Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7623–7627, October 1990
Biochemistry

# Hemophilia as a defect of the tissue factor pathway of blood coagulation: Effect of factors VIII and IX on factor X activation in a continuous-flow reactor

(blood flow/coagulation inhibitors/bleeding disorders)

DORIS REPKE*, CYNTHIA H. GEMMELL*, ARABINDA GUHA*, VINCENT T. TURITTO*†, GEORGE J. BROZE, JR.‡, AND YALE NEMERSON*§

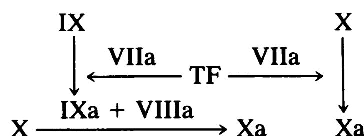
*Division of Thrombosis Research, Department of Medicine, Mount Sinai School of Medicine of the City University of New York, New York, NY 10029; and ‡Jewish Hospital at Washington University Medical Center, Division of Hematology, Saint Louis, MO 63110

*Communicated by Philip W. Majerus, July 11, 1990*

ABSTRACT **The effect of factors VIII and IX on the ability of the tissue factor–factor VIIa complex to activate factor X was studied in a continuous-flow tubular enzyme reactor. Tissue factor immobilized in a phospholipid bilayer on the inner surface of the tube was exposed to a perfusate containing factors VIIa, VIII, IX, and X flowing at a wall shear rate of 57, 300, or 1130 $sec^{-1}$. Factor Xa in the effluent was determined by chromogenic assay. The flux of factor Xa (moles formed per unit surface area per unit time) was strongly dependent on wall shear rate, increasing about 3-fold as wall shear rate increased from 57 to 1130 $sec^{-1}$. The addition of factors VIII and IX at their respective plasma concentrations resulted in a further 2- to 3-fold increase. The direct activation of factor X by tissue factor–factor VIIa could be virtually eliminated by the lipoprotein-associated coagulation inhibitor; however, when factors VIII and IX were present at their approximate plasma concentrations, factor Xa production rates were enhanced 15- to 20-fold. These results suggest that the tissue factor pathway, mediated through factors VIII and IX, produces significant levels of factor Xa even in the presence of an inhibitor of the tissue factor–factor VIIa complex; moreover, the activation is dependent on local shear conditions. These findings are consistent both with a model of blood coagulation in which initiation of the system results from tissue factor and with the bleeding observed in hemophilia.**

There is considerable evidence that the initiation of coagulation *in vivo* may be the result of the formation of a catalytic complex between tissue factor (TF), a transmembrane protein found in many cell types, and factor VII or VIIa, a zymogen of a serine protease and a serine protease, respectively (for a recent review see ref. 1). The TF–factor VII complex (TF:VII) is rapidly converted to TF:VIIa by activated factors X and IX, both products of the TF pathway of coagulation (2, 3). Thus, apart from the first catalytic cycles, the active promoter of coagulation appears to be TF:VIIa.

This complex catalyzes two reactions, the conversion of factor X to Xa and of factor IX to IXa. Each of these products leads to the formation of prothrombinase (4), the first directly and the latter indirectly (5–7); the indirect reaction involves factor VIIIa, the activated form of the antihemophilic factor, and factor IXa (8, 9).




The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

If coagulation is initiated by TF:VIIa, then the clinical manifestations of hemophilia (a deficiency of factor VIII or IX) dictate that the indirect path to factor Xa production must be the dominant one *in vivo*; yet direct measurements of the rates of factor IX and X activation *in vitro* have shown the latter to be a much more efficient reaction (6, 7). This observation has led to difficulty in integrating hemophilia into a formulation in which TF is the essential initiator of coagulation. Indeed, this very observation has lent credence to the concept of "contact" activation in which coagulation is initiated by factors XII and XI.

