipitate was dissolved in 2.2 ml of a 0.02 M Tris-HCl buffer, pH 6.5, containing 0.3 M glycine and 0.2 M sodium chloride. The final volume of the dissolved precipitate was 5.2 ml. Bentonite powder (20 mg/ml) was then added to remove fibrinogen. After *gentle* stirring for 3 min at room temperature, the bentonite was separated by centrifugation.

The factor VIII was further purified by gel filtration. Agarose in gel (Bio-Gel A-5 m, Bio-Rad Laboratories, Richmond, Calif.) (300 ml, 6%) was equilibrated with 10 l. of the above described Tris buffer. Then, a 2.5 × 79 cm column of agarose was prepared and equilibrated with 300 ml of the buffer. The factor VIII preparation (3 ml) was applied to the column. The column was eluted with the same buffer at a flow rate of 14 ml/hr and 2-ml fractions were collected. Factor VIII activity appeared with the void volume before the main peak of protein. The fractions in the first half of the factor VIII peak were pooled and stored at −20°. The preparation used for the experiments reported herein had a protein content of 0.26 mg/ml, an initial factor VIII activity of 21%, and no other measurable clotting factor activities (see Table I).

Like the factor VIII activity of whole plasma, the factor VIII activity of this preparation increased after exposure to traces of thrombin. For example, when 0.2 ml of the factor VIII reagent was incubated for 1 min at 4° with 0.025 ml of 1 unit/ml of thrombin, its clotting time in a one-stage factor VIII assay shortened from 65 sec (clotting time on incubation with buffer) to 45 sec. The blank time of the assay was unaffected by this concentration of thrombin.

The factor VIII reagent was stored frozen for months with slow loss of activity. When thawed, it remained stable over a working day if kept at 4°. However, it deteriorated rapidly at 4° after exposure to traces of thrombin.

We emphasize that the factor VIII reagent for these experiments was made from hereditary factor V deficiency plasma. When we made the same reagent from normal plasma, it contained enough residual factor V activity (despite its lack of measurable factor V activity in a one-stage assay) to influence the multistage, sequential incubations required for these experiments.

Factor IX_a Reagent. Normal human blood (125 ml) was clotted in a glass bottle with 3.75 ml of thromboplastin, stirred for 20 min at room temperature, and centrifuged for 10 min at 10,000 rpm. One part of 0.1 M potassium oxalate was added to five parts of the serum. The oxalated serum was then centrifuged at 28,000 rpm for 60 min in an ultracentrifuge to remove residual tissue thromboplastin. The supernatant was stored for 3 days at room temperature to inactivate thrombin and factor X_a activity. It was then absorbed with 100 mg/ml of $BaSO_4$ powder (Baker Chemical Co., Phillipsburg, N. J.) for 30 min at 4° with constant stirring. The pellet was washed twice in an equal volume of 0.85% sodium chloride and once with an equal volume of 0.01 M sodium citrate. It was then eluted in one-third volume of 0.14 M sodium citrate, and the eluate was dialyzed overnight against 0.85% sodium chloride. This was stored frozen at −20° until used for further purification.

A 2.5 × 82 cm column of Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) was equilibrated with 0.01 M sodium citrate according to the instructions of the manufacturer. Eluate (3 ml) was applied to the column. The column was eluted with 0.01 M sodium citrate at a flow rate of 12 ml/hr, and 2-ml fractions were collected. The coagulation factors were found in two main peaks—an initial peak containing factor IX and factor X, and a later peak containing factor IX_a. Factor VII was eluted between these peaks and overlapped both. The fractions containing

TABLE II: Evidence of an Interaction Between Factor IX_a, Factor $VIII_t$, Lipid, and Calcium.[a]

Line	Incubation Mixture Components: Factor IX_a	Factor $VIII_t$	Lipid	Factor X in Substrate	Substrate Clotting Time (sec): Incubation Time (sec) 10	20	40
1	+	+	1:50	+	48	33	29
2	+	+	1:200	+	54	41	35
3		+	1:50	+	71	72	72
4	+		1:50	+	69		75
5	+	+	1:50	+	36	29	27
6	+	+		+	57	57	58
7	+	+	1:50	+	43	37	39
8		+	1:50	+	64	62	62
9	+		1:50	+	69	69	68
10	+	+		+	54	53	53
11	+	+	1:50		>300	>300	>300

