The role of phospholipid and factor VIIIa in the activation of bovine factor X

Document Version:
Publisher's PDF, also known as Version of record

Please check the document version of this publication:

• A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
• The final author version and the galley proof are versions of the publication after peer review.
• The final published version features the final layout of the paper including the volume, issue and page numbers.

Link to publication

Download date: 04 Jan. 2021

The Role of Phospholipid and Factor VIIIa in the Activation of Bovine Factor X*

Gerbrand van Dieijen, Guido Tans, Jan Rosing, and H. Coenraad Hemker

From the Department of Biochemistry, Biomedical Centre, State University of Limburg, Maastricht, The Netherlands

The kinetic parameters of bovine factor X activation by bovine factor IXa have been determined in the absence and presence of Ca^{2+}, thrombin-activated bovine factor VIII (VIIIa), and phospholipid (dioleoylphosphatidylcholine/dioleoylphosphatidylserine, 75/25; mol/mol). Factor IXa in the absence of Ca^{2+}, factor VIIIa, and phospholipid is able to catalyze factor X activation. The K_m for factor X is 299 μM which is well above its concentration in bovine plasma, about 0.2 μM. The V_{max} of factor Xa formation is 0.0022 mol of $X_a \cdot min^{-1} \cdot mol$ of IX_a^{-1} under these conditions. Addition of Ca^{2+} has little effect on the kinetic constants of factor X activation by factor IXa. In the presence of 10 mM $CaCl_2$ the K_m for factor X is 181 μM, and the V_{max} is 0.0105 mol of $X_a \cdot min^{-1} \cdot mol$ of IX_a^{-1}. The presence of 10 μM phospholipid dramatically decreases the K_m for factor X to 0.058 μM, and the V_{max} becomes 0.0025 mol of $X_a \cdot min^{-1} \cdot mol$ of IX_a^{-1}. The V_{max} of factor X_a formation slightly increases when more phospholipid is present in our experiments, and there is a considerable increase of the K_m for factor X at higher phospholipid concentrations. Therefore, the K_m measured in the presence of phospholipid has to be regarded as an apparent K_m. The possible explanations for this phenomenon are discussed.

For the complete factor X-activating complex (i.e. factor IXa, factor VIIIa, Ca^{2+}, and 10 μM phospholipid) the K_m for factor X is 0.0063 μM, and the V_{max} is raised 200,000-fold to 500 mol of $X_a \cdot min^{-1} \cdot mol$ of IX_a^{-1}. In order to exert its stimulating effect on factor X activation factor VIII has to be activated with thrombin. Our results show that factor IXa is an enzyme which can activate factor X at a very low rate. The stimulating effect of phospholipid in factor X activation is mainly due to an effect on the K_m for factor X, bringing it within the range of the plasma concentration. The stimulatory effect of factor VIIIa is explained by its 200,000-fold increase of the V_{max} of factor X_a formation.

Blood coagulation factor X^1 is a plasma glycoprotein that, during the clotting process, is converted into the serine protease factor X_a through proteolytic cleavage of a single peptide bond (1, 2). Factor X_a is the enzyme that, during the coagulation process, activates prothrombin to thrombin in a reaction accelerated by factor V_a, calcium ions, and phospholipid.

Under physiological conditions, the activation of factor X can be accomplished in both the extrinsic and intrinsic pathways of blood coagulation. In the extrinsic pathway factor X is activated by a complex of factor VII, tissue factor, and calcium ions (for a review, see Ref. 3), whereas the activation of factor X via the intrinsic pathway involves the interaction of factor IXa, factor VIII, calcium ions, and phospholipid.

It is now generally accepted that factor IXa, factor VIII, phospholipid, and calcium ions form a complex (1, 4–11), in which factor IXa likely is the enzyme responsible for factor X activation, since it is highly homologous to other serine proteases involved in blood coagulation (12, 13), and factor VIII, phospholipid, and calcium ions function as cofactors (14). Thus it is tempting to speculate that the roles of the components of the intrinsic factor X activator are analogous to those of factors X_a, V_a, phospholipid, and calcium ions in the prothrombin-activating complex (7, 8, 9, 11, 14).

Since the activity of factor VIII is considerably enhanced by preincubation with small amounts of thrombin, it is likely that factor VIII participates in the factor X-activating complex in an activated form (15). Davie et al. (16, 18) reported that factor VIII can also be activated with factor X_a. Of interest is the finding of Vehar and Davie (17, 18) that thrombin-activated factor VIII is inhibited by diisopropylphosphorofluoridate and antithrombin III, which may have consequences for the way in which factor VIII functions in factor X activation.

In addition to factor IXa and factor VIII, the factor X-activating complex requires negatively charged phospholipid and calcium ions. It has been shown that the proteins have to be adsorbed to the phospholipid bilayer surface during the activation reaction (6–11, 14). Both vitamin K-dependent clotting factors IXa and X bind to the phospholipid surface via calcium bridges between the γ-carboxyglutamic acid-containing domains of the proteins and the polar head groups of the phospholipid (for a recent review on the role of phospholipid in blood coagulation, see Ref. 19).

The purpose of the experiments described in this paper is to examine the role of the various components of the intrinsic factor X-activating complex by an analysis of the kinetics of factor X activation. In the past, a direct study of this reaction has been difficult for a number of reasons. Methods for purification of the proteins involved were not available, and factor X activation could only be followed by a coagulation assay. Such an assay is, however, only possible in the presence of other coagulation factors and may be influenced by a number of feedback reactions.

A more detailed study of the activation reaction is now possible because methods are available to purify the proteins involved, and factor X_a formation can be measured directly either with the chromogenic substrate S 2222^2 (20) or through

---

[1] The nomenclature of the blood coagulation factors used is that recommended by the Task Force on Nomenclature of Blood Clotting Zymogens and Zymogen Intermediates.

[2] The abbreviations used are: S 2222, N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroanilide hydrochloride; S 2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride; p-NPGB, p-nitrophenyl-p′-guanidinobenzoate hydrochloride; RVV-X, purified factor X activator from Russell's viper venom; STI, soybean trypsin inhibitor.

the release of an acid-soluble radiolabeled activation peptide (21). Fujikawa *et al.* (1) have shown that the activation of factor X is the result of the cleavage of the same arginyl-isoleucine peptide bond in the heavy chain of factor X both with factor IX_a and factor VIII or tissue factor and factor VII_a as activator. Suomela and Blombäck (20) and Hultin and Nemerson (22) have shown that factor X is activated at a very low rate by factor IX_a in the presence of phospholipid and calcium ions. Addition of thrombin-activated factor VIII increased the rate of factor X_a formation more than 500-fold (22).

Brown *et al.* (23) reported a K_m for factor X of about 0.2 μM when factor X is activated by human factor IX_a and bovine factor VIII in the presence of calcium ions and phospholipid. No activation of factor X by factor IX_a in the absence of accessory components has yet been reported in the literature.

In this article, we present experiments designed to study the kinetics of intrinsic factor X activation. The approach is similar to that followed in a study on the kinetics of prothrombin activation reported earlier (24). Using purified clotting factors and an assay for factor X_a with chromogenic substrate, we are able to show factor X activation by factor IX_a alone and derive kinetic parameters (K_m for factor X and V_{max} for factor X_a formation) for this reaction. We determined the effects of phospholipid and factor VIII on these kinetic parameters. The results of this study allow a first attempt to explain the role of phospholipid and factor $VIII_a$ in the mechanism of factor X activation and an appreciation of the contribution of the intrinsic pathway to thrombin formation *in vivo*.

EXPERIMENTAL PROCEDURES

Materials

S 2222 and S 2238 were purchased from AB Kabi Diagnostica, Stockholm, Sweden. *p*-NPGB was from Nutritional Biochemicals. Russell's viper venom, heparin sodium salt (grade I, 170 USP units/mg), STI, and ovalbumin were obtained from Sigma. DEAE-Sephadex A-50, Sephadex G-100 and G-25, Sepharose 4B and 6B, and CNBr-activated Sepharose 4B were from Pharmacia. AcA 44 was purchased from LKB. Trasylol was from Bayer, Leverkusen, Germany. Kaolin light was from BDH. STI was coupled to CNBr-activated Sepharose 4B following the method of Cuatrecasas (25). Heparin was coupled to Sepharose 4B according to the method of Cuatrecasas (25) as described by Fujikawa *et al.* (26). All reagents used were of the highest grade commercially available.

Methods

Proteins—Bovine factor IX was purified as described by Fujikawa *et al.* (26). Bovine factors X_1 and X_2 were prepared according to Fujikawa *et al.* (27). The factor IX, X_1, and X_2 preparations were homogeneous as determined by gel electrophoresis in the presence of sodium dodecyl sulfate. The specific activities of these preparations, as determined with a clotting assay, were 100 units/mg for factor X and 145 units/mg for factor IX, assuming 1 unit of factor X and 1 unit of factor IX to be present per ml of normal bovine plasma.

