The initial surface reactions of the extrinsic coagulation pathway on live cell membranes were examined under flow conditions. Generation of activated coagulation factor X (fXa) was measured on spherical monolayers of epithelial cells with a total surface area of 41–47 cm² expressing tissue factor (TF) at >25 fmol/cm². Concentrations of reactants and product were monitored as a function of time with radiolabeled proteins and a chromogenic substrate at resolutions of 2–8 s. At physiological concentrations of fVIIa and fX, the reaction rate was 3.05 ± 0.75 fmol fXa/s/cm², independent of flux, and 10 times slower than that expected for collision-limited reactions. Rates were also independent of surface fVIIa concentrations within the range 0.6–25 fmol/cm². The transit time of fXa activated on the reaction chamber was prolonged relative to transit times of nonreacting tracers or preformed fXa. Membrane reactions were modeled using a set of nonlinear kinetic equations and a lagged normal density curve to track the expected surface concentration of reactants for various hypothetical reaction mechanisms. The experimental results were theoretically predicted only when the models used a slow intermediate reaction step, consistent with surface diffusion. These results provide evidence that the transfer of substrate within the membrane is rate-limiting in the kinetic mechanisms leading to initiation of blood coagulation by the TF pathway.

Blood coagulation reactions mediate fibrin deposition in hemostasis and many pathological processes. Blood clots are directly implicated in the lethal complications of cardiovascular disease and contribute significantly to the pathogenesis of infectious, autoimmune, and neoplastic diseases (1–6).

The blood coagulation process is initiated by an assembly of complexes comprised of an essential cofactor, TF (tissue factor) and a protease component, fVIIa. The functional complex, TF-fVIIa, cleaves the natural substrates, fVII, fX, fIX (factor IX), and fX at specific sites, generating fVIIa, fIXa, and fXa, respectively (4–6). Factors VII, IX, and X are vitamin K-dependent proteins, and their functional interaction with negatively charged procoagulant membranes has a calcium-dependent, electrostatic component (15–19). The interaction sites are located in highly homologous g-carboxyglutamic acid (Gla)-rich regions near the N terminus of all vitamin K-dependent coagulation proteins (4, 19). The specific binding and functional kinetics of interaction between coagulation proteins and biological membranes have been studied extensively under equilibrium steady-state conditions (20–27). Although equilibrium binding parameters vary significantly among vitamin K-dependent proteins, adsorption parameters are similar, suggesting nonspecific initial contact occurs (28, 29). Anionic phospholipid membranes modify the apparent kinetic parameters of coagulation reactions relative to kinetics in solution. The membrane effect is manifested by a large decrease in the apparent $K_d$ of substrates to values far below their respective plasma concentrations. The mechanisms by which this effect manifests itself during TF-mediated coagulation remain speculative. Achieving useful time resolutions has been one of the main obstacles to developing experimental systems to study the pre-steady-state transients of coagulation factor adsorption and activation on cell membranes. Blood clotting in vivo and in vitro can be completed faster than the sampling intervals of traditional batch systems used to measure membrane reactants.

Measurements of fVIIa binding and fXa generation on intact cell membranes under steady-state conditions indicate that TF-fVIIa functional activity is fully expressed before the binding interaction between fVIIa and TF reaches equilibrium (24, 25). Furthermore, under steady-state conditions, the overall rate of coagulation substrate activation on membranes pre-equilibrated with enzyme was close to the theoretical collisional limit (27). These findings suggest intermediate noncovalent steps on the membrane linking the initial adsorption step to the assembly and catalysis of substrate in the A-E-S (activator-enzyme-substrate).

Coagulation zymogens and active proteases are subject to local microcirculation controls (3, 7–10, 14), because they are found in extravascular lymphatic, synovial, and alveolar fluids. The importance of flow control in coagulation reactions has been demonstrated in vivo. Tracer studies with radiolabeled fibrinogen and vasoactive agents indicate a direct correlation between changes in vascular permeability and fibrin deposition (3). Several studies using lipid-coated capillaries also indicate that flow rates influence the activity of coagulation proteases.
Flow Kinetics of Coagulation Factor Xa

Radioactive Tracers—The control tracer used to measure concentrations in bulk aqueous phase was \(^{14}C\)-labeled ovalbumin (Sigma, St. Louis, MO) with a specific activity of 33 \(\mu\)Ci/mg. The test tracer for adsorption measurements was \(^{35}S\)IA radiolabeled with tritium using the technique of Van Lenten and Ashwell (40), with modifications (41).