[a] Factor IX_a reagent (1 ml), cephalin (0.1 ml), and 80 mM $CaCl_2$ (0.2 ml) were incubated together for 1 min at 37°. In a separate tube, factor VIII reagent (0.5 ml) and 1 unit/ml of thrombin (0.05 ml) were also incubated together for 1 min at 37°. Then, 0.4 ml of the first reaction mixture was combined with 0.2 ml of the second reaction mixture and subsamples were added at 10, 20, and 40 sec to the substrate mixture. The substrate mixture was prepared by allowing factor V–fibrinogen reagent (0.1 ml), prothrombin reagent (0.1 ml), and 1:25 cephalin (0.05 ml) to incubate together for 3 min at 37°. Then, in rapid succession, factor X reagent (0.1 ml) and a subsample from the incubation mixture (0.1 ml) plus 40 mM $CaCl_2$ (0.1 ml) were added and the clotting time was noted. In the experiment described in lines 5 and 6 conditions were identical except that the combined incubation mixture contained 0.4 ml of the first reaction mixture and 0.1 ml of the second reaction mixture. The experiment described in lines 7–11 was done at a later date with new factor IX_a and factor X reagents. The factor IX_a reagent, which was prepared from hereditary factor X deficiency plasma, contained 1.2% prothrombin, no measurable factor X, and no measurable factor V. The undiluted reagent gave a 32-sec clotting time in an intact factor IX assay. The factor X reagent contained 17% factor X, 4.2% prothrombin, and no measurable factor V. It also contained some factor X_a for the undiluted reagent gave a 59-sec clotting time in a factor X_a assay (blank time >240 sec). The factor VIII reagent had lost potency and contained 5% factor VIII activity. The experimental conditions were otherwise identical with those used in the experiments whose data are shown in lines 1–4.

factor IX_a were pooled and stored at 4°. They could not be dialyzed or concentrated without loss of activity, despite attempted stabilization with a variety of agents.

Fractions prepared and stored in this way had potent factor IX_a activity since they clotted rapidly in both an intact factor IX assay and a contact factor IX assay, yet contained only traces of factor X_a activity. The preparation used for the majority of these experiments clotted in 31 sec when tested undiluted in a contact factor IX assay (as compared with 52 sec for a 1:5 dilution of standard plasma). The reagent diluted 1:10 clotted in 97 sec in a factor X assay (blank time 130 sec). This represents <0.1% native factor X activity. However, a clotting time of 97 sec was also obtained when the Russell's viper venom reagent of the test was left out (factor X_a assay). Thus, the trace factor X activity of the reagent can be attributed primarily to a minute amount of contaminating factor X_a. As shown in Table I, the reagent had no other measurable clotting factor activities except for a 23% factor VII activity. (Since it contained factor IX_a, it could not be assayed for factor XI or factor XII activity.) It failed to clot a fibrinogen solution. This factor IX_a reagent was used for all experiments except those summarized in lines 7–11 of Table II. The reagent used in the latter is described in the footnote to Table II.

Factor X Reagent. Hereditary factor IX deficiency blood (hemophilia B) (200 ml) was collected in a plastic beaker, and 0.4 ml of thromboplastin was added with stirring to assure full consumption of prothrombin during clotting. After 5 min, 18 ml of 2% EDTA, pH 7.4, was added, and the serum was separated by centrifugation at 10,000 rpm for 10 min, followed by centrifugation in an ultracentrifuge at 40,000 rpm for 1 hr. The serum was stored for 48 hr at room temperature to inactivate thrombin and factor X_a. Serum (110 ml) was then absorbed for 30 min at 4° with 100 mg/ml of $BaSO_4$ powder. The pellet was washed twice with an equal volume of 0.85% sodium chloride and once with an equal volume of 0.01 M sodium citrate, and was eluted with 40 ml of 0.14 M sodium citrate. The eluate was dialyzed against 0.85% sodium chloride overnight.

Because the factor X_a activity of this preparation increased when it was incubated with calcium ions in control incubation mixtures free of added factor IX_a and factor VIII, 1 part of 2% EDTA was added to 10 parts of the eluate, and it was reabsorbed and washed again as described above. The pellet was eluted in half the volume of 0.14 M sodium citrate. The eluate was dialyzed overnight against 0.85% sodium chloride and stored at −20°.