Partially purified contact product was prepared according to Nossel (28) and further purified by heparin-agarose affinity chromatography as suggested by Østerud and Rapaport (29). Contact product was applied to the heparin-agarose column (1.5 × 15 cm) in a buffer containing 0.05 M sodium acetate and 0.3 M NaCl at pH 5.5 and was eluted with a linear gradient of 0.3 to 1.0 M NaCl in 0.05 M sodium acetate at pH 5.5.

RVV-X was purified from the crude venom as described by Schiffman *et al.* (30). Bovine factor X_a was prepared from bovine factor X_2 using RVV-X according to the method of Fujikawa *et al.* (31).

Bovine factor IX_a was prepared by incubating factor IX (2 mg/ml) at 37°C with the purified contact product (38 μg/ml) in a buffer containing 50 mM Tris-HCl, 50 mM NaCl at pH 8.5 in the presence of 10 mM $CaCl_2$ (32). After 60 min of incubation EDTA and benzamidine were added to result in final concentrations of 15 mM and 20 mM, respectively. The reaction mixture was then applied to a column of DEAE-Sephadex A-50 (1.5 × 30 cm) in 50 mM Tris-HCl, 50 mM NaCl,

and 20 mM benzamidine at pH 7.9. Factor IX_a was eluted with a linear gradient of 50 mM to 400 mM NaCl (2 × 250 ml) in 50 mM Tris-HCl, 20 mM benzamidine at pH 7.9.

Bovine β factor X was prepared from factor X_2 by incubation of factor X_2 (3 mg/ml) with factor X_a (36 μg/ml) in the presence of 100 μM phospholipid vesicles (for preparation see below) in a buffer containing 175 mM NaCl, 10 mM $CaCl_2$, and 50 mM Tris-HCl at pH 7.9 at 37°C (*cf.* Ref. 33). Gel electrophoresis in the presence of sodium dodecyl sulfate showed that after 90 min of incubation all factor X_2 present was converted into β factor X and a small amount of β factor X_a. So after 90 min of incubation EDTA was added to a final concentration of 15 mM, and the reaction mixture was applied to an AcA 44 column (2.5 × 90 cm) in 0.1 M sodium citrate at pH 6.0. β Factor X was eluted with 0.1 M sodium citrate, pH 6.0. After pooling, the β factor X preparation was dialyzed against 175 mM NaCl, 50 mM Tris-HCl buffer at pH 7.9 and chromatographed on a STI-Sepharose column (1.5 × 10 cm) to remove factor X_a. β Factor X preparations were homogeneous as determined by gel electrophoresis in the presence of sodium dodecyl sulfate.

Thrombin was purified as a prothrombin activation product as described earlier (24).

For the preparation of factor VIII, 9 liters of blood were collected in plastic containers containing 1 liter of 0.1 M oxalate, 100 mM benzamidine, 20,000 units of heparin, and 100,000 Kallikrein Inhibitor Units of Trasylol. Plasma was obtained by centrifugation of the blood at 0°C for 25 min at 2000 × *g* in a MSE Mistral 6L centrifuge. $BaSO_4$ (100 mg/ml) was added to the plasma, and the suspension was stirred for 20 min at 4°C. The $BaSO_4$ was removed by centrifugation at 0°C for 20 min at 2000 × *g*. $BaSO_4$-treated plasma was stored at −70°C in 3-liter plastic containers before further use. After thawing and ethanol precipitation as described by Schmer *et al.* (34), the suspension was centrifuged for 20 min at 2000 × *g* at −2°C in a MSE Mistral 6L centrifuge. The precipitate containing factor VIII was dissolved in a buffer of 0.017 M barbituric acid, 0.125 M NaCl, 0.05 M 6-aminohexanoic acid at pH 7.0 at room temperature. This was then applied to two coupled Sepharose 6B columns (5 × 100 cm) (35).

Factor VIII was eluted at room temperature with the same barbituric acid buffer containing 0.02% NaN_3 at pH 7.0. After about 950 ml of eluate factor VIII activity appeared well separated from a large protein peak containing fibrinogen. The front of the peak (60 ml) was pooled, and the pooled eluate was centrifuged at 190,000 × *g* for 21 h in a MSE Superspeed 65 ultracentrifuge at 4°C. The pellet (factor VIII) was dissolved in 50 mM Tris-HCl, 175 mM NaCl at pH 7.9 to a concentration of 40 units/ml. Following this procedure the total recovery of factor VIII is about 2%. The specific activity of this preparation, measured with a clotting assay, is about 25 units/mg. The activity is raised 15-fold upon incubation with thrombin. The specific activity of purified factor VIII measured with a clotting assay is a minimum value, since it is dependent on the dilution of the factor VIII preparation. Higher values were obtained when higher dilutions were tested.

Factor VIII activities were measured in a one-stage coagulation assay in a factor VIII-deficient plasma prepared according to Chantarangkul *et al.* (36). Fifty-μl buffer containing 0.029 M sodium barbiturate, 0.029 M sodium acetate, 0.116 M NaCl (pH 7.4), 0.25 mg/ml of inosithine, and 0.5 mg/ml of kaolin (light) were incubated for 6 min at 37°C with 50 μl of the factor VIII reagent. Fifty μl of a factor VIII-containing sample was added followed by the addition of 50 μl of 0.033 M $CaCl_2$, and the clotting time was measured. Activities of factor VIII were expressed in units/ml assuming 1 unit/ml present in normal bovine plasma.

All protein preparations were stored at −70°C after dialysis against 50 mM Tris-HCl, 175 mM NaCl at pH 7.9. Before storage at −70°C, the factor X_1, X_2, βX, and IX preparations were passed through a column of STI-Sepharose (0.9 × 20 cm) to reduce the small amounts of factor X_a that might be present in these preparations. No thrombin or factor X_a could be detected in our protein preparations as determined with the chromogenic substrate S 2238 or with S 2222.

Protein Concentrations—Factor X_a concentrations were determined by active site titration with *p*-NPGB according to Smith (37). Factor IX_a concentrations were also determined by active site titration with *p*-NPGB (38). Factor X_1, X_2, and βX concentrations were determined after complete activation with RVV-X followed by active site titration with *p*-NPGB. When protein concentrations are expressed in milligrams per ml, they were calculated from the A_{280} using an $A_{280}^{1\%}$ of 14.9 and 14.3 for factors IX and IX_a (32) and of 12.4 for factors X_1, X_2 (39), and βX.

Phospholipids and Phospholipid Vesicle Preparations—Phos-

pholipid vesicle preparations were made from a mixture of 1,2-di-oleoyl-*sn*-glycero-3-phosphoserine and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (25/75; mol/mol) in a buffer containing 50 mM Tris-HCl and 175 mM NaCl (pH 7.9) as described earlier (24).

Measurement of the Rates of Factor X_a Formation—Activation of factor X by factor IX_a at 37°C either in the presence or absence of phospholipid, $CaCl_2$, and/or factor $VIII_a$ (for further experimental conditions see legends to the figures) was followed by transferring small aliquots of the reaction mixture after different time intervals to a cuvette (thermostated at 37°C) containing a buffer of 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml of ovalbumin, and 10 mM EDTA at pH 7.9 in such amounts that the final volume became 2 ml; 192 μM of the factor X_a-specific chromogenic substrate S 2222 was also present. Further reaction of factor X_a with factor X is prevented by dilution and the presence of EDTA. Since factor IX_a has no amidase activity toward S 2222, the absorbance change recorded at 405–500 nm on an Aminco DW2 spectrophotometer (set in the dual wavelength mode) is a measure of the amount of factor X_a present in the aliquot.

From a calibration curve made with known amounts of active site-titrated factor X_a, determined under the same assay conditions as described above, the amount of factor X_a present in the aliquot can be calculated. The rate of factor X_a formation in the reaction mixture is calculated from the amounts of factor X_a present in the samples taken at different time intervals.

The amount of factor IX_a present in the incubation mixture was chosen such that less than 2% of the factor X added is converted during the experiment. Rates of factor X_a formation are expressed as mole factor X_a formed per min per mol of factor IX_a present as determined by active site titration with *p*-NPGB.

For the construction of Lineweaver-Burk plots the rates of factor X_a formation were averaged from three independent determinations at each factor X concentration. K_m and V_{max} were determined by statistical analysis of the data as described by Eisenthal and Cornish-Bowden (40), and lines were drawn accordingly.

Gel Electrophoresis—Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described in a previous paper (24).