**Reaction Scheme and Mathematical Model**—The surface reactions leading to Xa generation were analyzed according to the following scheme,

\[
\begin{align*}
& k_{-3} \quad \text{Fi} + \text{TF} \rightarrow \text{E} \\
& k_{2} \quad \text{FVIIa} + \text{TF} \rightarrow \text{E} \\
& k_{-3} \quad \text{E} + \text{FX} \rightarrow \text{FX}a + \text{E}
\end{align*}
\]

where \(E = \text{FVIIa}\), TF is the FVIIa and TF complex (“enzyme”) that forms and dissociates with rate constants \(k_{+1}\) and \(k_{-1}\), respectively. The substrate-enzyme complex denoted by \(E'X\) associates and dissociates with a second-order rate constant, \(k_{+2}\), and a first-order rate constant, \(k_{-2}\), respectively. The effective rate of product (\(Xa\)) formation from the complex and its irreversible release are denoted by the first-order rate constant, \(k_{+3}\). Denoting the surface concentrations of each species by \(I_{i}\), \(G_{i}\), \(F_{i}\), \(E_{i}\), \(T_{i}\), \(F_{i}X_{a}\), \(X_{a}\) and \(3H_{i}\), \(2\), \(1\), \(4\), \(5\), \(6\). The full kinetic equations consistent with Eqs. 1 and 2 are

\[
\begin{align*}
\Gamma_{1} &= -k_{-1}I_{1} + k_{+1}I_{2} + k_{2}I_{2} = k_{-2}I_{2} + \alpha_{F}C_{X}(t) \\
\Gamma_{2} &= -k_{-2}I_{2} + k_{+2}I_{3} + k_{3}I_{3} - \beta_{2}I_{3} + \alpha_{F}C_{X}(t) \\
\Gamma_{3} &= -k_{-3}I_{3} + k_{+3}I_{4} + k_{3}I_{4} - \beta_{2}I_{4} + \alpha_{F}C_{X}(t) \\
\Gamma_{4} &= k_{4}I_{4} + \alpha_{E}C_{X} - \beta_{5}I_{5} \\
\Gamma_{5} &= k_{5}I_{5} + \alpha_{E}C_{X} - \beta_{5}I_{5} \\
\Gamma_{6} &= k_{6}I_{6} + \alpha_{E}C_{X} - \beta_{5}I_{5} \\
\end{align*}
\]

where \(I_{i}(t) = dI_{j}(t)/dt\), with \(i = 1, 2, 3, 4, 5, 6\). The constants \(k_{+1}\) and \(k_{-1}\) correspond to effective rates of FVIIa- and FX-binding interactions with TF and E, respectively. These coefficients include the time delays of all intermediary processes on the membranes before the interactions.

The time distributions of these unspecified processes are accounted for in our numerical predictions of the time course for the overall Xa activating process. The possibility of inhibition or Xa/Xa-destroying sinks is precluded from our data, because, within experimental error, all the absorbed Xa is recovered as Xa.

In the above nonlinear differential equations, \(\beta_{1}\) and \(\alpha_{1}\) are desorption and adsorption rates, respectively. Because the total area fraction of adsorbed species is negligible under our experimental conditions, species adsorption from bulk is simply proportional to the bulk concentration, \(C_{i}(t)\), at the surface of each microsphere. As the fluid passes through the ensemble of microcarriers, certain flow lines are faster or slower than the mean flow velocity, resulting in a distribution of reactant velocities. A “lagged normal density curve” (LNDC) has been successfully used to approximate the dispersion resulting from the combined effects of random velocity distribution and molecular diffusivity in the human circulatory system (36, 37). We find good agreement between a fitted lagged density curve and the sequentially measured concentrations in the outflow of the reaction chamber (Fig. 3). Therefore, to simplify the modeling process, we assume that the dispersion and diffusion of all species are equal and use the LNDC to approximate the source, \(S_{i}(t)\), surrounding each microcarrier. The parameters used in the lagged density curve will reflect chamber packing characteristics, bulk diffusion constants, and the imposed volume flow rate, \(J_{0}\). Additional details, analysis, and simplifications of Eqs. 3–8 are provided under “Appendix.”