A 1:10 dilution of this doubly absorbed reagent clotted in 20 sec in a factor X assay, which was equivalent to 50% factor X activity. The *undiluted* reagent clotted in 71 sec

in a factor X_a assay as compared with a blank time of 240 sec. Thus, the reagent still contained a trace of factor X_a activity. However, it no longer generated added factor X_a activity in control incubation mixtures. The reagent contained 38% factor VII activity, a slight apparent factor VIII and factor IX activity (attributable to the trace of factor X_a) and no measurable factor V or prothrombin activity (see Table I). It failed to clot a fibrinogen solution. This factor X reagent was used for all experiments except those summarized on lines 7–11 of Table II. The reagent used for the latter experiments is described in the footnote to that table.

Thrombin. Bovine thrombin (Parke Davis) was absorbed with $BaSO_4$ powder as described elsewhere (Rapaport *et al.*, 1963) and then chromatographed on diethylaminoethylcellulose (Whatman DE 23) according to Kerwin and Milstone (1967) to remove traces of factor X_a. Those fractions that gave the same clotting time in a slightly modified factor X_a assay when the lipid reagent was replaced with buffer (*e.g.*, 84 sec with lipid, 83 sec with buffer) were considered to be free of contaminating traces of factor X_a. They were pooled and stored frozen in a concentration of 8 NIH units/ml. This stock thrombin reagent was thawed and diluted in barbital buffer to a concentration of 1 NIH unit/ml just prior to use.

Factor VIII Antibody. A factor VIII antibody preparation was made available through the courtesy of Dr. Donald I. Feinstein. This preparation had been made from serum from a hemophilic patient who had developed a powerful factor VIII antibody after transfusions. The patient's citrated serum had been heated to 56° for 1 hr and then absorbed with $Al(OH)_3$ gel to remove clotting factor activities. The antibody had been concentrated by precipitation at 50% saturation with ammonium sulfate and further purified by starch block electrophoresis. The eluate from the starch block had been dialyzed against citrate–saline and stored at −20°. This powerful antibody completely neutralized factor VIII activity in plasma within 1 min, but had no significant effect upon prothrombin, factor V, factor IX, or factor X (see Table III).

Factor IX Antibody. Plasma from normal blood collected in 2% EDTA anticoagulant was absorbed with 100 mg/ml of $BaSO_4$ powder. After the pellet was washed three times with 0.85% sodium chloride, it was eluted in approximately one-tenth of the plasma volume of 0.14 M sodium citrate. The eluate was dialyzed overnight against 0.85% sodium chloride. The dialyzed eluate (1 ml) was then mixed with 1 ml of Freund's adjuvant and injected into the footpad of a rabbit. The remaining eluate was frozen. Each week for 4 weeks, 0.5 ml was thawed and injected subcutaneously into the animal. (It is important to emphasize that the initial injection was made with fresh eluate and that subsequent injections were made with eluate which had been stored frozen. If the eluate is stored at 4°, its factor IX slowly activates, and, in our hands, factor IX_a has failed to stimulate the formation of a factor IX antibody in rabbits.)

Rabbits were bled after 5 weeks. The serum was incubated at 56° for 1 hr, following which 9 parts of serum were absorbed twice with 1 part of aluminum hydroxide gel diluted 1:2 with distilled water. The antiserum was then incubated with an equal volume of factor IX deficiency plasma for 1 hr at 37° and 14 hr at 4°. Precipitated antigen–antibody complexes were removed by centrifugation at 10,000 rpm for 10 min.

TABLE III: Specificity of the Factor VIII Antibody.[a]

Clotting Factor Assay	Clotting Time (sec) of Mixture of Normal Plasma and: Citrate–Saline	Factor VIII Antibody
Prothrombin	20	20
Factor V	33	34
Factor IX	63	63
Factor X	24	24
Factor VIII	75	112 (<0.1)[b]

[a] Either citrate–saline (control) or factor VIII antibody diluted 1:2.5 in citrate–saline was incubated with an equal volume of normal plasma for 1 min at 37°. The mixture was then diluted 1:10 in citrate–saline and added as the test substance in the assays listed. The final concentration of factor VIII antibody in the above incubation mixture was the same as its final concentration in experimental reaction mixtures (see footnote to Table II). [b] Per cent of normal plasma activity.