RESULTS

The Kinetics of Factor X Activation in the Absence of Phospholipid and Factor VIII—Although there are no reports in the literature which demonstrate that factor IX_a alone is able to activate factor X, our kinetic experiments on prothrombin activation in solution (24), which showed a high K_m for the substrate prothrombin and a low V_{max}, made clear that it might be possible to activate factor X in solution at high factor X and factor IX_a concentrations. Fig. 1 shows that factor IX_a is indeed able to activate factor X in the absence of calcium ions, factor VIII, and phospholipid. Calcium ions stimulate the activation about 7-fold, and there is a rather broad optimum around 10 mM Ca^{2+}. The Ca^{2+} titration curves are independent of the amounts of factor IX_a and factor X present. Pretreatment of factor IX_a with 10 mM diisopropyl-phosphorofluoridate for 1 h followed by dialysis did not affect the rate of activation. Since factor IX_a is probably the only serine protease involved in blood coagulation that is not sensitive to diisopropylphosphorofluoridate (5, 41), it is unlikely that factor X activation in the above experiment is accomplished by traces of other serine proteases contaminating our factor IX_a preparations.

To allow a kinetic analysis of the reaction, it is necessary to demonstrate that the rate of factor X_a formation is constant in time and increases linearly with the amount of factor IX_a present. That this is the case is shown in Fig. 2. We also obtained constant rates of factor X_a formation proportional to the amount of factor IX_a at higher factor X concentrations and in the absence of calcium ions (data not shown).

The rate of factor X_a formation at various factor X concentrations was determined in the absence of calcium ions and in the presence of 10 mM $CaCl_2$. The data are presented in the form of Lineweaver-Burk plots (Fig. 3, *A* and *B*). In the absence of calcium ions the K_m for factor X is 299 μM, and the V_{max} of factor X_a formation is 2.2×10^{-3} mol of $X_a \cdot min^{-1} \cdot$
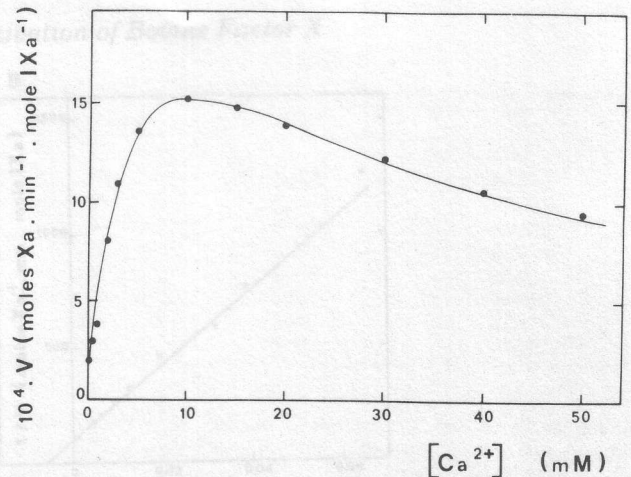

FIG. 1. **The Ca^{2+} dependence of factor X activation by factor IX_a in solution.** Factor X_2 (31.4 μM) was incubated at 37°C with factor IX_a (1.1×10^{-3} μmol/ml) in 200 μl of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml of ovalbumin at pH 7.9 in the presence of varying amounts of $CaCl_2$. Varying amounts of extra NaCl were present to compensate for the changes in ionic strength due to the variation of the $CaCl_2$ concentration. When no $CaCl_2$ was added the presence of 5 mM EDTA did not influence the rate of factor X_a formation. The rate of factor X_a formation was calculated from the amounts of factor X_a present after 7.5 and 15 min of incubation.
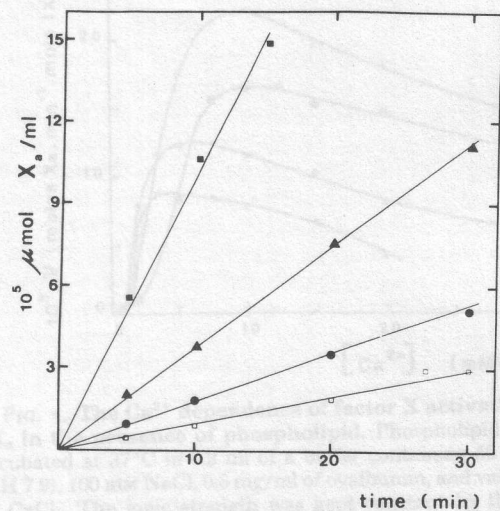

FIG. 2. **Time course of factor X activation with different amounts of factor IX_a.** Factor X_2 (31.4 μM) was incubated at 37°C with varying amounts of factor IX_a in 200 μl of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 10 mM $CaCl_2$, and 0.5 mg/ml of ovalbumin (pH 7.9). After the time intervals indicated, a sample was taken and assayed for factor X_a as described under "Experimental Procedures." The amounts of factor IX_a present were: □——□, 0.55×10^{-3} μmol/ml; ●——●, 1.1×10^{-3} μmol/ml; ▲——▲, 2.2×10^{-3} μmol/ml; ■——■, 6.6×10^{-3} μmol/ml.

mol of IX_a^{-1}. In the presence of 10 mM $CaCl_2$ the K_m is 181 μM, and the V_{max} is 10.5×10^{-3} mol of $X_a \cdot min^{-1} \cdot$ mol of IX_a^{-1}. The factor X used in these experiments was factor X_2. Hence all subsequent experiments reported in this paper are carried out with factor X_2 as substrate.

The Kinetics of Factor X Activation in the Presence of Phospholipid and Calcium Ions—The time course of factor X activation by factor IX_a in the presence of calcium ions and varying factor X and phospholipid concentrations is shown in Fig. 1*.[3] To obtain a linear time course of factor X activation

[3] Portions of this paper (including Figs. 1*–5*) are presented in miniprint at the end of this paper. Miniprint is easily read with the
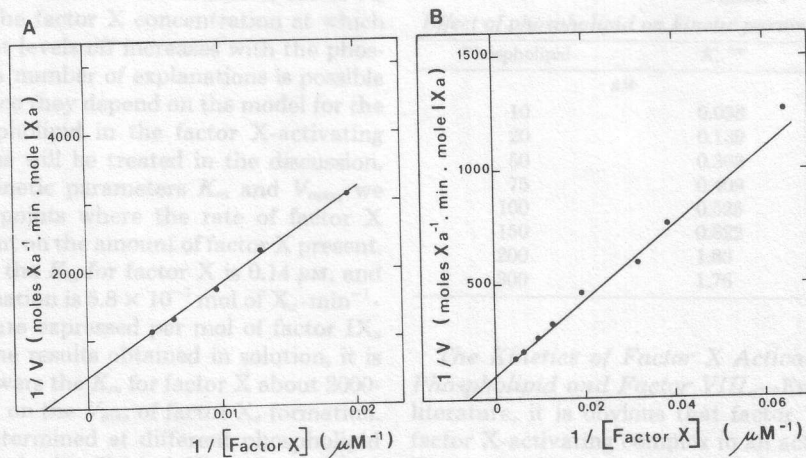
FIG. 3. **Lineweaver-Burk plots of factor X activation by factor IX$_a$ in the presence and absence of CaCl$_2$.** Factor X$_a$ formation at varying concentrations of factor X$_2$ was measured at pH 7.9 at 37°C in 200 μl of a reaction mixture containing 50 mM Tris-HCl, 175 mM NaCl, ovalbumin (0.5 mg/ml), factor IX$_a$ (1.1×10^{-3} μmol/ml). *A,* in the presence of 5 mM EDTA, and *B,* in the presence of 10 mM

it is essential to preincubate the phospholipid vesicles for 10 min at 37°C in the presence of 50 mM CaCl$_2$. As can be seen the rate of factor X activation is linear at factor X concentrations well below and above the K_m determined at the respective phospholipid concentrations (see below).

Jesty *et al.* (33, 42) and Fujikawa *et al.* (43) have shown that factor X$_a$ is able to catalyze a number of feedback reactions on the substrate factor X, especially in the presence of phospholipid and calcium ions. Factor X$_a$ can cleave a small glycopeptide from the carboxyl-terminal end of the heavy chain of factor X, giving rise to so-called β factor X. Factor X$_a$ is also able to convert both factor X and β factor X auto-catalytically into, respectively, α factor X$_a$ and β factor X$_a$ by hydrolyzing a specific peptide bond in the NH$_2$-terminal region of the heavy chain. This second feedback reaction especially may interfere with the determination of the rate of factor X activation by factor IX$_a$. It is possible, however, to select experimental conditions such that factor X activation by factor X$_a$ is negligible compared to the contribution by factor IX$_a$. The low amounts of factor X$_a$ formed in our experiments (0.05–2 pmol/ml) can easily be detected on the Aminco DW2 spectrophotometer. When these amounts of factor X$_a$ are incubated with calcium ions, phospholipid, and factor X under the conditions employed in our kinetic experiments, no extra factor X$_a$ formation is detectable. As an extra control an aliquot of the reaction mixture (see legend to Fig. 1*) taken after 30 min was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The only protein that could be seen on the gel was factor X (data not shown), and no β factor X or factor X$_a$ is detectable. From these control experiments we conclude that the factor X$_a$ formation we measure in our experiments results from the action of factor IX$_a$ on α factor X.