Calculation of Reactant Concentrations in Membranes—The proportion of Xa and FVIIa adsorbed from the flowing phase into the membrane was determined from the difference between the normalized concentrations of control, \(^{14}C\)-labeled ovalbumin, and \(^{3}H\)-labeled FVIIa. Concentrations of factor VIIa adsorbed at time \(t\) were estimated using

\[
\Gamma_{i}(t) = ([^{14}C(0)] - [^{3}H(0)]Q)S_{i}^{-1}
\]

where \([^{14}C]\) and \([^{3}H]\) are the fraction of the total nonadsorbed
RESULTS

Rate of fXa Generation at Different fVIIa Concentrations—
The generation of fXa from fX on live coagulating cell membranes was examined in reaction chambers filled with spherical cell monolayers. The geometrical and flow characteristics of these reaction chambers are summarized in Fig. 1 and Table I, respectively. The distribution of concentrations of reactants in the flowing bulk aqueous phase was followed using control tracer [14C]ovalbumin. Reactions were initiated with fX and fVIIa, and the product, fXa, was measured by amidolytic assay. Reactions were followed until 70–90% of the nonreacting control tracer was recovered in the effluent. The amounts of [14C] and [3H] tracer collected and the amount of [3H] adsorbed to the cell are shown as functions of time in Fig. 2A.

The time evolution of reactants inside the reaction chamber can be fairly well approximated by the LDNC, as shown in Fig. 3. This agreement indicates that dispersion of reactants in the chamber due to the random flow distribution and diffusion is qualitatively similar to that encountered in human circulation (36–38, 43, 46–47). Fig. 3 also illustrates the time/concentration distribution of fXa generated in the 1.23 ml reaction chamber in a typical reaction initiated with 500 ng of fVIIa and 9000 ng of fX. No fXa was detected in the control experiments where microcarriers were used in the absence of cells.

Under the conditions of these experiments, aqueous-phase concentrations ranged from 0.3 to 10 ± 2 nm and from 4 to 137 ± 29 nm for fVIIa and fX, respectively. A time trace measuring the total amount of fXa collected and fX adsorbed on cell membranes is shown in Fig. 2B. Factor Xa profiles were weakly sigmoidal with a linear middle segment. Table II shows that the average fXa production rate, calculated from the linear segment, did not change when average concentrations of fVIIa in aqueous phase were decreased by 10-fold, from 5 to 0.5 nm (membrane concentrations ranged from 0.7 to 25.0 fmol/cm²).

No fXa was generated in the absence of fVIIa and reaction rates did not differ significantly when fVII was substituted for fVIIa. The observation that maximal constant catalytic activity is reached at very low concentrations of TF–fVIIa complexes allows for simplifying substitutions in the model equations (Eqs. 3–8) for $\Gamma_1$ and $\Gamma_4$ (“Appendix”).

Comparison between the Reaction Rate and the Theoretical
Flow Kinetics of Coagulation Factor Xα

**TABLE II**

| Enzyme density* | Rate* | Flux* |
|-----------------|-------|-------|
| fVIIa fmol/cm³   | fmol fXa cm⁻³ s⁻¹ | fmol/s |
| 5.0–25           | 2.9 ± 0.05 | 180    | 3241 |
| 2.2–9.0          | 3.7 ± 0.04 | 52     | 779  |
| 1.9–3.4          | 3.7 ± 0.08 | 23     | 1728 |
| 0.7–1.8          | 3.7 ± 0.06 | 11     | 608  |
| 0               | 0      | 0      | 2000 |

*Membrane density and adsorption rate of fVIIa were derived from the flux and adsorption rate coefficients previously measured (29). Values are the initial and final concentrations measured during the linear interval of the progression curve. Tissue factor density in these experiments was estimated at >25 fmol/cm² from cell protein and specific activity assays.

Average rate was calculated from the concentration of fXa measured by chromogenic assay in samples collected during the linear segment of the reaction progression curve.

Average fluxes of fVIIa and fX in each experiment were determined from the concentration of ¹⁴C-control tracer in the effluent. The total amounts of fVIIa and fX initially added to the 1.23-ml reaction chamber were 48–500 ng and 2800–9000 ng, respectively.

The collisional limit (29), activation rates below this limit support the second possibility.