Until recently, virtually all studies of coagulation reactions have been conducted in thermodynamically closed, static systems. Our laboratory recently introduced an open, dynamic system in which product formation could be evaluated as a function of local flow conditions as well as constant reactant concentrations (10). In the present study, we used this flow reactor to evaluate the role of the antihemophilic factors VIII and IX in the production of factor Xa. We employed a glass microcapillary tube whose inner surface was coated with a phospholipid bilayer containing TF. This reactor was perfused with factors VIIa, IX, VIII, and X, and the outlet concentration of factor Xa was determined. The concentration of factor Xa was converted to a flux from the following relationship: flux = (volumetric flow rate × [factor Xa])/surface area. We have also evaluated the role of a TF inhibitor called the lipoprotein-associated coagulation inhibitor (LACI) (11) or, alternatively, the extrinsic pathway inhibitor (12) (for a recent review see ref. 13). In essence, we find that factor Xa generation at the TF surface is enhanced by increasing wall shear rate. Further, we show that in the presence of LACI the direct activation of factor X is severely inhibited; in contrast, in the presence of the antihemophilic factors, VIII and IX, factor Xa production continues at appreciable rates. Thus, we conclude that relative to the requirement for factors VIII and IX in the activation of factor X, coagulation may be essentially a TF-dependent process.

## MATERIALS AND METHODS

**Proteins.** Recombinant human factor VIII (14) was kindly supplied by M. Mozen, Miles Laboratories, Berkeley, CA. The factor VIII was allowed to activate *in situ* because preliminary experiments showed that preactivation of this preparation with either thrombin or factor Xa did not increase factor Xa production (data not shown). Recombinant human LACI (15) was generously provided by Tze-Chein Wun,

Abbreviations: LACI, lipoprotein-associated coagulation inhibitor; AT III, antithrombin III; TF, tissue factor; TBS, Tris-buffered saline.

†Present address: Department of Biomedical Engineering, Memphis State University, Memphis, TN 38152.
§To whom reprint requests should be addressed.

Monsanto. It was used at 1 nM unless otherwise indicated. TF was purified from human brain by immunoaffinity chromatography (16, 17). Factors IX and X (18) and factor VII (19) were purified from human plasma. Factor VII was activated using factor XII fragments (20).

Inasmuch as our experiments could be biased by small amounts of contaminating procoagulant proteins, we developed the following procedures to establish the functional purity of our preparations. Factor X was passed through a TF-coated reactor for 20 min at 300 $sec^{-1}$. If no factor Xa was detected at the outlet, the preparation was judged to be free of factor VII(a). The presence of factor IXa was evaluated by perfusing a phospholipid-coated reactor (≈0.6 mM phosphatidylserine/phosphatidylcholine, 30:70, wt/wt) with factors VIIa, VIII, and X. If no factor Xa appeared at the outlet after a 20-min perfusion at a wall shear rate of 300 $sec^{-1}$, we concluded that no factor IXa was present. The presence of factor IX in the other procoagulant proteins was evaluated by perfusing a TF-coated tube with factors VIIa, VIII, and X. If the flux of Xa was not enhanced by the presence of factor VIII, we considered the preparations to be free of factor IX. The presence of factor Xa was estimated by a chromogenic assay (see below).

**Removal of Factors IXa and Xa from Factors IX and X.** To achieve purity as defined by these criteria, we used the following procedures: Fifty milligrams of human antithrombin III (AT III; kindly supplied by M. Mozen, Miles Laboratories, Berkeley, CA) was dissolved in 10 ml of cold distilled water and dialyzed against 1 liter of 0.1 M $NaHCO_3$ at 4°C overnight. Affi-Gel 15 (BioRad; 25 ml) was prepared according to the manufacturer's directions. The gel was stirred with AT III overnight at 4°C, after which unreacted groups were blocked by stirring with 5 ml 1.0 M ethanolamine for 1 hr. The suspension was then transferred to a fritted-glass funnel and successively washed with 100 ml of cold water, 100 ml of 4 M guanidine hydrochloride in water, and 200 ml of cold water and then equilibrated with Tris-buffered saline (TBS: 50 mM Tris Cl, pH 7.5/0.1 M NaCl). Over 90% of the AT III was coupled to the Affi-Gel. Heparin (Sigma; 100 mg) was stirred with 10 ml of AT III-Affi-Gel at room temperature for 4 hr. The slurry was stored at 4°C. Prior to use, 4 ml of the heparin-AT III-Affi-Gel in a 1 × 5-cm column was washed with 50 ml of TBS at room temperature; 2–5 mg of factor IX or X in 1 ml was then loaded and allowed to equilibrate for 2 hr. The protein was eluted with about 8 ml of TBS. The protein peak contained trace amounts of heparin as detected by a toluidine blue assay (21); the heparin was removed by passage over a 2-ml Mono Q column (Pharmacia). The protein was eluted with 0.5 M NaCl/50 mM Tris Cl, pH 7.5, and under these conditions heparin remained bound to the column. The recovery of the proteins was over 90%.