The Ca^{2+} dependence of the rate of factor X$_a$ formation at various phospholipid concentrations is shown in Fig. 4. The titration curves are sigmoidal, and the optimal Ca^{2+} concentration slightly increases with increasing phospholipid concentrations. The Ca^{2+} optimum is, however, independently of the
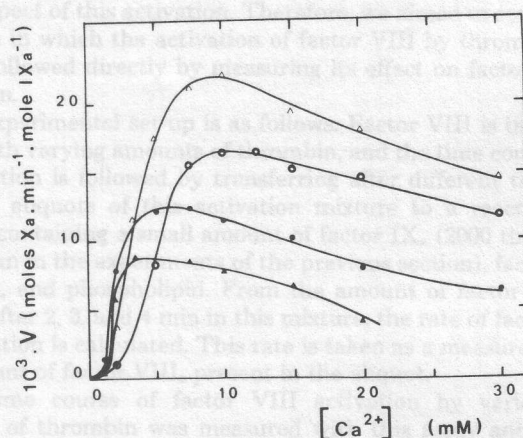
CaCl$_2$. The reaction was started by the addition of factor IX$_a$. After 7.5 and 15 min samples were taken and assayed for factor X$_a$ as described under "Experimental Procedures." From the amounts of factor X$_a$ found, the rate of factor X$_a$ formation was calculated. The kinetic constants calculated are summarized in Table III.

FIG. 4. **The Ca^{2+} dependence of factor X activation by factor IX$_a$ in the presence of phospholipid.** Phospholipid vesicles were incubated at 37°C in 0.3 ml of a buffer containing 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 0.5 mg/ml of ovalbumin, and varying amounts of CaCl$_2$. The ionic strength was kept constant by the addition of extra NaCl. After 10 min, 0.95 ml of factor X (2.0 μM) was added, and 4 min later, factor X activation was started by the addition of 0.25 ml of factor IX$_a$ (0.09 μM). Both proteins were dissolved in a buffer containing 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, and 0.5 mg/ml of ovalbumin. The final reaction mixture contained 50 mM Tris-HCl (pH 7.9), 0.5 mg/ml of ovalbumin, an amount of NaCl to bring the ionic strength at 0.21, CaCl$_2$ as indicated in the figure, 1.27 μM factor X, 1.5 $\times$ 10^{-5} μmol/ml of factor IX$_a$ and ▲——▲, 25 μM phospholipid; ●——●, 50 μM phospholipid; △——△, 200 μM phospholipid; and ○——○, 400 μM phospholipid. The rates of factor X$_a$ formation were calculated from the amount of factor X$_a$ present in the reaction mixture after 7.5 and 15 min. Phospholipid vesicles were prepared as described under "Experimental Procedures."

amounts of factor IX$_a$ and factor X present. When the factor IX$_a$ concentration is varied at a constant amount of factor X, calcium ions, and phospholipid, the rate of factor X activation observed is directly proportional to the factor IX$_a$ concentration (Fig. 2*).

The dependence of the rate of factor X$_a$ formation on the factor X concentration was determined at 10 mM CaCl$_2$ and various phospholipid concentrations. A set of Lineweaver-Burk plots was obtained, two of which are shown in Fig. 5. An interesting feature of the Lineweaver-Burk plots shown in Fig. 5 is the hockey stick shape of the plot. Above a certain factor

X concentration, no further increase of the rate of factor X_a formation is observed. The factor X concentration at which the Lineweaver-Burk plot levels off increases with the phospholipid concentration. A number of explanations is possible for this phenomenon. Since they depend on the model for the mode of action of phospholipid in the factor X-activating complex, the explanations will be treated in the discussion. For calculation of the kinetic parameters K_m and V_{max}, we only used experimental points where the rate of factor X activation is still dependent on the amount of factor X present.

At 20 μM phospholipid the K_m for factor X is 0.14 μM, and the V_{max} of factor X_a formation is 5.8×10^{-3} mol of $X_a \cdot min^{-1} \cdot$ mol of IX_a^{-1}. The rates are expressed per mol of factor IX_a added. Compared with the results obtained in solution, it is seen that phospholipid lowers the K_m for factor X about 2000-fold with only little effect on the V_{max} of factor X_a formation. The kinetic constants determined at different phospholipid concentrations are summarized in Table I. There is a gradual increase of the K_m for factor X when the phospholipid concentration is raised. The V_{max} of factor X_a formation also increases with the phospholipid concentration. The variation of V_{max} may be due to the fact that the V_{max} is calculated from the amount of factor IX_a added, while it is actually the amount of factor IX_a bound to the phospholipid surface at each phospholipid concentration that determines the rate of factor X activation. The kinetic parameters of factor X activation in solution are such that free factor IX_a does not contribute to factor X_a formation. Therefore, a variation of the amount of factor IX_a bound due to the variation of the phospholipid concentration will then be reflected in the observed value of V_{max}. In that case extrapolation of a double reciprocal plot ($1/V_{max}$ *versus* $1/$[phospholipid]) at a constant factor IX_a to infinite phospholipid concentration yields the V_{max} for bound factor IX_a (Fig. 3*). The V_{max} calculated from this plot is 0.08 mol of $X_a \cdot min^{-1} \cdot$ mol of IX_a^{-1} (bound).

TABLE I

Effect of phospholipid on kinetic parameters of factor X activation

Phospholipid	$K_m{}^{app}$	V_{max}
μM		$mol\ X_a \cdot min^{-1} \cdot mol\ IX_a^{-1}$
10	0.058	0.00247
20	0.139	0.00579
50	0.363	0.0226
75	0.409	0.0219
100	0.525	0.0231
150	0.822	0.0295
200	1.83	0.0474
300	1.76	0.0437

The Kinetics of Factor X Activation in the Presence of Phospholipid and Factor VIII$_a$—From many reports in the literature, it is obvious that factor VIII participates in the factor X-activating complex in an activated form (15, 18, 22). The activity of factor VIII in the intrinsic factor X activation is considerably enhanced by prior incubation with trace amounts of thrombin. The effect of thrombin on the activity of factor VIII is evaluated in general with a clotting assay. Feedback reactions by thrombin and other serine proteases accumulating during clotting hamper a study of the quantitative aspect of this activation. Therefore, we aimed to set up a system in which the activation of factor VIII by thrombin can be followed directly by measuring its effect on factor X activation.

The experimental set-up is as follows. Factor VIII is incubated with varying amounts of thrombin, and the time course of activation is followed by transferring after different time intervals aliquots of this activation mixture to a reaction mixture containing a small amount of factor IX_a (2000 times lower than in the experiments of the previous section), factor X, CaCl$_2$, and phospholipid. From the amount of factor X_a formed after 2, 3, and 4 min in this mixture, the rate of factor X_a formation is calculated. This rate is taken as a measure of the amount of factor VIII$_a$ present in the aliquot.

The time course of factor VIII activation by various amounts of thrombin was measured with this assay and is shown in Fig. 4*. Incubation of factor VIII with thrombin results in a rapid increase of factor VIII$_a$ activity followed by a decrease. Both the rates of activation and inactivation increase with the thrombin concentration. When either factor VIII or thrombin is omitted from the activation mixture no factor X_a is formed in the reaction mixture. This experiment stresses again that factor VIII has to be activated before it can exert its stimulating effect on the activation of factor X by factor IX_a.

We were unable to show activation of factor VIII by factor X_a either in the presence or in the absence of phospholipid and calcium ions. This is in contrast with earlier reported findings (16, 18) that factor X_a can activate factor VIII. We have to emphasize, however, that the amount of factor X_a is limited to 0.5 μg/ml, since higher amounts of factor X_a cause autocatalytic factor X activation in the assay system.

In our further experiments, factor VIII is activated by incubation with 0.04 μg of thrombin/ml for 5 min at 37 °C.