Theoretical steady-state collisional rates were calculated from the aqueous-phase concentrations of fX and the radius of the spherical microcarriers using Smoluchowski’s relationship for steady-state diffusion (42, 57).

\[ k_{\text{coll}} = D_{1,2} C_{1,2} R^{-1} \]  

(Eq. 11)

where \( k_{\text{coll}} \) is the rate of collisions between reactant molecules and a unit area of membrane (collisions/cm²/s), \( D_{1,2} \) is the diffusion constant for fVIIa, fX in water (\( \sim 5 \times 10^{-7} \) cm/s), \( R = 7.5 \times 10^{-6} \) cm is the microcarrier radius, and \( C_{1,2} \) is the fVIIa, fX concentration (molecules/cm³).

Fig. 3 contrasts the number of fXa molecules released by the monolayer and the aqueous phase concentration, \( C_α(t) \), of fX as a function of time. Because the collisional rate follows Eq. 11, collision-limited rates are expected to be directly proportional to \( C_α(t) \). However, the rate of fX activation on the monolayer was not correlated with fX-membrane collisions. The rate of fXa production (molecules/cm²/s) reached maximal values after the peak in \( C_α(t) \) and collisional rates. Furthermore, high fXa rates were sustained during the rapid decrease in collisions between fX and the membrane, following the concentration peak. Averaged over 13 experiments, the activation rate was 3.05 ± 0.72 fmol/s/cm².

**FIG. 2.** Adsorption of reactants and factor Xa generation under flow. The reaction chamber was maintained at 37 °C and perfused at 13.4 μl/s with HEPES buffered medium, pH 7.2, containing 0.15 mM NaCl, 3 mM CaCl₂, and 0.1 mM nonlabeled ovalbumin. Maximal initial concentrations of reactant were 5 nM [³⁵S]fVIIa and 130 nM fXa. The TF density on the monolayer surface was estimated at >25 fmol/cm² from both functional and immunological assays. A, total amounts of reactant (either fVIIa or fX, □) adsorbed to the monolayer were determined from the difference between the normalized concentrations of control tracer, ¹⁴C (○), and test tracer, ³⁵S ³⁵S (△), collected in effluent samples. Tracer amounts were normalized as the fraction of the total added to the reaction chamber. B, the amount of fX on the membrane (○) was determined from the difference between fX adsorbed (□) and fXa (○) released. The average rate of fXa generation for this experiment calculated from the slope of the middle linear segment of the progression curve (101–150 s) was 2.1 ± 0.02 fmol of fXa/s/cm². The mean from 13 similar experiments was 3.05 ± 0.72 fmol/s/cm².

**FIG. 3.** The lagged normal density curve and distributions of substrate and product concentrations. The open circles correspond to concentrations of fX (in nanomolar) in aqueous phase determined from the concentration of nonreacting, nonadsorbing control tracer. The zero of the time axis was chosen to correspond to initial detection of ¹⁴C. The qualitative fit to the lagged normal density curve (LNDC) yields the parameters \( a_i = 21 \) s, \( τ_i = 91 \) s, and \( T_i = 19 \) s. The filled circles correspond to the fXa concentration released into each aliquot in the reaction system (×10 in the figure to facilitate comparison with fX values).

compared with the theoretical collisional rate between reactants and microspheres, given the aqueous phase concentrations of fX used and the flow rates, \( J_\text{fr} \), imposed. Because average adsorption rates were shown to approach or to exceed the reaction chamber.

amounts were normalized as the fraction of the total added to the reacting aliquots. The fXa concentration released into each aliquot in the similar experiments was 3.05 ± 0.02 fmol of fXa/s/cm². The mean from 13 similar experiments was 3.05 ± 0.72 fmol/s/cm².

FIG. 3. The lagged normal density curve and distributions of substrate and product concentrations. The open circles correspond to concentrations of fX (in nanomolar) in aqueous phase determined from the concentration of nonreacting, nonadsorbing control tracer. The zero of the time axis was chosen to correspond to initial detection of ¹⁴C. The qualitative fit to the lagged normal density curve (LNDC) yields the parameters \( a_i = 21 \) s, \( τ_i = 91 \) s, and \( T_i = 19 \) s. The filled circles correspond to the fXa concentration released into each aliquot in the reaction system (×10 in the figure to facilitate comparison with fX values).