Factor X (160 nM) was judged to be free of factor Xa as measured in the chromogenic assay and factor IX did not generate factor Xa in the flow reactor as described above.

**Tubular Reactor.** Tubes were prepared by a modification of the technique of Gemmell *et al.* (10). Fisher microcapillary tubes (5 μl, 0.27-mm internal diameter, and 127-mm length; catalogue no. 21-164-2A) were treated with chromic acid for 1 hr, following which they were rinsed extensively with tap water by using a vacuum pump to ensure adequate flow. The tubes were boiled in Sparkleen (Fisher, ≈2 g per liter) for at least 3 hr, rinsed several times in hot water, and then boiled in several changes of distilled, deionized water for several hours. The tubes were then rinsed with deionized water with the help of a vacuum pump. After drying at 110°C they were stored in a sealed container under $N_2$. Tubes were prepared for experiments by filling them with a suspension of TF (4 nM) in vesicles containing ≈0.6 mM phosphatidylserine/phosphatidylcholine, 30:70 (wt/wt), prepared as described (22). After 20 min at room temperature the tubes were rinsed with buffer (10 mM Hepes, pH 7.5/140 mM NaCl/0.1% bovine serum albumin) for 5 min at a wall shear rate of 1500 $sec^{-1}$ and used immediately thereafter. The perfusion was performed as described (10) except that a Hamilton gas-tight syringe was used (no. 1002-LT). The syringe and microcapillaries were maintained at 37°C by enclosing them in a thermostatted water jacket mounted on a syringe pump (Sage no. 351). A fresh capillary was used for each experiment.

All shear rates were determined at the capillary wall from the tube diameter and volumetric flow rate, assuming a Newtonian velocity profile (23). All proteins and vesicles were in the Hepes buffer described above. Factor Xa concentrations were determined using the chromogenic substrate Spectrozyme Xa (American Diagnostica, New York). The chromophore, *p*-nitroaniline, was measured by its absorbance at 405 nm. Appropriate amounts of effluent were added to a 50 mM EDTA/0.05% bovine serum albumin. Reactions were conducted at 37°C in Hepes buffer with Spectrozyme Xa at 500 μM. After 5 or 10 min (depending on the factor Xa concentration) the reactions were quenched by adding acetic acid to a final concentration of 5% (vol/vol). The assay was linear with Xa concentration under 5 nM. At the Spectrozyme Xa and LACI levels employed in this study, LACI did not affect the factor Xa assay.

Plasma concentrations of factors VII, IX, and X were taken to be 10, 60, and 160 nM, respectively (18, 19). Unless otherwise stated, these concentrations were used. Factor VIII was assayed using an activated partial thromboplastin time technique. Unless otherwise indicated, it was used at 1.0 unit/ml. The concentration of LACI was determined by the bicinchoninic acid (BCA) protein assay (Pierce) according to the manufacturer's directions.

## RESULTS

**Effect of LACI and Factor VIII on Factor Xa Flux.** In our initial experiments, we evaluated the effect of LACI on factor Xa production in a system containing factor VIIa, IX, and X with and without factor VIII at a wall shear rate of 300 $sec^{-1}$. LACI concentration was varied from 0 to 2.5 nM. As expected, the flux of factor Xa fell as the LACI concentration was increased. At 1.6 nM LACI, the direct activation of factor X was virtually undetectable, whereas in the presence of factor VIII, the flux of factor Xa was maintained at about 7% of the control (Fig. 1). Because we were interested in determining the relative rates of factor Xa production as a function of factor VIII and IX concentration, we chose to use 1 nM LACI for our subsequent experiments as this allowed us to make relatively precise estimates of the effect of the antihemophilic factors in the system.