Nine volumes of the supernatant were reabsorbed twice with one volume of aluminum hydroxide suspension diluted 1:4 with distilled water and heated for 60 min at 56° to inactivate clotting factors. Then, solid ammonium sulfate was added to a final concentration of 40% saturation, and, after 1 hr at 4°, the precipitate was separated by centrifugation. The precipitate was redissolved in 0.85% sodium chloride in one-fifth of its original volume, dialyzed against 0.85% NaCl, and stored at −20°.

For our experiments this factor IX antibody preparation had to be free of contamination with significant antibody against either factor X or prothrombin. As shown in Table IV, when the antibody was incubated with an equal volume of either full strength or diluted normal plasma, factor IX activity, and factor VII activity were lost but factor X activity and prothrombin activity were unaffected. The antibody neutralized all but a trace of the factor IX_a activity of our factor IX_a reagent within 1 min. Thus, the clotting time of a mixture of equal parts of factor IX_a reagent and control reagent was 48 sec in an intact factor IX assay, whereas the clotting time of a mixture of factor IX_a reagent and factor IX antibody were after 10-sec incubation, 102 sec; after 1-min incubation, 117 sec; and after 5-min incubation, 124 sec. The blank time of this assay was about 160 sec.

A protein solution for use as a control reagent was made exactly as described above from normal rabbit serum.

Clotting Procedures. Prothrombin was measured in a one-stage assay (Hjort *et al.*, 1955) that was modified by adding a $BaSO_4$ eluate of serum clotted with thromboplastin to provide factor X. Factor V was determined in a one-stage assay which measures the ability of a test substance to shorten the clotting time of a hereditary factor V deficiency substrate plasma in the presence of thromboplastin (Owren, 1947). Factor VII was measured in a similar one-stage assay utilizing hereditary factor VII deficiency substrate plasma (Hall *et al.*, 1964). Factor VIII was assayed in a one-stage activated

TABLE IV: Specificity of the Factor IX Antibody.[a]

Clotting Factor Assay	Clotting Time (sec) of Mixture of: Undiluted Normal Plasma and Saline	Undiluted Normal Plasma and Factor IX Antibody	1:5 Normal Plasma and Saline	1:5 Normal Plasma and Factor IX Antibody
Prothrombin	31	30	59	58
Factor X	25	25	40	37
Factor VII	35 (78)[b]	89 (4.5)	60 (75)	123 (<0.1)
Factor IX	59 (66)	85 (4.4)	75 (75)	94 (5.0)

[a] Normal plasma (full strength or diluted 1:5 in citrate–saline) was incubated at 37° for 1 hr with an equal volume of either saline (control) or factor IX antibody. Subsamples were then diluted 1:5 in citrate–saline and tested in the assays listed above. [b] Figures in parentheses are per cent normal plasma activity.

partial thromboplastin time assay utilizing hemophilia A plasma as the substrate (Schiffman *et al.*, 1965). Factor IX was assayed by a similar technique (contact factor IX assay) utilizing as substrate hemophilia B plasma reinforced with absorbed ox plasma as an added source of factor V and factor VIII (Schiffman *et al.*, 1965). Factor IX_a activity was measured in an intact factor IX assay, utilizing hemophilia B plasma as substrate, a cephalin reagent without kaolin powder, and a plastic clotting tube (Schiffman *et al.*, 1965). Factor X was determined in a one-stage assay modified from Hougie (1962). This assay is based upon the ability of a test substance to shorten the clotting time of the following mixture, bentonite absorbed plasma (prothrombin source)–absorbed ox plasma (factor V and fibrinogen source)–Russell's viper venom (factor X activator)–cephalin (lipid source)–calcium. The same clotting mixture minus the Russell's viper venom was used as an assay for factor X_a activity.

For simplicity, the details of the multistage experimental clotting systems constructed for specific experiments are described in the footnotes to the tables.

Results

Evidence for a Rapid Reaction among Factor IX_a, Factor $VIII_t$, Lipid, and Calcium. Kinetic data in the literature suggesting a reaction between factor IX_a and factor VIII can be questioned (see Discussion). Therefore, before examining the clotting properties of a presumed product (factor X activator) of an interaction among factor IX_a, factor $VIII_t$, lipid, and calcium, we had to establish whether or not a reaction involving these factors could be demonstrated with our reagents. An experiment was designed to answer the following specific question: if factor IX_a is allowed to react with lipid and calcium, and if factor VIII is allowed to react with weak thrombin, will a further time-consuming reaction occur when the products of these initial reactions are brought together?