compared with the theoretical collisional rate between reactants and microspheres, given the aqueous phase concentrations of fX used and the flow rates, \( J_\text{fr} \), imposed. Because average adsorption rates were shown to approach or to exceed the reaction chamber.
and catalytic cleavage is expected to delay the transit of the substrate that is adsorbed and catalyzed as compared with bulk aqueous-phase reactants. A mean transit time, $T_D$, was determined from the concentration of FXa and control tracer in 72 consecutive samples of the effluent according to the expression,

$$T_D = \frac{\sum_n^{12} [\text{C}(t_n)] \times t_n}{\sum_n^{12} [\text{C}(t_n)]} \quad \text{(Eq. 12)}$$

where $[\text{C}(t_n)]$ is the fraction (of the total amount added or theoretical maximum) of either control tracer or FXa collected in aliquot $n$ at time $t_n = n \times (2 - 8) \, \text{s}$, depending on the particular experiment. Results shown in Table III indicate that the mean transit time of FX, $T_D(\text{FX})$, activated in the reaction chamber is increased relative to the $T_D$ of aqueous-phase control tracer. In contrast, $T_D(\text{FXa})$ for FXa formed before being introduced in the reaction chamber is indistinguishable from that of the control tracer. Furthermore, the increase in $T_D(\text{FX})$ is inversely correlated with flow rate. These results are consistent with a slow membrane step following the fast, flow-dependent adsorption step.

**Kinetic Modeling of Surface Reactions** — The hypothesis that the reaction pathway proceeds with fast equilibration of enzyme activity followed by a rate-limiting step involving reactant surface diffusion was also tested by comparing experimental measurements to the solutions of the kinetic equations (Eqs. 3–8). Eqs. 3–8 were solved numerically using initial estimates for intrinsic rate constants based upon results of previous steady-state kinetic studies (15, 20, 21, 25–27). Heuristic arguments for initial guesses for all the rate parameters are provided under “Appendix.” A continuous function for aqueous-phase concentrations $C_N(t)$ is derived from a least-squares fit to a LNDC (“Appendix”) shown in Fig. 3. The remaining parameters in the model were then adjusted until the best visual fit of $\Gamma_i(t)$ to FXa collected was achieved. Because $\Gamma_i(t)$ was indirectly measured and subject to larger experimental errors, we used only varied rate parameters to get an order-of-magnitude agreement between the measured FX (Fig. 3) and $\Gamma_i(t)$ (Fig. 4B), using measured FXa and $\Gamma_i(t)$ to more precisely fit the parameters. The solutions and the associated best-fit parameters are shown in Fig. 4. We found that the magnitudes of $\Gamma_i(t)$ match the measurements only when the amount of TF assumed in the simulations was 0.32 fmol/cm$^2$, much smaller than the actual amount expressed on the cell membranes. This finding is consistent with our hypothesis that enzyme complexes form domains, which is further developed under “Discussion.”

The model also shows that, within reasonable ranges, the shape and magnitude of the product curve, $\Gamma_f(t)$, is sensitive to $k_{-a}$, $k_a$, and $\beta_i$, but less sensitive to the other parameters. If the association step, $k_{+a}$, was fast, the theoretical model would predict a premature overproduction of FXa, as shown in Fig. 5A. The sensitivity to a slow intermediate membrane step associated with $k_{+a}$ is shown in Fig. 5C, and the corresponding predicted values for effective enzyme, TF, and FX on the membranes are shown in Fig. 5D. Note also that for parameters differing from those used in Fig. 4, the magnitudes of $\Gamma_f(t)$ change dramatically and are no longer close to the measured FX (Fig. 3).

For the parameters used to fit the measurements in Fig. 2, the numerical solution for $E(\Gamma_f)$ plateaus to a value $-\Gamma_f$ after $t \sim 40 \, \text{s}$ and remains nearly constant for the duration of the 300-s interval under consideration. This quasi-steady state exists even for the cases where $k_{+a}$ is too large (Fig. 5B) or too small (Fig. 5D). We show under the “Appendix” that this quasi-steady-state behavior allows us to define an approximate effective rate constant (s$^{-1}$),

$$k_{\text{eff}} = \frac{k_a k_{+a} \Gamma_f}{k_{-a} + k_{+a}} \quad \text{(Eq. 13)}$$

which approximately describes the rate of FXa production on the cell membranes via,

$$\Gamma_i = k_{\text{eff}} \Gamma_f + \text{adsorption/desorption terms} \quad \text{(Eq. 14)}$$