**Requirement of Factors VIII and IX for the Production of Factor Xa in the Presence of LACI.** For these experiments, we held factor IX at 60 nM and varied factor VIII from 0 to 4 units/ml. The increase in factor Xa flux was directly related to the increase in factor VIII concentration (Fig. 2). In a similar experiment, we held factor VIII at 1 unit/ml while varying factor IX from 0 to 130 nM. The results paralleled those obtained with factor VIII: the factor Xa flux varied directly with factor IX concentration. In the absence of either factor VIII or factor IX, factor Xa flux was at most 5% of that obtained in the presence of plasma concentrations of these factors (Fig. 2).

**Effect of Wall Shear Rate on Factor Xa Flux.** In these experiments we employed concentrations of clotting factors approximating those found in plasma. The flow rates were 6.0, 34.7, and 130.4 μl/min, corresponding to wall shear rates of 57, 300, and 1130 $sec^{-1}$, thereby encompassing much of the physiological range (23, 24). To evaluate the role of the antihemophilic factors under these conditions, we omitted only factor VIII. We did this because of the symmetry

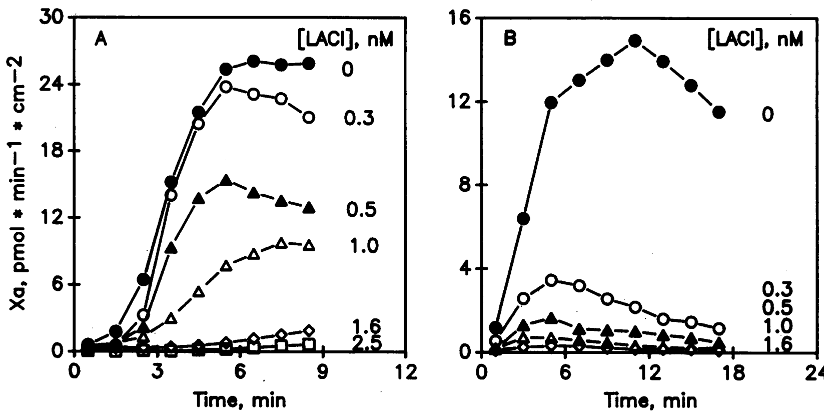



FIG. 1. LACI titration in the presence (*A*) or absence (*B*) of factor VIII at a wall shear rate of 300 $sec^{-1}$. The perfusates contained factors VIIa (10 nM), IX (60 nM), VIII (1 unit/ml), and X (160 nM), $Ca^{2+}$ (5 mM), and the indicated concentrations of LACI. Note the difference in scales.

established above whereby the reaction velocities were similar when we omitted either factor VIII or factor IX.

The first set of experiments were done in the absence of LACI (Fig. 3 *A*, *C*, and *E*). The steady-state production of factor Xa increased with increasing wall shear rate. This increase most likely reflects a faster delivery of substrate to the wall-bound enzymatic complexes with the consequent more rapid conversion of factor X to Xa combined with a faster removal of factor Xa by the flowing buffer. This was independent of the presence of factor VIII. The production rates of factor Xa were increased about 2.5-fold relative to the rates observed in the absence of factor VIII. However, the relative increases in production rate as a function of factor VIII were independent of wall shear rate.

When LACI was included in the perfusate, a similar dependence of factor Xa flux on wall shear rate was noted (Fig. 3 *B*, *D*, and *F*). However, in the absence of factor VIII, the activation of factor X was just detectable. In its presence, factor Xa flux was appreciable although reduced by the presence of the inhibitor (Fig. 3, compare *A*, *C*, and *E* with *B*, *D*, and *F*). Inspection of Fig. 3 indicates that the dependence of factor Xa production on the antihemophilic factors was increased from 2- to 3-fold in the absence of LACI to about 20-fold in its presence.