Two preliminary incubation mixtures were made at 37°—one containing factor IX_a reagent, cephalin, and calcium and the other containing factor VIII reagent and weak thrombin. After 1 min the preliminary incubation mixtures were combined and subsamples were added as rapidly as possible over the next 40 sec to a substrate mixture containing factor X, factor V, lipid, prothrombin, fibrinogen, and calcium. In control experiments the appropriate buffer was substituted for factor IX_a reagent, factor VIII reagent, or cephalin. Details are given in the footnote to Table II.

This experiment was performed on several occasions, and each time a very rapid reaction—complete within 40 sec—could be demonstrated after the preliminary incubation mixtures were combined. Representative data are shown in lines 1, 2, 5, and 7 of Table II. In contrast, when either factor IX_a reagent or factor VIII reagent was replaced by control buffer, no time-consuming reaction was observed (lines 3 and 4). Moreover, when lipid was left out of the incubation mixture, no reaction occured (lines 6 and 10).

Similar data were obtained when the preliminary incubation times were lengthened to 2 min before the incubation mixtures were combined. Thus, further progress of either of the preliminary reactions—the reaction between factor IX_a, lipid, and calcium, or the reaction between factor VIII and thrombin—could not account for the shortening of the clotting time observed upon combining the preliminary mixtures after 1 min.

A further control experiment was carried out at a later date with new factor IX and factor X reagents. Its purpose was to assure ourselves that the shortening observed on combining the preliminary mixtures did not stem from generating contaminating factor X_a in the combined mixture (lines 7–11). As line 11 illustrates, when factor X reagent was eliminated from the substrate mixture, no clotting was observed within 5 min. This indicates that significant amounts of factor X_a did not form in the combined incubation mixture.

Evidence That a Factor IX Antibody Can Inhibit the Clotting Activity of the Product of the Interaction of Factor IX_a, Factor $VIII_t$, Lipid, and Calcium. On four occasions factor IX_a reagent, factor VIII reagent, thrombin, cephalin, and calcium were incubated together as described in detail in the footnote in Table V. Then, either factor IX antibody or a control protein solution was added to the incubation mixture. After allowing 1 min for the antibody to act, a source of factor X was added to the incubation mixture. Subsamples were removed over the next 20 min and added to the substrate mixture containing factor V, lipid, prothrombin, fibrinogen, and calcium. As a further control the experiment was also performed with the factor X reagent being added 30 sec before the factor IX antibody.

Representative data are shown in Table V. The marked difference observed between the clotting times after adding control protein (line 2) and the clotting times after adding factor IX antibody (line 3) establishes that a factor IX antibody inhibits the clotting activity of the product of the interaction of factor IX_a, factor $VIII_t$, lipid, and calcium. Note also in line 2 that the clotting time shortens from 25 sec at 10-sec incubation to 16 sec after 4-min incubation. This indicates continuing formation of factor X_a during the first

TABLE V: The Effect of Factor IX Antibody upon the Product of the Interaction of Factor IX_a, Factor $VIII_t$, Lipid, and Calcium.[a]

Initial Incubation Mixture			Substrate Clotting Time (sec)		
			Incubation Time		
IX_a	$VIII_t$	Materials added to Incubation Mixture	10 sec	4 min	20 min
	+	Control protein then factor X	83	82	89
+	+	Control protein then factor X	25	16	19
+	+	Factor IX antibody then factor X	56	55	58
+	+	Factor X then factor IX antibody	26	28	29

[a] The following reagents were incubated together for 1 min at 37°: 0.3 ml of factor IXa reagent or control buffer, 0.1 ml of factor VIII reagent, 0.05 ml of 1:50 cephalin, 0.025 ml of 1 unit/ml of thrombin, and 0.025 ml of 80 mM $CaCl_2$. Then, in the experiments listed in the first 3 lines, 0.1 ml of either control protein or factor IX antibody was added to the incubation mixture. One minute later 0.1 ml of factor X reagent was added, and, at the incubation times shown, subsamples were transferred to a substrate mixture. The substrate mixture consisted of 0.1 ml of factor V–fibrinogen reagent, 0.1 ml of prothrombin reagent, and 0.05 ml of 1:25 cephalin. These had incubated together for 3 min at 37° before the addition of 0.1 ml of 40 mM $CaCl_2$ and 0.1 ml of the subsample. In the experiment shown on line 4, 0.1 ml of factor X reagent was added to the factor X activator incubation mixture 30 sec before 0.1 ml of factor IX antibody was added. One minute more was allowed to elapse for the antibody to act and then subsamples were transferred to the substrate at the further incubation times listed.