For large $k_a$ (the fast chemical step), $k_{+a}$ becomes the limiting rate, because $k_{\text{eff}} \sim k_{+a}$. The experimental data are consistent with model predictions both qualitatively and quantitatively, when $k_{+a}$ is in the range expected for lateral diffusion of proteins on membranes. An estimate for a mechanistically relevant diffusion length can be derived from

$$\ell_0 = \left(\frac{D_{\text{surf}}}{k_{\text{eff}}}\right)^{1/2} \quad \text{(Eq. 15)}$$

where $D_{\text{surf}}$ is the surface diffusion constant of FX in the cell.
The membrane hinders surface diffusion and reduces the rate of Xa generation due to the reduced mass transfer. The aqueous-phase concentration of substrate is dependent on the flow rates and membrane concentrations of Xa. The instantaneous fraction of membrane X converted to Xa is generated in lower quantities and at later times. If the surface diffusion is slower than optimal, with a typical value of $k_{\text{eff}} = 0.01$, factor Xa is generated in lower quantities and at later times. The yield of Xa is expressed as the percentage of the total X added to the reaction chamber. Average reaction rates were determined from the steepest linear segment of the progression curves as in Fig. 2B.

**Table IV**  

| $J_v$ (µl/s) | $dG_{v(t)}/dt$ | Yield | Average rate |  
|-------------|----------------|-------|--------------|
| 4.6         | $5.0 \pm 0.2$  | 17.6  | 2.75         |
| 6.0         | $1.0 \pm 0.5$  | 17.5  | 3.21         |
| 6.4         | $2.0 \pm 0.9$  | 17.1  | 2.29         |
| 13.3        | $1.0 \pm 0.4$  | 7.8   | 2.29         |
| 14.2        | $1.0 \pm 0.5$  | 5.2   | 2.34         |
| 27.0        | $1.0 \pm 0.15$ | 6.0   | 2.85         |
| 28.0        | $1.0 \pm 0.04$ | 3.9   | 2.91         |

*The fraction of membrane-bound X converted to Xa increased linearly with time giving the slopes (roughly 0.001 s⁻¹) indicated. The density of X on the membrane was calculated from the difference between X adsorbed on membrane and released as Xa. The concentration of the Xa released was measured directly in the effluent samples by amidolytic assay.*

**DISCUSSION**

In this paper, we have analyzed the surface reaction kinetics of blood coagulation initiated by interaction among coagulation factors on biological membranes. Reactions were initiated on live epithelial cells expressing TF with physiological concentrations of FVIIa/FVa and Xa. The aqueous and membrane concentration of reactants as well as product formation were measured at time resolutions relevant to plasma clotting. Although adsorption of vitamin K-dependent proteins on procoagulant membrane surfaces has been shown to be fast and correlated to aqueous-phase flux (29), the rate of Xa generation was independent of both enzyme density on the membrane and flow rate, $J_v$. Moreover, using tracer dilution analyses we found that the transit time of the X participating in the reaction was prolonged relative to transit times of nonreacting control tracers. Rate coefficients calculated from reaction rates and either the aqueous or membrane concentration of reactants changed with time. Flow velocities influenced the total amount of reactants adsorbed to the membrane and the total yield but not the intrinsic rate of product formation. Taken together, the experimental results provide evidence for a kinetic mechanism limited by a slow transfer of substrate between initial membrane adsorption sites and reaction sites. The hypothesis of a slow membrane step was further tested by numerically solving a set of nonlinear kinetic equations describing the evolution of all membrane reaction species. The experimental results were reproduced when the rate-limiting step followed the adsorption of substrate to the membrane and preceded the chemical catalysis. Testing the alternative hypothesis of either slow adsorp-
tion or slow catalytic steps resulted in product yield and profiles markedly different from those observed experimentally.

Maximal surface catalytic activity was observed when the membrane concentration of FVIIa was at least two orders of magnitude lower than the surface concentration of TF, estimated either by immunoassay or functional tests. The experimentally measured FX concentrations best matched solutions of the kinetic equations (Eqs. 3–8) when the intrinsic rate constants shown in Fig. 4 were used along with a maximal enzyme concentration of $\Gamma_4 = 0.32 \text{ fmol/cm}^2$. This observation suggests that only a fraction of the available membrane E = FVIIa:TF is involved in catalysis as would be expected if catalytic sites were in large molar excess over the substrate. However, the substrate concentration reached, $>100 \text{ fmol/cm}^2$, is much higher than TF or enzyme concentrations.