## DISCUSSION

The results of these experiments, conducted in a tubular reactor and intended to approximate much of the range of wall shear rates encountered physiologically (23), demonstrate that factors VIII and IX are required for optimal factor Xa production when coagulation is initiated by TF. Indeed, at all wall shear rates studied, the presence of these factors (in the absence of LACI) led to a 2- to 3-fold enhancement in factor Xa production under conditions in which the perfusates contained the approximate plasma concentrations of each reactant (Fig. 3 *A*, *C*, and *E*). When LACI was added at 1 nM, the dependence of factor Xa production on factor VIII rose to about 20-fold (Fig. 3 *B*, *D*, and *F*). At higher LACI concentrations the direct activation of factor X was decreased to barely detectable levels, whereas measurable levels were produced in the presence of factors VIII and IX. Because the direct activation of factor X was so low, the relative enhancement in rate could not be estimated.

The mechanism by which LACI increases the dependence on factor VIII is unknown, but we suggest that the effect of LACI in this system is equivalent to reducing the wall concentration of TF. This interpretation is in accord with an earlier study of the involvement of factor VIII in TF-dependent activation of factor X in plasma, in which plasma levels of factor VIII led to an ≈10-fold increase in the rate of activation of factor X (25). In that study, in which tritiated factor X was used to evaluate the formation of factor Xa in a static system, the enhancement was observed only when very small amounts of TF were used. Other experiments, in which the clotting time of whole plasma was determined as a function of the amount of TF added, revealed a similar phenomenon: hemophilic plasma clotting times exceeded those of normal plasma only when very dilute suspensions of TF were used (26). These previous findings have been interpreted as resulting from the large molar excess of factors IX and X over their respective cofactors, factors VIII and V (1). Thus, for example, if all the factor IX were converted to factor IXa, it would be in ≈50-fold molar excess over factor VIII (27). Accordingly, the vast majority of the factor IXa molecules could be kinetically inert and the bulk of the activation of factor X would proceed via the direct pathway of activation. This interpretation implies that the rates of factor IX and X activation are each a function of the TF concentration and that they would vary in a similar fashion with the TF concentration. We believe this to be a reasonable assumption inasmuch as both factors are activated by TF:VIIa and it has been shown that in the presence of factor VIIa, factor X activation rises with the TF concentration,

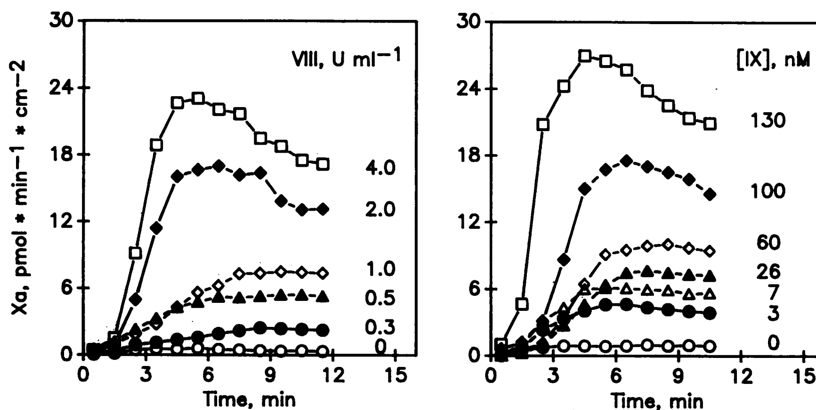



FIG. 2. Factor VIII and factor IX titration in the presence of LACI (1 nM). The perfusates were constituted as in Fig. 1. U, unit(s).