The time course of factor X activation in the presence of factor VIII$_a$, calcium ions, and 5, 10, or 25 μM phospholipid (Fig. 6). Factor VIII$_a$, activated as described above, was added to a mixture of phospholipid (pretreated with CaCl$_2$), CaCl$_2$, factor IX_a, and factor X preincubated 3 min at 37 °C. A typical time course of factor X activation shows a lag period of 1–2 min, a 4-min period with an apparent constant rate of factor X_a formation followed by a decrease of the activation rate. We have been unable to shorten the lag period by changing the
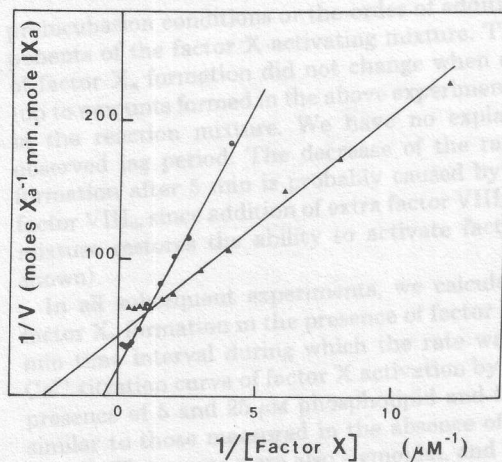

FIG. 5. **Lineweaver-Burk plots of factor X activation by factor IX_a in the presence of phospholipid.** Phospholipid vesicles were preincubated at 37 °C in a buffer containing 50 mM Tris-HCl, 175 mM NaCl, and 50 mM CaCl$_2$ at pH 7.9. After 10 min, 0.1 ml of the phospholipid mixture was transferred to 0.35 ml of a buffer containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 0.5 mg/ml of ovalbumin, and varying amounts of factor X. Four min later, factor X activation was strated by the addition of 0.05 ml of factor IX_a (0.11 μM). The final reaction mixture contained: 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 0.4 mg/ml of ovalbumin, 10 mM CaCl$_2$, 1.09×10^{-5} μmol/ml of IX_a, varying amounts of factor X, and ▲——▲, 50 μM phospholipid; ●——●, 200 μM phospholipid. The rate of factor X_a formation was calculated from the amount of factor X_a present in the reaction mixture after 7.5 and 15 min. Phospholipid vesicles were prepared as described under "Experimental Procedures." The kinetic parameters calculated are summarized in Table I.
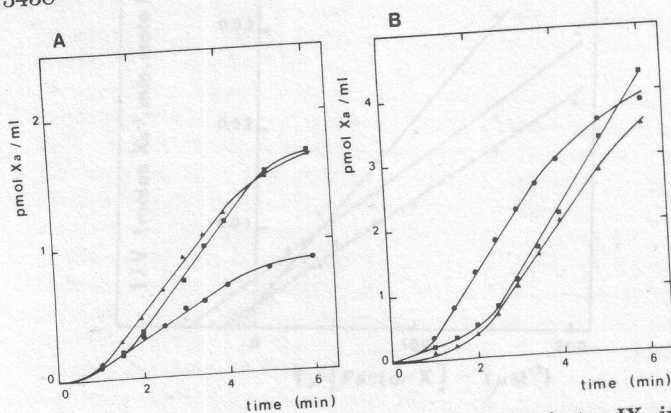
FIG. 6. **Time course of factor X activation by factor IX_a in the presence of phospholipid, factor $VIII_a$, and $CaCl_2$.** Factor VIII (20 units/ml) was activated at 37°C with 0.04 mg/ml of thrombin. After 5 min 0.5 ml of the activation mixture was added to 0.5 ml of a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 10 mM $CaCl_2$, 1 mg/ml of ovalbumin, 0.68×10^{-8} µmol/ml of factor IX_a, and various amounts of phospholipid and factor X at 37°C. Before use, phospholipid vesicles were incubated 10 min at 37°C in a buffer containing 50 mM Tris, 175 mM NaCl, and 50 mM $CaCl_2$ at pH 7.9. After the time intervals indicated in the figure aliquots from this activation mixture were taken and assayed for factor X_a. The final reaction mixture contained 50 mM Tris (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/ml of ovalbumin, 0.34×10^{-8} µmol/ml of IX_a, factor $VIII_a$, and ●——●, 5 µM phospholipid, 0.005 µM factor X; ▲——▲, 10 µM phospholipid, 0.01 µM factor X; ■——■, 25 µM phospholipid, 0.025 µM factor X; ●——●, 5 µM phospholipid, 0.05 µM factor X; ▲——▲, 10 µM phospholipid, 0.1 µM factor X (A) or ●——●, 5 µM phospholipid, 0.05 µM factor X; ■——■, 25 µM phospholipid, 0.25 µM factor X (B). Phospholipid vesicles were prepared as described under "Experimental Procedures."

preincubation conditions or the order of addition of the components of the factor X-activating mixture. The time course of factor X_a formation did not change when extra factor X_a (up to amounts formed in the above experiment) was included in the reaction mixture. We have no explanation for the observed lag period. The decrease of the rate of factor X_a formation after 5 min is probably caused by inactivation of factor $VIII_a$, since addition of extra factor $VIII_a$ to the reaction mixture restores the ability to activate factor X (data not shown).

In all subsequent experiments, we calculated the rate of factor X_a formation in the presence of factor $VIII_a$ over the 4-min time interval during which the rate was constant. The Ca^{2+} titration curve of factor X activation by factor IX_a in the presence of 5 and 25 µM phospholipid and factor $VIII_a$ were similar to those measured in the absence of factor $VIII_a$ (*cf.* Fig. 4). The curves were also sigmoidal, and the optimal Ca^{2+} concentration under these conditions is about 7.5 mM at both phospholipid concentrations.

When the factor IX_a concentration is varied at a constant amount of factor X, $CaCl_2$, phospholipid, and factor $VIII_a$, the rate of factor X activation observed is directly proportional to the factor IX_a concentration (Fig. 5*).

Having established the best experimental conditions for measurement of factor X activation with the complete factor X-activating complex, Lineweaver-Burk plots were made at 10 µM phospholipid with various amounts of factor $VIII_a$ present (Fig. 7). The kinetic parameters that are obtained from these plots are summarized in Table II. At high factor $VIII_a$ concentrations the K_m for factor X is 0.063 µM, and the V_{max} is 500 mol of $X_a \cdot min^{-1} \cdot$ mol of IX_a^{-1}. When the same experiment was carried out in the absence of factor $VIII_a$, the K_m was 0.058 µM and the V_{max} was 0.0025 mol of $X_a \cdot min^{-1} \cdot$ mol of IX_a^{-1} (see Table I). It is obvious that the presence of factor $VIII_a$ hardly affects the K_m for factor X but increases
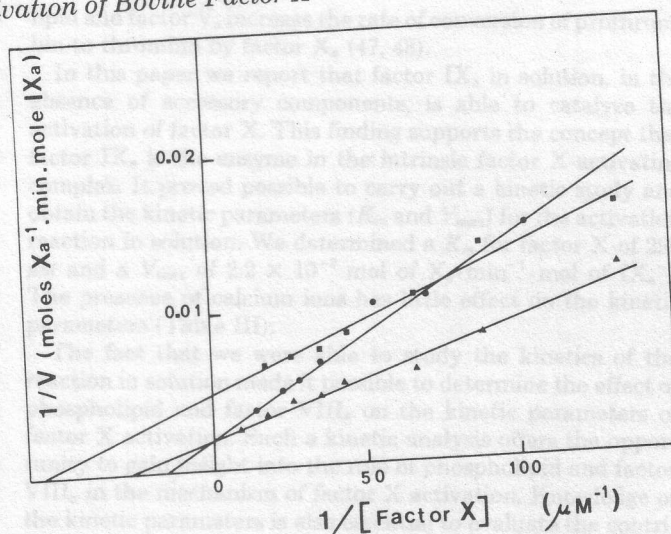

FIG. 7. **Lineweaver-Burk plots of factor X_a formation by factor IX_a in the presence of phospholipid and varying amounts of factor VIII.** Factor VIII (20 units/ml) was activated at 37°C with 0.04 µg/ml of thrombin in a buffer containing 50 mM Tris-HCl and 175 mM NaCl at pH 7.9. After 5 min of incubation varying amounts of this incubation mixture were added to a reaction mixture containing the further components for factor X activation in such amounts that the final concentrations in the reaction mixture (1 ml) became 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 7.5 mM $CaCl_2$, 0.5 mg/ml of ovalbumin, 0.34×10^{-8} µmol/ml of factor IX_a, 10 µM phospholipid, varying amounts of factor X, and ■——■, 2 units/ml of factor VIII, ▲——▲, 8 units/ml of factor VIII, and ●——●, 11 units/ml of factor VIII. At 2, 3, and 4 min after addition of factor VIII to the reaction mixture, aliquots were taken and assayed for factor X_a. The rate of factor X_a formation was calculated from the amounts of factor X_a present in these aliquots. The phospholipid vesicles present in the reaction mixture were prepared as described under "Experimental Procedures" and were incubated for 10 min at 37°C in 50 mM Tris-HCl (pH 7.9) and 175 mM NaCl before addition to the reaction mixture. The kinetic parameters calculated are summarized in Table II.