These results can be explained using a model in which the enzyme concentration, $\Gamma_4$, is in relative local excess, and only a fraction of the surface enzyme effectively participates in catalysis. If enzyme complexes FVIIa:TF form domains, the fast catalytic cleavage reaction would be expected to occur only near the perimeter of these enzyme domains, where the substrate catalytic cleavage reaction would be expected to occur only near this experimental system are relevant to reactions on biological membranes after exposure of TF to flowing plasma coagulation proteins. The experimental approaches and mathematical model used here to identify the early kinetic mechanisms of FXa generation will also be useful for studying novel pharmacokinet- nic mechanisms occurring on biomembranes.

**APPENDIX**

In this section, we give details of the mathematical model (Eqs. 3–8) and the associated approximations used in its analysis. Because no measurable amount of FXa is generated by FVIIa and FX in bulk solution, we have assumed that chemical reactions can only occur when molecules are adsorbed on the surface of each cell-covered sphere.

The kinetic equations (Eqs. 3–8) are solved numerically using finite difference approximations. All surface densities, $\Gamma_i$, are in units of $\text{fmol/cm}^2$, whereas all bulk concentrations, $C_i$, are measured in units of $\text{pmol/cm}^3$. With this convention, the rate constants take on the following units: $[\beta] = [k_{\text{on}}] = [k_{\text{off}}] = [k_s] = s^{-1}$, $[k_{\text{on},s}] = [k_{\text{off},s}] = \text{cm}^2/(\text{fmol}s)$, and $[\alpha] = 10^{-3} \text{ cm/s}$.

We assume that the adsorption rates of species from the bulk onto the microsphere surfaces are proportional to the local bulk concentration. For the sake of completeness, and to motivate more quantitative modeling, we write the governing equations for surface adsorption of reactants. In the bulk phase, the concentration of species $i$ follows the convection-diffusion equation,

$$\partial_t C_i + \mathbf{v} \cdot \nabla C_i = D_i \nabla^2 C_i \quad r \geq R \quad (\text{Eq. 16})$$

where $C_i = C_1, C_2, C_6$, and $D_i$ are the bulk solution concentrations (number per volume) and associated diffusion constants of FVIIa, FX, and FXa, respectively. Although a closure relation is required to specify the detailed velocity field, $\mathbf{v}$, around each sphere, we will assume that the identical microspheres each are subject to an equivalent, averaged, effective flow velocity, $\mathbf{V}$. The boundary conditions within continuum theory at the sphere surface are found by balancing the diffusive flux with the desorption and adsorption rates on the surface of each microsphere (at $r = R$),

$$D_{i,\text{eff}} C_i(r = R,f) = \Phi C_i(R,t) - \beta_i \Gamma_i(t) \quad (\text{Eq. 17})$$

where the area fraction available for adsorption $\Phi = 1$ at low coverage.

The above equations constitute the exact continuum equations for the species in solution. The complete set of equations consists of the convection-diffusion equation (Eq. 16), the boundary conditions (Eq. 17), and the surface reaction equations (Eqs. 3–8).

Significant simplifications and decoupling of some of Eqs. 3–8 can be realized by assuming that the bulk concentrations at the spheres’ surfaces $C_i(R,f)$ can be approximated by the LNDc (37). As the fluid passes through the ensemble of cell-covered microspheres, certain flow lines are faster or slower than the mean flow velocity. The reactants in the aqueous phase are randomly advected through the microsphere chamber at a distribution of velocities. The LNDc is a convolution of random advection velocities with molecular diffusion and has been used to approximate advection-diffusion in blood flow (37). Although the source concentration, $C_i(t)$, may depend on the position of the microsphere within the reaction chamber, we assume that the dispersion and diffusion of all species are equal and use the lagged density curves to approximate the source, $C_i(t)$, surrounding each microsphere. The parameters used in the LNDc depend upon average microsphere packing, the bulk diffusion constants, and the imposed constant volume flow rate, $J_f$. 

