Surface-Mediated Enzymatic Reactions: Simulations of Tissue Factor Activation of Factor X on a Lipid Surface

Rodney Gentry,* Liqiang Ye,* and Yale Nemerson‡
*Department of Mathematics and Statistics, University of Guelph, Guelph, Ontario, Canada N1G 2W1; and ‡Division of Thrombosis Research, Departments of Medicine and Biochemistry, Mt. Sinai School of Medicine of the City University of New York, New York, New York, 10029 USA

ABSTRACT Blood coagulation proceeds via reactions in which zymogen coagulation factors are activated to proteases. An essential step is the activation of factor X by a complex of tissue factor and factor VIIa. This complex usually is studied using phospholipid vesicles into which tissue factor is inserted. Because factor X exists free in solution and bound to the lipid-surface, it is difficult to establish experimentally the kinetic contribution of surfaces. We therefore developed a stochastic model to simulate such reactions and generate initial velocity data from which Michaelis-Menten parameters are estimated. Simulated K_m values decrease slightly when substrate binding to lipid is increased and by a factor of four when the rates of surface diffusion are increased to that of fluid phase-diffusion. Simulations with various size planar surfaces established an enzyme capture radius of 32–64 nm. Simulations with different modes of enzyme-substrate complex assembly show that if the true substrate is lipid-bound, under certain conditions, the true k_{cat} is not measured; rather, the product "leaving rate" from the complex is the rate-limiting step that is measured as substrate is taken to infinity. This model is applicable to any surface-bound enzyme reaction.

INTRODUCTION

The specific binding of ligands to receptor molecules embedded in cell membranes mediates many biological functions. Blood coagulation is an essential biological process that entails surface-bound enzymatic complexes and substrates that can interact by either fluid phase (three-dimensional) diffusion, directly or by three-dimensional diffusion to the lipid surface, followed by lateral surface diffusion (two-dimensional) (Nemerson, 1988; Giesen et al., 1991; Krieg et al., 1987). It is generally agreed that surface diffusion can enhance the enzymatic rates, but the relative contributions of the two diffusion paths differ with different reaction mechanisms. This recently has been considered theoretically for both diffusion-limited reaction sinks on a cell surface and for reaction-limited targets (Wang et al., 1992; Axelrod and Wang, 1994). It has been suggested that surface binding per se enhances the local concentration of the substrate in a shell around the lipid vesicle, thereby increasing the effective local substrate concentration in the vicinity of the enzyme (Nesheim et al., 1984). Alternatively, it has been suggested that the enhancement of lipid reactions seen in coagulation reactions is simply a factor of the greater efficiency of 2D diffusion even though the mean velocity is slower than in fluid diffusion (Goldstein, 1989). On the other hand, there is direct experimental evidence that when the lipids are below their transition temperatures, the enzymic rates are not altered (Higgins et al., 1985). This observation suggests that lipid viscosity is not a limiting factor in substrate delivery, and one could conclude that surface diffusion is not a significant path for substrate delivery. The confounding aspects of lipid binding and overall reaction rates have been difficult to elucidate experimentally, as emphasized by Hermens et al. (1988), for prothrombin generation. The most general model for a reaction occurring on a surface would allow formation of enzyme-substrate (E-S) complexes with access of substrate to the enzyme either directly from the fluid or from the surface. An alternative model is required if the substrate cannot complex with the enzyme directly from the fluid, or the substrate activation site is not accessible for unbound substrate. In this case, if the complexed substrates (and subsequent products) remain bound to the surface, the complexes are actually lipid-enzyme-substrate (L-E-S) complexes. If the newly formed product is always bound to the surface, it can significantly impede subsequent formation of a new productive complex. The magnitude of this effect obviously would depend on the overall leaving rate of the product from the immediate vicinity of the enzyme. Such alternative models have been suggested for the initial step in blood coagulation in which tissue factor, a transmembrane protein, forms catalytic complexes with factor VIIa, a soluble serine protease (Nemerson, 1988; Mann et al., 1988). This complex, in turn, proteolytically activates factor X, a γ-carboxyglutamic acid containing zymogen, to another serine protease, factor Xa. For the purposes of this study, we have considered the tissue factor:factor VIIa complex to be nondissociable, which is justified by their very low dissociation constant, ~10 pM (Waxman et al., 1992), and by the fact that experimentally, virtually complete occupancy of tissue factor is ensured by keeping factor VIIa concentrations very high (Nemerson and Gentry, 1986). This reaction

Received for publication 2 February 1995 and in final form 11 May 1995.
Address reprint requests to Dr. Rodney D. Gentry, Department of Mathematics and Statistics, University of Guelph, Guelph, Ontario, Canada N1G 2W1. Tel.:519-824-4120 (ext. 3573); Fax: 519-837-0221; E-mail: rgentry@msnet.mathstat.uoguelph.ca.