TABLE II

Effect of factor $VIII_a$ and phospholipid on the kinetic parameters of factor X activation

Phospholipid	$VIII_a$	K_m^{app}	V_{max}
µM	*units/ml*	*µM*	*mol $X_a \cdot min^{-1} \cdot$ mol IX_a^{-1}*
10	2	0.018	207
10	4	0.022	309
10	8	0.036	497
10	11	0.063	500
5	8	0.024	198
10	8	0.045	439
25	8	0.083	436

the V_{max} of factor X_a formation about 200,000-fold. The V_{max} increases with the amount of factor $VIII_a$ added (Table II). At high concentrations of factor VIII the rate of activation is not further increased, indicating that it is possible to add saturating amounts of factor $VIII_a$.

In a model in which factor $VIII_a$ is the cofactor of the enzyme factor IX_a, factor IX_a is saturated with factor $VIII_a$, and an optimal concentration of factor IX_a-factor $VIII_a$ complex is formed. It is a question, however, whether the model is as simple as that, since the amount of factor $VIII_a$ present also influences the K_m for factor X that is measured (Table II). For this phenomenon we have no obvious explanation yet.

Lineweaver-Burk plots in the presence of factor $VIII_a$ (8 units/ml) were made at three different phospholipid concentrations (Fig. 8). The kinetic constants obtained from these
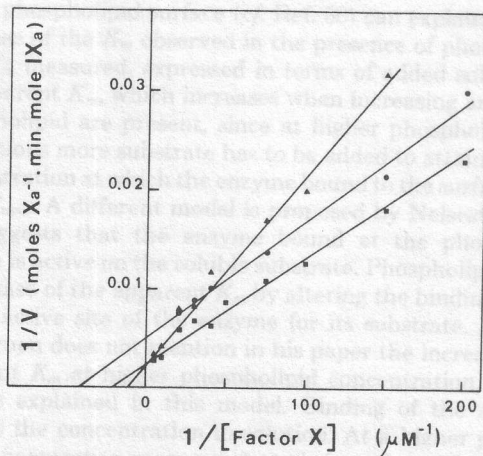
FIG. 8. **Lineweaver-Burk plots of factor X_a formation by factor IX_a in the presence of factor $VIII_a$ and varying amounts of phospholipid.** The rate of factor X_a formation at various factor X concentrations was measured at ●——●, 5 μM phospholipid, ■——■, 10 μM phospholipid, and ▲——▲, 25 μM phospholipid in the presence of activated factor VIII (8 units/ml) and $CaCl_2$ (7.5 mM). For experimental details, see legend to Fig. 7.

plots are also summarized in Table II. The K_m for factor X appears to be dependent on the phospholipid concentration to the same extent as observed in the experiments carried out in the absence of factor $VIII_a$ (cf. Table I).

Kinetics of Activation of Factor X_1 and β Factor X—Since all experiments reported in this paper were carried out with factor X_2, we were interested whether factor X_1 and β factor X are kinetically identical. Therefore, a number of experiments were repeated using factor X_1 and factor βX as substrate. Both factor X_1 and βX showed the same kinetics of activation as factor X_2.

DISCUSSION

Previous studies on the intrinsic activation of factor X suggest that the activator is a complex composed of factor IX_a, factor $VIII_a$, phospholipid, and calcium ions (4–11, 22, 23). Several properties of factor IX_a indicate that it is a serine protease and hence will be the enzyme that actually activates factor X. Factor IX_a is highly homologous to other serine proteases involved in blood coagulation (13) and has an esterase activity toward synthetic arginine esters (44). Its enzymatic activity is also inhibited by well known serine protease inhibitors like antithrombin III (44, 45) and hirudin (46).

When it is accepted that factor IX_a is the enzyme in the factor X-activating complex it is plausible to assume that factor VIII, phospholipid, and calcium ions act as cofactors to factor IX_a. In this concept the roles of factor IX_a, factor VIII, and phospholipid are analogous to those of, respectively, factor X_a, factor V_a, and phospholipid in the prothrombin-activating complex.

If this model is correct one would expect that factor IX_a can activate factor X in the absence of accessory components. Although no activation of factor X by factor IX_a in solution has been reported yet, Hultin and Nemerson (22) have shown that factor IX_a in the presence of phospholipid and calcium ions, but without factor VIII, slowly activates factor X. The rate of factor X activation was substantially increased when thrombin-activated factor VIII was included in the reaction mixture. A rate enhancement caused by factor VIII has been reported in several other papers (1, 20, 22, 23). These findings extend the analogy with the prothrombin-activating complex. The accessory components phospholipid and factor $VIII_a$ stimulate factor X activation by factor IX_a, just as phospho-

lipid and factor V_a increase the rate of conversion of prothrombin to thrombin by factor X_a (47, 48).

In this paper we report that factor IX_a in solution, in the absence of accessory components, is able to catalyze the activation of factor X. This finding supports the concept that factor IX_a is the enzyme in the intrinsic factor X-activating complex. It proved possible to carry out a kinetic study and obtain the kinetic parameters (K_m and V_{max}) for the activation reaction in solution. We determined a K_m for factor X of 299 μM and a V_{max} of 2.2×10^{-3} mol of $X_a \cdot min^{-1} \cdot$ mol of IX_a^{-1}. The presence of calcium ions has little effect on the kinetic parameters (Table III).

The fact that we were able to study the kinetics of the reaction in solution made it possible to determine the effect of phospholipid and factor $VIII_a$ on the kinetic parameters of factor X activation. Such a kinetic analysis offers the opportunity to gain insight into the role of phospholipid and factor $VIII_a$ in the mechanism of factor X activation. Knowledge of the kinetic parameters is also essential to evaluate the contribution of intrinsic factor X activation to factor X_a formation at physiological conditions.

The kinetic parameters for different factor X-activating mixtures are summarized in Table III. Both phospholipid and factor $VIII_a$ cause important changes of the kinetic parameters of factor X activation. In the presence of 10 μM phospholipid the K_m drops from 181 μM to 0.058 μM, while there is little change of the V_{max}. The effect of factor $VIII_a$ is mainly restricted to the V_{max}. In the presence of factor $VIII_a$ the V_{max} increases about 200,000-fold.

The data presented in this report have important implications for the mode of action of phospholipid in the mechanism of intrinsic factor X activation. Phospholipid has little effect on the catalytic activity of factor IX_a. For free factor IX_a a V_{max} of 0.01 mol of $X_a \cdot min^{-1} \cdot$ mol of IX_a^{-1} is measured (Table III), and for phospholipid-bound factor IX_a the V_{max} is 0.08 mol of $X_a \cdot min^{-1} \cdot$ mol of IX_a^{-1}. The K_m for factor X is dramatically decreased in the presence of phospholipid. There is, however, a considerable increase of the K_m at higher phospholipid concentrations, so a K_m measured in the presence of phospholipid has to be regarded as an apparent K_m. In a previous paper (24), we have shown that in the prothrombinase complex phospholipid causes a marked decrease of the K_m for prothrombin, and the K_m is also raised at increasing phospholipid concentrations. Hence it seems plausible that the mechanistic basis for the mode of action of phospholipid in both complexes is identical. Two models have been proposed to explain the role of phospholipid in prothrombin activation. Because of the similarity between prothrombin and factor X activation with respect to phospholipid involvement, these models are also applicable to factor X activation. In previous papers (24, 49), we suggested that the enzyme and substrate are bound to the phospholipid surface and that the amount of bound substrate determines the rate of activation. An increased local substrate concentration at or in the vicinity

of the phospholipid surface (*cf.* Ref. 50) can explain the large decrease of the K_m observed in the presence of phospholipid. The K_m measured, expressed in terms of added substrate, is an apparent K_m, which increases when increasing amounts of phospholipid are present, since at higher phospholipid concentrations more substrate has to be added to attain the local concentration at which the enzyme bound to the surface works at ½ V_{max}. A different model is proposed by Nelsestuen (51). He suggests that the enzyme bound at the phospholipid surface is active on the soluble substrate. Phospholipid causes a decrease of the apparent K_m by altering the binding affinity of the active site of the enzyme for its substrate. Although Nelsestuen does not mention in his paper the increase of the apparent K_m at higher phospholipid concentration, this can also be explained in this model. Binding of the substrate reduces the concentration in solution. At a higher phospholipid concentration more substrate is bound, and hence more substrate has to be added to reach a substrate level in solution at which the enzyme functions at ½ V_{max}. It will be obvious that both models can qualitatively explain the effect of phospholipid on the K_m for factor X of the intrinsic factor X activator reported in this paper. The flattening of the Lineweaver-Burk plots observed at high factor X concentrations is most easily explained in the model where bound factor X is the substrate. At high factor X concentrations the binding sites for factor X at the phospholipid vesicles become saturated. The addition of extra factor X cannot further increase the bound factor X concentration, and consequently there is no further increase in the rate of activation. At higher phospholipid concentration more factor X is required to saturate the binding sites, and hence the leveling off occurs at a higher factor X concentration. It is interesting to notice that the break point in the Lineweaver-Burk plots is observed at factor X concentrations about equal to the concentration of binding sites for factor X present on the kind of phospholipid vesicles used in our experiments (0.011 μM binding sites/μM phospholipid; Ref. 52). A qualitative explanation of the above phenomenon in the model where soluble factor X is the substrate is not readily available. In order to determine which of the two models is valid, the exact binding parameters of factor X binding to the phospholipid vesicles under the conditions of our kinetic experiments have to be known. In that case the amounts of bound and free factor X at each individual point of the Lineweaver-Burk plot can be calculated, and it can be verified whether the reaction obeys Michaelis-Menten kinetics with bound or soluble substrate. At the moment experiments are underway in our laboratory in which binding and kinetic parameters for various phospholipid mixtures are determined under identical conditions with the aim to discriminate between the two models.