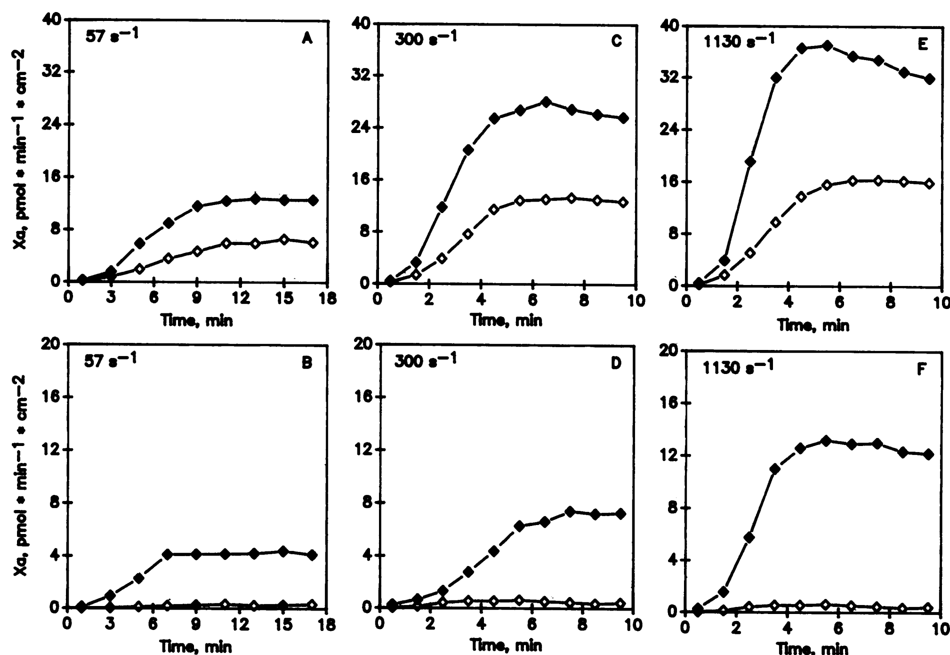



FIG. 3. Influence of factor VIII concentration and wall shear rate on the production of factor Xa in the capillary tube reactor. Factors VIIa (10 nM), X (160 nM), and IX (60 nM) were perfused at wall shear rates of 57, 300, or 1130 $sec^{-1}$ in the presence (◆) or absence (◇) of factor VIII (1.0 unit/ml). Experiments depicted in *A*, *C*, and *E* were performed in the absence of LACI, and those in *B*, *D*, and *F* with 1 nM LACI. Note the difference in vertical scales.

reaching a maximum asymptotically as it surpasses the factor VIIa concentration (28). It follows from these considerations that as TF concentration is reduced, a kinetic regime will be reached in which the indirect pathway becomes significant.

In the present studies we examined the effect of shear rate on factor Xa production and, consequently, differing reaction velocities, without varying the bulk phase concentration of reactants. In the absence of LACI and factor VIII, as the wall shear rates were increased from 57 to 300 to 1130 $sec^{-1}$, the absolute rate of Xa formation rose considerably (Fig. 3 *A*, *C*, and *E*), from ≈6 to ≈12 to ≈16 $pmol{\cdot}min^{-1}{\cdot}cm^{-2}$. When factor VIII (1 unit/ml) was added, formation of factor Xa was still dependent on the wall shear rate, rising from ≈12 to ≈34 $pmol{\cdot}min^{-1}{\cdot}cm^{-2}$ over the same range of shear rates. In the presence of LACI, a similar dependence on wall shear rate was observed (Fig. 3 *B*, *D*, and *F*), although the absolute flux of factor Xa was reduced by about 60%. The relative dependence on the antihemophilic factors did not change with increasing Xa flux, suggesting that this dependence is related to the concentrations of the reactants and not to the reaction rates.

The effect of LACI on factor Xa formation is clearly concentration-dependent (Fig. 1). We chose to use a LACI concentration of 1 nM because we could measure the flux of factor Xa under all our experimental conditions. In the presence of LACI the flux of factor Xa was shown to be a function of the concentration of both factor VIII and IX (Fig. 2). Thus, this model system appears to reflect the physiological dependence of hemostasis on adequate levels of the antihemophilic factors. Indeed, both factors affected the reaction rates more or less equally with respect to varying their concentrations from 0 to 2 to 4 times their plasma concentrations. This dependence on the antihemophilic factors may be viewed as a route to escape the LACI-modulated blockade of TF.