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**Flow Kinetics of Coagulation Factor Xa**

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**FIG. 6.** Diagram representing protein distribution on the cell surface. If the formation of FXa occurs upon nearly each encounter of E and FX on the membrane surface, only E molecules near the perimeter of the domains will participate in catalysis of FXa.
The concentration of the ith species in a random flow environment is assumed to obey
\[ \frac{dC_i(R,t)}{dt} = \frac{1000m_i}{J_i(2\pi\sigma_i)^{3/2}} \exp\left(-\frac{1}{2}(T_i/\sigma_i)^2\right) \]  
(Eq. 18)
where \( m_i \) is the total number of femtomoles of species \( i \) added via the bolus injection, and \( J_i \) is the constant flow rate measured in \( \mu l/s \). The intrinsic delay time, \( T_i \), is inversely related to the mean \( \ln V \), whereas \( \sigma_i \) and \( \tau_i \) describe the width and effects of molecular dispersion, respectively. The amount of spreading embodied in \( \sigma_i \) is proportional to the bulk diffusion constant, \( D_i \). The solution to the initial value problem (Eq. 18) is
\[ C_i(t) = \frac{1000m_i}{2\pi\sigma_i} e^{-\sigma_i^2/4\tau_i} \left[ \text{Erf}\left(\frac{\sigma_i}{2\sqrt{\tau_i}}\right) - \frac{\sigma_i}{2\sqrt{\tau_i}} \left( t - \frac{\sigma_i^2}{4\tau_i} \right) \right] \]  
(Eq. 19)

The concentration, \( C_i \), above is given in nanomolar units. These solutions determine the sources, \( \alpha_i C_i(R,t) \), for the surface kinetic equations (Eqs. 3–8). We take the entire reaction chamber and the inlet and outlet tubes to constitute a single, effective flow system. The zero used in Eq. 19 corresponds to the inlet and outlet tubes to constitute a single, effective flow system. The zero used in Eq. 19 corresponds to the membrane enzyme (Eq. 19) reaches a plateau. This behavior permitted simplification of Eqs. 3–8 and approximate analytic solutions for the surface concentrations \( \Gamma_{i,j} \) (Eq. 21). For large \( k_{-a} \), the concentration \( \Gamma_{i,j} \) (Eq. 22) is always small. From a typical simulation (Figs. 4 and 5) we observed a short transient in \( \Gamma_3 \) (TF). At times beyond this transient, \( \Gamma_3 \approx 0 \), and the enzyme concentration, \( \Gamma_4 \), reaches a nearly steady value,
\[ \Gamma_4 = \frac{k_{+a}}{k_{-a}} \Gamma_3 \]  
(Eq. 23)

From the equation for \( \Gamma_4 \), we see that, over long time intervals, \( \Gamma_{i,j} \) approximately follows the adsorption and desorption processes,
\[ \Gamma_i = \alpha_i C_i(t) - \beta_i \Gamma_i \]  
(Eq. 24)

It is evident from Figs. 4 and 5 that \( \Gamma_4 \) (E) also reaches a quasi-steady state shortly after TF. Therefore, setting \( \Gamma_4 = 0 \),
\[ \Gamma_3 = \frac{k_{+a}}{k_{-a}} \Gamma_3 \]  
(Eq. 25)

The remaining time-dependent surface quantities at these quasi-steady-state times obey
\[ \Gamma_i(t) \approx \frac{\Gamma_i(0)}{k_{-a} + k_{+a}} \frac{1}{k_{-a} \Gamma_4} \]  
(Eq. 26)

where \( k_{-a} \) given by Eq. 13 is the effective rate of conversion from \( \text{IX} \) to \( \text{IXa} \) during quasi-steady-state times when the enzyme concentration is \( \Gamma_4 \approx 0 \). Assuming that \( \alpha_i C_i \) is negligible, Eqs. 26 admit analytic solutions, and, considering the approximate nature of our model, a further simplification can be made: Using Eq. 22, the second equation in Eq. 26 becomes
\[ Q_8(t) = \frac{k_{+a}}{k_{-a} \Gamma_4} \left( \frac{S_T}{J_T} \Gamma_4 - \frac{1}{k_{-a}} \frac{Q_8}{J_T} \right) \]  
(Eq. 27)

Therefore, an independent measurement of \( \Gamma_2 \) and the collected product \( Q_8 \) can be used to estimate the unknowns \( \beta_6 \) and \( k_{-a} \). Although in our analyses we have numerically solved the full kinetic equations (Eqs. 3–8), a simplified set of equations (Eqs. 24 and 26) provide an analytic model to the reaction kinetics for times beyond the initial short transient (\( t > 40 \) s for the run shown in Fig. 4).

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Flow Kinetics of Coagulation Factor Xa

7835

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