Factor VIII$_a$ enormously stimulates the V_{max} of factor X activation. No activation of factor X can be detected by factor VIII$_a$ alone. Since factor IX$_a$, even in the absence of accessory components, can catalyze the formation of factor X$_a$, we conclude that factor VIII$_a$ acts as a co-factor to accelerate an enzymatic reaction which already occurs in its absence. Factor VIII has to be activated with thrombin to accomplish its stimulatory effect on the V_{max}. When factor VIII$_a$ is replaced by factor VIII in an experiment carried out at a saturating factor VIII$_a$ concentration, the rate of factor X activation is below 1% of that measured with factor VIII$_a$. The remaining activity cannot be explained by the action of factor IX$_a$ alone. Since our unactivated factor VIII preparation may contain small amounts of factor VIII$_a$, we cannot determine whether native factor VIII can support factor X activation. The V_{max} rises when increasing amounts of factor VIII$_a$ are present. At high concentrations of factor VIII$_a$ the V_{max} is not further

increased, which in the co-factor model for factor VIII$_a$ would mean that factor IX$_a$ becomes saturated with co-factor. Under these conditions factor IX$_a$ has a proteolytic activity comparable to factor X$_a$ in the prothrombin-activating complex (24, 53) and to factor VII$_a$ in the extrinsic factor X-activating complex (21) determined in the presence of saturating amounts of their respective co-factors V$_a$ and tissue factor. For the increase of the K_m for factor X observed at increasing factor VIII$_a$ concentrations we have no interpretation yet. A more extensive kinetic analysis will be required to explain this phenomenon. The high molecular weight form of factor VIII was used in the experiments described in this paper. This preparation, generally referred to as factor VIII/von Willebrand factor (factor VIII/vWF) is a complex of low molecular weight factor VIII coagulant activity and high molecular weight von Willebrand platelet aggregating activity. Recently, Vehar and Davie (18) succeeded in obtaining a highly purified factor VIII coagulant activity. It will be interesting to compare the effects of factor VIII coagulant activity and factor VIII/vWF on the kinetics of factor X activaton.

The physiological significance of the alteration of kinetic parameters will be obvious. Intrinsic factor X activation requires phospholipid because it lowers the K_m for the substrate to within the range of the plasma concentration (0.2 μM). At high phospholipid concentrations the K_m rises, however, above the plasma factor X concentration. It is possible that this phenomenon plays a role in the physiologic regulation of factor X$_a$ formation. Factor VIII$_a$ via its effect on V_{max} increases the rate of factor X activation to a level where sufficient factor X$_a$ is formed to control hemostasis. It is interesting to compare the kinetic parameters that we found for the intrinsic factor X activator with those of the extrinsic factor X activator determined by Silverberg *et al.* (21). They found a K_m for factor X of 0.34 μM and a V_{max} of 1900 mol of X$_a$ · min^{-1} · mol of VII$_a$$^{-1}$. Thus the catalytic efficiency, V_{max}/K_m, for both activator systems is about equal. These data will have to be taken into consideration in a discussion about the contribution of the intrinsic and extrinsic factor X activation to factor X$_a$ formation *in vivo*.

REFERENCES

1. Fujikawa, K., Coan, M. H., Legaz, M. E., and Davie, E. W. (1974) *Biochemistry* **13**, 5290–5299
2. Jesty, J., and Nemerson, Y. (1974) *J. Biol. Chem.* **249**, 509–515
3. Davie, E. W., and Fujikawa, K. (1975) *Annu. Rev. Biochem.* **44**, 799–829
4. MacFarlane, R. G., Biggs, R., Ash, B. J., and Denson, K. W. E. (1964) *Br. J. Haematol.* **10**, 530–541
5. Lundblad, R. L., and Davie, E. W. (1964) *Biochemistry* , 1720–1725
6. Schiffman, S., Rapaport, S. I., and Chong, M. M. Y. (1966) *Proc. Soc. Exp. Biol. Med.* **123**, 736–744
7. Hougie, C., Denson, K. W. E., and Biggs, R. (1967) *Thromb. Diath. Haemorrh.* **18**, 211–222
8. Barton, P. G. (1967) *Nature* **215**, 1508–1509
9. Hemker, H. C., and Kahn, M. J. P. (1967) *Nature* **215**, 1201–1202
10. Østerud, B., and Rapaport, S. I. (1970) *Biochemistry* **9**, 1854–1861
11. Varadi, K., and Hemker, H. C. (1976) *Thromb. Res.* **8**, 303–317
12. Bucher, K., Nebelin, E., Thomsen, J., and Stenflo, J. (1976) *FEBS Lett.* **68**, 293–296
13. Katayama, K., Ericsson, L. H., Enfield, D. L., Walsh, K. A., Neurath, H., Davie, E. W., and Titani, K. (1979) *Proc. Natl.*

Acad. Sci. U. S. A. **76**, 4990–4994

14. Hemker, H. C., Kahn, M. J. P., and Devilee, P. P. (1970) *Thromb. Diath. Haemorrh.* **24**, 214–223

15. Østerud, B., Rapaport, S. I., Schiffman, S., and Chong, M. M. Y. (1971) *Br. J. Haematol.* **21**, 643–660

16. Davie, E. W., Fujikawa, K., Legaz, M. E., and Kato, H. (1975) *Cold Spring Harbor Conf. Cell Proliferation* **2**, 65–72

17. Vehar, G. A., and Davie, E. W. (1977) *Science* **197**, 374–376

18. Vehar, G. A., and Davie, E. W. (1980) *Biochemistry* **19**, 401–410

19. Zwaal, R. F. A. (1978) *Biochim. Biophys. Acta* **515**, 163–205

20. Suomela, H., and Blombäck, B. (1977) *Thromb. Res.* **1**, 267–281

21. Silverberg, S. A., Nemerson, Y., and Zur, M. (1977) *J. Biol. Chem.* **252**, 8481–8488

22. Hultin, M. B., and Nemerson, Y. (1978) *Blood* **52**, 928–940

23. Brown, J. E., Baugh, R. F., and Hougie, C. (1978) *Thromb. Res.* **13**, 893–900

24. Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Zwaal, R. F. A., and Hemker, H. C. (1980) *J. Biol. Chem.* **255**, 274–283

25. Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059–3065

26. Fujikawa, K., Thompson, A. R., Legaz, M. E., Meyer, R. G., and Davie, E. W. (1973) *Biochemistry* **12**, 4938–4945

27. Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972) *Biochemistry* **11**, 4882–4891

28. Nossel, H.. L. (1964) *The Contact Phase of Blood Coagulation*, Blackwell Scientific Publication, Oxford

29. Østerud, B., and Rapaport, S. I. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5260–5264

30. Schiffman, S., Theodor, I., and Rapaport, S. I. (1969) *Biochemistry* **8**, 1397–1405

31. Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972) *Biochemistry* **11**, 4892–4899

32. Fujikawa, K., Legaz, M. E., Kato, H., and Davie, E. W. (1974) *Biochemistry* **13**, 4508–4516

33. Jesty, J., Spencer, A. K., and Nemerson, Y. (1974) *J. Biol. Chem.* **249**, 5614–5622

34. Schmer, G., Kirby, E. P., Teller, D. C., Davie, E. W. (1972) *J. Biol. Chem.* **247**, 2512–2521

35. Van Mourik, J. A., and Mochtar, I. A. (1970) *Biochim. Biophys.*

Acta **221**, 667–679

36. Chantarangkul, V., Ingram, G. I. C., Thorn, M. B., and Darby, S. C. (1978) *Br. J. Haematol.* **40**, 471–488

37. Smith, R. L. (1973) *J. Biol. Chem.* **248**, 2418–2423

38. Byrne, R., Link, R. P., and Castellino, F. J. (1980) *J. Biol. Chem.* **255**, 5336–5341

39. Jackson, C. M., Johnson, T. F., and Hanahan, D. J. (1968) *Biochemistry* **7**, 4492–4505

40. Eisenthal, R., and Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715–720

41. Aronson, D. L. (1974) *Thromb. Diath. Haemorrh.* **559**, 11–26

42. Jesty, J., Spencer, A. K., Nakashima, Y., Nemerson, Y., and Konigsberg, W. (1975) *J. Biol. Chem.* **250**, 4497–4504