At high reaction rates, a steady state was not obtained, and after reaching a maximum, factor Xa flux gradually declined. We do not know the reason for this, but we have ruled out loss of surface coat, by flushing a reactor in which a reaction had decreased significantly with a $Ca^{2+}$-free buffer and repeating the experiment. The curves were virtually superimposable (not shown), thus ruling out a physical loss of TF. We have repeatedly observed that the rate at which the system reaches the maximum flux of factor Xa determines whether a steady state is maintained. This is illustrated in Fig. 2: at the highest levels of both factors VIII and IX there is a decay in the factor Xa flux with time. We have observed the same phenomenon in experiments using only factors VIIa and X in which the same level of factor Xa flux was obtained irrespective of the factor VIIa concentration; however, when 10 nM factor VIIa was employed the flux decayed slowly, whereas at 2 nM factor VIIa a steady state was maintained for at least 2 hr (data not shown). Inasmuch as the same phenomena are observed whether the reaction velocity is modulated by factor VII, factor VIII, or factor IX, we do not believe that it is caused by a contaminant. Accordingly, this damping phenomenon may be intrinsic to the system and needs further study.

At concentrations of factors VIII and IX equal to or less than their respective plasma concentrations, a steady state was reached and was a direct function of the concentration of these factors (Fig. 2). This is different from what was observed in a system containing only factors VIIa and X, in which it was shown that the steady state was independent of the factor VIIa concentration (10). This difference could result from the TF:VIIa complex having a lower $K_d$ than the VIIIa:IXa:phospholipid complex. If this is true, then platelets, which might present a more efficient surface for these reactions than phospholipids (29), could alter this result. Alternatively, the degradation of factor VIIIa, both spontaneous and catalyzed by factor Xa, could account for this difference.

It is important to note that these experiments require six purified proteins. The experiments presented were performed with a single preparation of each species. When other batches were used, the observed production rates of factor Xa varied by as much as 50%; in each instance, however, the ratios of production rates were similar to those presented in this study. Presumably this variation is due to the obvious difficulty of obtaining each protein at a totally reproducible specific activity and to the multiplicative nature of enzyme cascades.

The reactions occurring within the capillary are numerous, the most important being the activations of factors VIII, IX, and X. To simplify the system, we used factor VIIa instead of its zymogen, although preliminary data (unpublished) suggest that there is little quantitative difference between these species, probably owing to the rapidity of the conversion of factor VII to factor VIIa in association with TF (2, 3).

We chose to use recombinant factor VIII in these experiments because of its purity. It would be of interest to study the effect of combining the recombinant protein with von Willebrand factor. We expect that decreasing the diffusion of factor VIII consequent to its increase in apparent molecular weight would increase the dependency on wall shear rate because the diffusion to the surface is related to the diffusion coefficient to the 2/3 power (30, 31). Thus, the relative dependencies of these reactions on shear rate when all other conditions are held constant would be indicative of the importance of the diffusion of factor VIII to the surface. In addition, LACI, which circulates mainly as a lipoprotein (32), was used in its free, purified form. The relative effectiveness of free and lipoprotein-bound LACI is not known, but we have observed the LACI effects considerably below its estimated plasma concentration of 2.3 nM (Fig. 1). The delivery of native LACI to the wall might be more dependent on shear rate than that of the form we used.

In summary, we have used an open, flow system to demonstrate that the presence of factors VIII and IX significantly accelerates factor X activation, particularly in the presence of LACI. We further have demonstrated that increased wall shear rates enhance steady-state factor X activation. If this model system reflects the physiology of hemostasis, then we suggest that not only is the TF initiation of coagulation consistent with the diseases accompanying factor VIII and factor IX deficiencies, but that another parameter, wall shear rate, must be considered when analyzing blood coagulation. Finally, we wish to emphasize that like the deposition of platelets on the subendothelium in a flow system (33, 34), the production of an important coagulation intermediate, factor Xa, also rises with increasing wall shear rates.

We thank Dr. M. Mozen for his generous gift of factor VIII and AT III and Dr. T. C. Wun for recombinant LACI. This research was supported in part by U.S. Public Health Service Grants HL29019, HL34462, and HL38933.