4 min after adding factor X to the incubation mixture. In contrast, in the experiment in which the factor IX antibody was added 30 sec after the factor X reagent (line 4) a similar initial clotting time of 26 sec failed to shorten on further incubation. We interpret this to mean that factor X activator could act upon factor X only during the 30-sec interval before the factor IX antibody was added to the incubation mixture.

Evidence That a Factor VIII Antibody Can Inhibit the Clotting Activity of the Product of the Interaction of Factor IX_a, Factor $VIII_t$, Lipid, and Calcium. The effect of a factor VIII antibody upon the clotting activity of the product of the interaction of factor IX_a reagent, factor VIII reagent, thrombin, lipid, and calcium was studied on three occasions in an experiment similar to that described in the preceding section. As clearly shown in Table VI, factor VIII antibody also blocked the clotting activity of the product of the interaction. In the experiment in which factor X was added before the factor VIII antibody (line 3), 1 min was allowed for activation of factor X before the antibody was added.

Discussion

It is established that both factor VIII and factor IX are needed to form an intrinsic activator of factor X (Spaet and Cintron, 1963; Macfarlane *et al.*, 1964). It is also established that factor IX must first be activated by factor XI_a (Ratnoff and Davie, 1962; Schiffman *et al.*, 1963; Lundblad and Davie, 1964) to generate intrinsic factor X activator effectively. In the "cascade" or "waterfall" theory of intrinsic clotting (Macfarlane, 1964; Davie and Ratnoff, 1964) factor IX_a was then postulated to activate factor VIII, and factor $VIII_a$, acting alone, was postulated to activate factor X. However, kinetic data interpreted earlier as supporting this sequence of reactions (Lundblad and Davie, 1964; Macfarlane *et al.*, 1964) must be reinterpreted with the knowledge that minute amounts of contaminating thrombin (Rapaport *et al.*, 1965; Biggs *et al.*, 1965) or lipid (Schiffman *et al.*, 1966) markedly influence the velocity of formation of factor X_a in reaction mixtures not fortified with these reactants.

Indeed, a critical reexamination of earlier data yields only scanty evidence (Schiffman *et al.*, 1966) for a time-consuming reaction involving factor IX_a and factor VIII when the following experimental conditions are met: first, that the activity of factor VIII has been enhanced by its prior exposure

TABLE VI: The Effect of Factor VIII Antibody upon the Product of the Interaction of Factor IX_a, Factor $VIII_t$, Lipid, and Calcium.[a]

Materials Added to Initial Incubation Mixture	Substrate Clotting Time (sec)		
	Incubation Time		
	10 sec	4 min	20 min
Citrate–saline then factor X	23	16	18
Factor VIII antibody then factor X	56	56	59
Factor X then factor VIII antibody	20	21	22

[a] Factor X activator was formed as described in Table V, footnote *a*. Then, 0.1 ml of citrate–saline (control) or factor VIII antibody was added to the incubation mixture. One minute later 0.1 ml of factor X reagent was added, and, at the incubation times shown, subsamples were transferred to the substrate mixture described in Table V, footnote *a*. In the experiment shown on line 3, 0.1 ml of factor X reagent was added 1 min before 0.1 ml of factor VIII antibody. One more minute was allowed to elapse and then subsamples were transferred to the substrate at the further incubation times listed.

to a known minute amount of thrombin; second, that the reaction mixture contains an adequate amount of lipid; and third, that the reaction mixture is free of significant contamination with native factor X. Therefore, before a study of the clotting properties of a presumed product of a factor IX_a–factor VIII interaction had meaning, it was necessary to reexamine the hypothesis that these factors did participate together in a time-consuming reaction that gave rise to an intrinsic factor X activator.