43. Fujikawa, K., Titani, K., and Davie, E. W. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72**, 3359–3363

44. Kurachi, K., Fujikawa, K., Schmer, G., and Davie, E. W. (1976) *Biochemistry* **15**, 373–377

45. Rosenberg, J. S., McKenna, P. W., and Rosenberg, R. D. (1975) *J. Biol. Chem.* **250**, 8883–8888

46. Davie, E. W., Fujikawa, K., Kurachi, K., and Kisiel, W. (1979) *Adv. Enzymol.* **48**, 277–318

47. Jobin, F., and Esnouf, M. P. (1967) *Biochem. J.* **102**, 666–674

48. Esmon, C. T., Owen, W. G., and Jackson, C. M. (1974) *J. Biol. Chem.* **249**, 8045–8047

49. Zwaal, R. F. A., Rosing, J., Tans, G., Bevers, E. M., and Hemker, H. C. (1980) in *The Regulation of Coagulation* (Mann, K. G., and Taylor, F. B., eds) pp. 95–112, Elsevier/North-Holland, New York

50. Nemerson, Y., Zur, M., Bach, R., and Gentry, R. (1980) in *The Regulation of Coagulation* (Mann, K. G., and Taylor, F. B., eds) pp. 193–202, Elsevier/North-Holland, New York

51. Nelsestuen, G. L. (1980) in *The Regulation of Coagulation* (Mann, K. G., and Taylor, F. B., eds) pp. 31–41, Elsevier/North-Holland, New York

52. Nelsestuen, G. L., and Broderius, M. (1977) *Biochemistry* **16**, 4172–4177

53. Nesheim, M. E., Taswell, J. B., and Mann, K. G. (1979) *J. Biol. Chem.* **254**, 10952–10962

Supplementary material to

THE ROLE OF PHOSPHOLIPID AND FACTOR VIII$_a$ IN THE ACTIVATION OF BOVINE FACTOR X

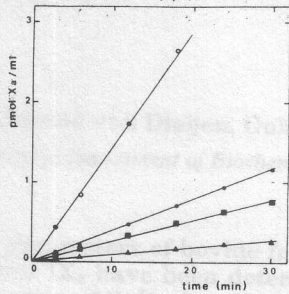Gerbrand van Dieijen, Guido Tans, Jan Rosing and H. Coenraad Hemker

Fig. 1* - Time course of factor X activation by factor IX$_a$ in the presence of phospholipid and CaCl$_2$. Phospholipid vesicles were incubated 10 min at 37°C in a buffer containing 50 mM Tris-HCl, 175 mM NaCl and 50 mM CaCl$_2$ (pH 7.9). 0.15 ml of this phospholipid suspension was added to 1.2 ml of a reaction mixture containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin and factor X (amounts indicated below) at pH 7.9 and 37°C. After 3 min, factor X activation was started by the addition of 0.15 ml factor IX$_a$ (0.067 μM). The final reaction mixture contained: 50 mM Tris-HCl, 175 mM NaCl, 10 mM CaCl$_2$, 0.45 mg/ml ovalbumin, 6.7x10^{-6} μmoles/ml factor IX$_a$ and ▲—▲, 10 μM phospholipid, 0.02 μM factor X; ■—■, 10 μM phospholipid, 0.1 μM factor X; ●—●, 100 μM phospholipid, 0.2 μM factor X; ○—○, 100 μM phospholipid, 1.0 μM factor X. After the time intervals indicated in the figure, samples were taken and assayed for factor X$_a$. Phospholipid vesicles were prepared as described in the experimental procedures.
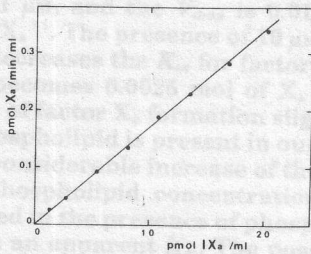

Fig. 2* - The effect of factor IX$_a$ on the rate of factor X activation in the presence of phospholipid. Phospholipid vesicles were preincubated at 37°C in 50 mM Tris-HCl, 175 mM NaCl, 50 mM CaCl$_2$ at pH 7.9. After 10 min, 50 μl of this suspension was transferred to 300 μl of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin and 0.833 μM factor X at pH 7.9. After 4 min incubation at 37°C, factor X activation was started by the addition of 150 μl 50 mM Tris-HCl (pH 7.9), 175 mM NaCl buffer containing 0.5 mg/ml ovalbumin and various amounts of factor IX$_a$. The final reaction mixture contained 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 10 mM CaCl$_2$, 50 μM phospholipid, 0.5 μM factor X, 0.5 mg/ml ovalbumin and amounts of factor IX$_a$ as indicated in the figure. The rate of factor X activation was calculated from the amounts of factor X$_a$ present in the reaction mixture after 5 and 10 min. The phospholipid vesicles were prepared as described in the experimental procedures.
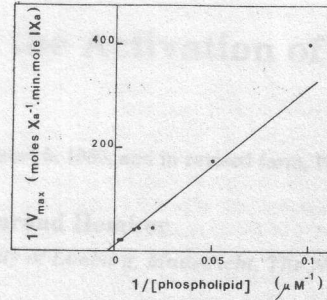

Fig. 3* - Double reciprocal plot of V$_{max}$ as a function of the phospholipid concentration. The factor X activating mixture consisted of factor IX$_a$, CaCl$_2$ and phospholipid. This plot contains data summarized in Table I.
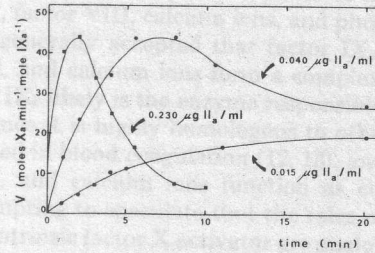

Fig. 4* - Time course of activation of factor VIII with various amounts of thrombin. Factor VIII (10 U/ml) was incubated at 37°C with various amounts of thrombin in a buffer containing 50 mM Tris-HCl, 175 mM NaCl at pH 7.9. The amounts of thrombin present are indicated in the figure. After the time intervals indicated in the figure, 0.1 ml of this incubation mixture was transferred to 0.9 ml of a reaction mixture containing all further components required for factor X activation. The final concentrations in the reaction mixture (1 ml) were: 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 10 mM CaCl$_2$, 0.5 mg/ml ovalbumin, 0.34 x 10^{-8} μmoles/ml factor IX$_a$, 0.015 μM factor X and 12.5 μM phospholipid. The phospholipid vesicles were preincubated for 10 min at 37°C in a buffer containing 50 mM Tris-HCl, 175 mM NaCl and 50 mM CaCl$_2$ before addition to the reaction mixture. 2, 3 and 4 min after the addition of factor VIII$_a$ aliquots from the reaction mixture were assayed for factor X$_a$. The rate of factor X$_a$ formation was calculated from the amounts of factor X$_a$ present in these aliquots. Phospholipid vesicles were prepared as described under "Experimental Procedures".
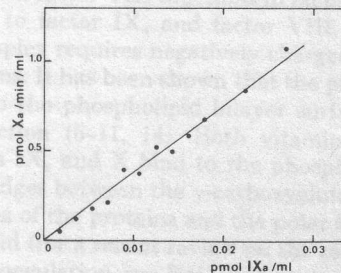

Fig. 5* - The effect of factor IX$_a$ on the rate of factor X activation in the presence of factor VIII$_a$ and phospholipid. Factor VIII (10 U/ml) was activated at 37°C with 0.04 μg/ml thrombin in a buffer containing 50 mM Tris-HCl (pH 7.9) and 175 mM NaCl. After 5 min, 0.1 ml of the activation mixture was added to 0.9 ml of a reaction mixture. The final concentrations in the reaction mixture were: 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 10 mM CaCl$_2$, 0.5 mg/ml ovalbumin, 0.01 μM factor X, 10 μM phospholipid, factor VIII$_a$ and factor IX$_a$ as indicated. Before use, the phospholipid vesicles were preincubated for 10 min at 37°C in 50 mM Tris-HCl, 175 mM NaCl, 50 mM CaCl$_2$ at pH 7.9. After 2, 3 and 4 min aliquots were taken from the reaction mixtures and were assayed for factor X$_a$. The rate of factor X$_a$ formation was calculated from the amount of factor X$_a$ present in these aliquots. Phospholipid vesicles were prepared as described under "Experimental Procedures".