1. Nemerson, Y. (1988) *Blood* **71**, 1–8.
2. Nemerson, Y. & Repke, D. (1985) *Thromb. Res.* **40**, 351–358.
3. Rao, L. V. & Rapaport, S. I. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6687–6691.
4. Mann, K. G., Jenny, R. J. & Krishnaswamy, S. (1988) *Annu. Rev. Biochem.* **57**, 915–956.
5. Osterud, B. & Rapaport, S. I. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5260–5264.
6. Zur, M. & Nemerson, Y. (1980) *J. Biol. Chem.* **255**, 5703–5707.
7. Silverberg, S. A., Nemerson, Y. & Zur, M. (1977) *J. Biol. Chem.* **252**, 8481–8488.
8. Vehar, G. A. & Davie, E. W. (1980) *Biochemistry* **19**, 401–410.
9. Hultin, M. B. & Nemerson, Y. (1978) *Blood* **52**, 928–940.
10. Gemmell, C. H., Turitto, V. T. & Nemerson, Y. (1988) *Blood* **72**, 1404–1406.
11. Broze, G. J., Jr., Warren, L. A., Novotny, W. F., Higuchi, D. A., Girard, J. J. & Miletich, J. P. (1988) *Blood* **71**, 335–343.
12. Rao, L. V. & Rapaport, S. I. (1987) *Blood* **69**, 645–651.
13. Rapaport, S. I. (1989) *Blood* **73**, 359–365.
14. Toole, J. J., Knopf, J. L., Wozney, J. M., Sultzman, L. A., Buecker, J. L., Pittman, D. D., Kaufman, R. J., Brown, E., Shoemaker, C., Orr, E. C., Amphlett, G. W., Foster, W. B., Coe, M. L., Knutson, G. J., Fass, D. N. & Hewick, R. M. (1984) *Nature (London)* **312**, 342–347.
15. Wun, T. C., Kretzmer, K. K., Girard, T. J., Miletich, J. P. & Broze, G. J., Jr. (1988) *J. Biol. Chem.* **263**, 6001–6004.
16. Bach, R., Nemerson, Y. & Konigsberg, W. (1981) *J. Biol. Chem.* **256**, 8324–8331.
17. Guha, A., Bach, R., Konigsberg, W. & Nemerson, Y. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 299–302.
18. Miletich, J. P., Broze, G. J., Jr., & Majerus, P. W. (1981) *Methods Enzymol.* **80**, 221–228.
19. Broze, G. J., Jr., & Majerus, P. W. (1980) *J. Biol. Chem.* **255**, 1242–1247.
20. Radcliffe, R., Bagdasarian, A., Colman, R. & Nemerson, Y. (1977) *Blood* **50**, 611–617.
21. Jacques, L. B. & Bell, H. J. (1959) *Methods Biochem. Anal.* **7**, 253–309.
22. Bach, R., Gentry, R. & Nemerson, Y. (1986) *Biochemistry* **25**, 4007–4020.
23. Goldsmith, H. L. & Turitto, V. T. (1986) *Thromb. Haemostasis* **55**, 415–435.
24. Turitto, V. T. & Baumgartner, H. R. (1987) in *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, eds. Colman, R., Hirsh, J., Marder, V. & Salzman, E. (Lippincott, Philadelphia, PA), p. 555.
25. Marlar, R. A., Kleiss, A. J. & Griffin, J. H. (1982) *Blood* **60**, 1353–1358.
26. Biggs, R. & MacFarlane, R. G. (1951) *J. Clin. Path.* **4**, 445–459.
27. Fulcher, C. A. & Zimmerman, T. S. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1648–1652.
28. Nemerson, Y. & Gentry, R. (1986) *Biochemistry* **25**, 4020–4033.
29. Neuenschwander, P. & Jesty, J. (1988) *Blood* **72**, 1761–1770.
30. Koyayashi, T. & Laidler, K. J. (1974) *Biotechnol. Bioeng.* **16**, 99–118.
31. Laidler, K. J. & Bunting, P. S. (1980) *Methods Enzymol.* **64**, 227–248.
32. Hubbard, A. R. & Jennings, C. A. (1987) *Thromb. Res.* **46**, 527–537.
33. Turitto, V. T. & Baumgartner, H. R. (1979) *Microvas. Res.* **17**, 38–54.
34. Turitto, V. T., Weiss, H. J. & Baumgartner, H. R. (1980) *Microvas. Res.* **19**, 352–365.