0006-3495/95/08/362/10 $2.00

is studied experimentally by embedding the tissue factor-factor VIIa complex into lipid surfaces. We treat this complex as a single lipid-bound enzyme. The reaction's kinetics can be varied by altering the composition of the lipid, and thereby the extent of factor X binding to the surface.

To differentiate these competing models experimentally has proved to be elusive. An alternative approach, which is considered in this study, is to simulate the reaction. We have developed a flexible computer program that models the activation of substrate by a surface-bound enzyme. Although our specific objective was to simulate the tissue factor-factor VIIa activation of factor X, the model is generally applicable to any similar surface mediated reaction. The simulation program combines a structural representation of the reaction milieu within a grid, with substrate and product transport processes, and models the kinetics of activation using our microscopic kinetic model (Gentry et al., 1995). Two variations of the model are considered in this report, one for reactions with lipid vesicles, referred to as the V-model, and one in which the lipid is a surface analogous to a planar bilayer, which is referred to as the P-model. Substrate and product diffusion, both in the fluid and on the lipid, is simulated as random walks on square grid lattices. Simulation of specific reactions in parallel to laboratory biochemical experiments can be achieved by choosing corresponding model parameters such as the concentrations of reactants, diffusion constants, and probability constants corresponding to rates. Such simulations can provide more detailed observations of the reaction than is normally feasible for actual biochemical reactions, and thus provide an additional perspective for examining various theories of reaction mechanisms.

To introduce the simulation model, we briefly discuss some theoretical aspects of reaction-diffusion systems. The general model's structure and assumptions are introduced, and three series of simulations are used to explore aspects of surface mediate reactions. The first explores the kinetic effect of lipid binding per se. The second establishes the lipid-radius of capture of an enzyme by varying the extent of the lipid surfaces. The third series was designed to examine different models of substrate delivery to the enzyme.

Theoretical and modeling aspects of diffusion- and surface-mediated reactions

Diffusion is a stochastic process that can be modeled, on average, as a deterministic process described by the diffusion equation for the concentration at time t and spatial coordinates X, $C(X, t)$:

$$C_t(\mathbf{X}, t) = -D\nabla^2 C(\mathbf{X}, t) .$$

The diffusion constant D is the mean-square displacement of molecules per unit time. When the diffusion process is coupled with a kinetic process that either removes or creates molecules, the resulting system is described by a reaction-diffusion equation of the form

$$C_t(\mathbf{X}, t) - v(\mathbf{X}, t) = -D\nabla^2 C(\mathbf{X}, t)$$

where $v(X, t)$ is the velocity at which the diffusing substance is converted to a product (Britton, 1986; Fife, 1979; Murray, 1977). The nature of solutions $C(\mathbf{X}, t)$ depends on the geometry of the diffusion region, the initial state of the system, and boundary conditions that must be satisfied at the surface or enzyme. Theoretical considerations of the role of reduction of dimensionality in mediating surface reactions have generally assumed spherically symmetric domains, in which molecules diffuse to targets on spheres (Berg, O. G. and von Hippel, 1985; Adam and Delbrück, 1968; Hardt, 1979). A basic premise in these analyses is that the reaction is diffusion-limited, so that the reactive target can be viewed as an annihilation sink, at which the substrate concentration is zero. This has the effect of removing the reaction velocity term from the reaction-diffusion model. Although such assumptions allow the determination of analytical solutions for special cases, techniques do not exist for solving most diffusion equations with more complex boundary conditions or geometries. To describe such systems, either numerical solution of the differential equations or stochastic simulations must be used.

The stochastic or random nature of diffusion can be modeled as a random walk (Karlin, 1975). The use of random walks to model diffusion in biological processes has been clearly set out by H. C. Berg (1983). We chose this approach for a discrete simulation model because it allows one to investigate reactions involving a single-enzyme site rather than ensembles. Furthermore, it allows modeling of reactions that are not governed simply by a reaction-diffusion equation because of other factors, such as facilitated or limited transport or the presence of inhibitors. The model introduced in this study is novel in that it can be used to investigate the reduction in dimensionality hypothesis, because it couples three-dimensional diffusion in solution to two-dimensional diffusion along a surface. Furthermore, it can be used to explore the inter-relations between intrinsic kinetic parameters and the diffusion and lipid-binding parameters.