The experiments summarized in Table II demonstrate that a very rapid reaction occurs between factor IX_a, factor $VIII_t$, lipid, and calcium. Within seconds a more potent clotting activity was generated when a subsample of a preliminary incubation mixture of factor IX_a, lipid, and calcium was combined with a subsample of a preliminary incubation mixture of factor VIII and very weak thrombin. Increasing the duration of the preliminary incubations did not eliminate the generation of this more potent activity. Moreover, a more potent activity failed to form when one of the reactants—factor IX_a, factor VIII, or lipid—was left out of the combined reaction mixture. Thus, the activity generated when all three were present could not have represented an effect of further incubation of two of the reactants in the absence of the third.

The data summarized in lines 7 through 11 of Table II deserve further comment. The clotting times of >300 sec when factor X reagent was left out of the substrate mixture (line 11) establish that contaminating factor X_a was not formed in the reaction mixture, and thus could not account for its increased clotting activity. The difference between this very long clotting time and the 60–70-sec clotting times when either factor IX_a or factor VIII was left out of the reaction mixture (lines 8 and 9) can be explained by the known traces of contaminating factor X_a in our factor X reagent. The clotting times in the 50-sec range (line 10) when lipid was left out of the *reaction* mixture (clotting times which are intermediate between the 30- and 40-sec times obtained when all reactants were present and the 60- and 70-sec times obtained when either factor IX_a or factor VIII was left out) are thought to reflect the generation of intrinsic factor X activator after the factor IX_a and factor $VIII_t$ from the reaction mixture came in contact with lipid in the *substrate* mixture.

Thus, all of the data of Table II fit the hypothesis that factor IX_a, factor $VIII_t$, lipid, and calcium interact to form an intrinsic factor X activator. When conditions are optimal they interact so rapidly that a time-consuming reaction cannot be demonstrated unless two people work together in transferring subsamples from the incubation mixtures to the reaction mixture and from the latter to the substrate mixture.

We then turned to the question of whether formed intrinsic factor X activator still requires factor IX_a, factor $VIII_t$, or both for its clotting activity. If the "cascade" or "waterfall" theory were correct—and the role of factor IX_a were limited to activating factor $VIII_t$—then the product of their interaction should no longer require factor IX_a. This hypothesis was tested in an experiment (Table V) in which the product of the interaction of factor IX_a, factor $VIII_t$, lipid, and calcium was exposed to a factor IX antibody. Although the antibody reagent also contained antibody against factor VII, this factor plays no known role in intrinsic clotting. Therefore, the data—which showed that the antibody reagent blocked the activity of fully formed intrinsic X activator—mean that intrinsic factor X activator still requires factor IX_a for its activity. The earlier theory that factor IX_a converts factor VIII from a proenzyme into the enzymatic activator of factor X must be abandoned.

A converse hypothesis, that factor $VIII_t$ converts factor IX_a into the intrinsic factor X activator, was tested in an experiment utilizing a factor VIII antibody (Table VI). The data from this experiment show that the intrinsic factor X activator also required factor $VIII_t$ for its activity. Thus, the experiments with the two antibodies establish that intrinsic factor X activator requires both factor IX_a and factor $VIII_t$ for its activity.

The prothrombin activator consists of a complex of lipid, factor X_a, factor V, and calcium ions (Papahadjopoulos and Hanahan, 1964; Cole *et al.*, 1965; Jobin and Esnouf, 1967). Several investigators have postulated that the intrinsic factor X activator consists of an analogous complex of lipid, factor IX_a, factor VIII, and calcium ions (Hougie *et al.*, 1967; Barton, 1967; Hemker and Kahn, 1967). This hypothesis has appeal because factor IX_a has common properties with factor X_a and because factor VIII has common properties with factor V. However, data supporting this hypothesis are as yet limited. Hougie and coworkers (1967) reported that the factor IX_a activity of a mixture of factor IX_a, lipid, and calcium ions subjected to gel fractionation on G-200 Sephadex appeared in the void volume with the lipid. In the absence of calcium the factor IX_a activity appeared after the lipid. This suggested binding of factor IX_a to lipid in the presence of ionic calcium. Hemker and Kahn (1967) found that when a mixture of either factor VIII or factor IX_a with lipid and calcium was subjected to centrifugation at 100,000*g* for 30 min, much of the factor VIII or IX_a activity disappeared from the supernatant and was presumably absorbed onto the precipitated lipid. Our data, which establish that fully formed factor X activator still requires both factor IX_a and factor $VIII_t$ for its clotting activity, provide further indirect but strong support for the hypothesis that the intrinsic factor X activator consists of a complex of factor IX_a, factor $VIII_t$, lipid, and calcium ions.

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