The reaction system and model assumptions

The modeled reaction system can be applied to a variety of surface-mediated biochemical reactions with a reaction milieu consisting of an aqueous environment containing substrate, S, and product, P, and a surface, which shall be referred to as lipid, L, that may be either planar bilayer sheets or vesicles, on which the enzyme, E, is attached. In an unstirred environment, substrate and product are assumed to move via simple diffusion in the fluid and can bind to and diffuse on the lipid.

Only a small portion of the reaction environment is modeled, assuming a steady-state system with uniformly

structured lipid and randomly distributed protein molecules. The simulation volume is a cubic region containing a single lipid vesicle or planar sheet. A lipid vesicle is modeled by a smaller cube centered in a modeled region while a bilayer is assumed to lie on a face of the region. Symmetry is used to extrapolate the simulation results to bulk system kinetic behavior. The simulation uses a lattice representation of the region. Noting that an equidistant three-dimensional grid cannot be established (Loeb, 1976), regular cubic and square grids are used to model the fluid and lipid surface, respectively. Substrate and product diffusion is achieved via a random walk governed by transition rules applied iteratively. Molecules can move randomly to adjacent nodes or remain at the same node. A "collision" on the surface is deemed to occur when a molecule is designated to move to an occupied position, in which case the molecule remains in its original position. Initial and periodic random ordering of the sequence of molecular movement avoids bias over many iterations (typically $>10^6$). Reflective boundary conditions are applied at the boundary of the simulated cube to maintain conservation of mass.

Lipid surface attachment involves initial binding, lateral diffusion and, eventually, dissociation from the surface. When a molecule encounters the surface is randomly controlled by the diffusion process; the probability that the molecule then binds to the lipid, $P_{on,L}$, depends on the properties of the surface, the molecule, and the energy and orientation of the collision. The probability of dissociation from the surface, $P_{off,L}$, is the reciprocal of the off rate measured experimentally.

The probability, $P_{on,E}$, that a complex is formed when a substrate or product encounters a vacant enzyme can be different for bound and fluid-phase substrate or product. Similarly, the residence time, T_{res}, for an enzyme-complex may be different for E-S and E-P complexes. The probability of activation, P_{act}, is invoked after the residence time of an E-S complex to determine product formation. When a complex dissociates, the released molecule randomly moves to an adjacent grid node. Each simulation cycle corresponds to a period of time of the chemical reaction being modeled, which depends on the diffusion rates and the assumed grid length between adjacent nodes. The cycle time is the minimum of the mean time required for the fastest diffusing molecule to "traverse" a grid length, the shortest residence time of molecules on the lipid surface, and the residence time of complexes. For fine grids, the fluid diffusion coefficients will generally establish the reaction time-simulation cycle correspondence.

Computer code for the model has been implemented in FORTRAN 77 and has been compiled and run on different platforms, including PCs, Unix, and a CRAY parallel processor, which only marginally decreased run time because the code is interactive and essentially not vectorizable. Slight variations are required for each platform as the random number generator calls the operating system time periodically to re-seed the algorithm. An external input file is used to supply model parameters for specific reaction systems. The primary output consists of periodic reporting of the reaction's progress, the number of collisions involving the diffusing molecules, the enzyme, and surface collisions and occupancy. Output options include the ability to track all or specific molecules and to record the number of cycles since a molecule last contacted the surface, enzyme, or another molecule.

Specific model parameters are established on the basis of known physical and chemical properties of the reactants and correspond to experimental conditions used in actual biochemical experiments conducted in our laboratory. The volume, V, of a simulation cell is determined from the assumption that the modeled cell contains a single vesicle or lipid sheet:

$$V = 1/N_v \approx 1.66044/[LV]\ \mathrm{nm}^3 .$$

To conserve computer memory and processing time, in the V-model we assume that the reaction system is locally radially symmetric about each vesicle and we model only one octant with volume $V_m = V/8$. The cell is modeled by a regular cubic grid lattice with N_g nodes per edge, a distance, δ, apart. For the V-model, the grid step length is

$$\delta = V_m^{1/3}/(N_g - 1) \approx \{0.59208/(N_g - 1)\}/[LV]^{1/3}\ \mathrm{nm} .$$

The one-eighth of the vesicle contained in the modeled octant has volume $V_l = \pi r^3/6$, where r is the radius of the vesicle. To use the same grid structure for the fluid and the lipid surface, the vesicle is modeled by a cubic sub-grid with N_l nodes per edge, where

$$N_l = \mathrm{Int}\{(N_g - 1) \cdot [V_l/V_m]^{1/3}\} + 1$$

and Int{ . . . } is the greatest integer less than the value within the braces.

The number of substrate molecules, N_S, in a modeled octant corresponding to a bulk substrate concentration, $[S]$, is

$$N_S = \alpha N_A [S][LV]^{-1},$$

where α is 1 for a P-model and 1/8 for the V-model. Physically, the mean time, τ, required for a molecule with diffusion coefficient D to travel a distance δ is $\tau = \delta^2/D$ (Berg, H. C., 1983). Normally, the fluid diffusion constant, D_f, is several orders of magnitude greater than the lipid surface diffusion constant, D_l. Consequently, if a molecule can move one grid step in the fluid per iteration, to move the same distance on the surface will take D_f/D_l iterations, approximately.

In the P-model, the lipid is represented by a square two-dimensional lattice centered on one face of the cubic lattice corresponding to the fluid region. The lipid's size or extent is indicated by the number of nodes per edge, N_l. The simplest model places a single enzyme centered at the central node of the lipid (requiring N_l to be odd to maintain symmetry). The mass conservation boundary conditions invoked when a molecule via the random walk algorithm exits the cube, randomly places another molecule of the

same type on the same surface, except on the lipid containing face of the P-model, where molecules exiting are replaced at the same distance from the center of the face to avoid bias.

Simulations of factor X activation by tissue factor-factor VIIa

The model parameters used in the reported simulation were chosen to correspond to experimental reaction conditions used in in vivo studies of the tissue factor system. The enzyme is thus a tissue factor-factor VIIa complex, which we assume to be nondissociable, the substrate, factor X, and the product, factor Xa. A commonly used vesicle preparation uses a 0.05 nM concentration of 80-nm radius vesicles (Forman, 1986). Thus, the average volume of the reaction region containing one vesicle is 33.2088×10^9 nm^3 and $V_m = (1.60714 \times 10^3 \text{ nm})^3$. The radius of gyration of a factor X molecule has been estimated by centrifugal techniques to be ~3.4 nm, so we assume the substrate diameter is ~7 nm (Forman, 1986). Arbitrarily choosing the number of grid nodes per edge to be $N_g = 100$ gives a grid step length of $\delta = 16.23$ nm and makes the molecules on adjacent grid nodes separated by ~9 nm. (There was no appreciable difference in simulations with finer grids, $N_g = 200$.) The octant of an 80-nm radius vesicle then has five nodes per edge.

The number of substrate molecules included in the simulation volume is $N_S = 2.5 \times 10^9$ [S], and the simulated [E] is then 0.4 nM when a single enzyme is simulated per octant or per lipid sheet. Simulated K_m determinations, corresponding to the typical laboratory experiment protocols, involve series of simulations with substrate concentrations ranging from nanomolar to micromolar values; in all cases, high [S] exceed 10 K_m and V_{max} is clearly approached.

The diffusion coefficient of factor X in an aqueous fluid is $\sim 5 \times 10^7$ nm^2/s (Forman and Nemerson, 1986). For grid length $\delta = 16.23$ nm, the average time for molecules to move one grid step in the fluid is then $\tau_f \approx 2.58 \times 10^{-6}$ s. Estimates for the lipid diffusion coefficient of factor X range from 100 to 5×10^5 nm^2/s (Alberts et al., 1983). Thus, our default "normal" ratio of D_f:D_l is 1000, corresponding to $D_l = 5 \times 10^4$ nm^2/s, with the average time required for a factor X molecule to diffuse on the lipid through a region of area δ^2 being $\tau_l \approx 2.58 \times 10^{-3}$ s. This is taken as the average time for a molecule to move to an adjacent grid node on the lipid surface. With these parameters, 10^6 simulation iterations correspond to ~2.6 s of real reaction time.

Simulations and results and analysis

The effect of lipid binding on K_m for a vesicle model

The kinetics of factor X activation by tissue factor-factor VIIa can be significantly altered by varying the composition of the lipid to change the charge of the lipid and, thereby, the extent of substrate binding. To explore these effects, a set of simulations were run with different lipid binding parameters. In this set of simulations, the *product is assumed to leave the enzyme either directly into the fluid phase or to the surface.*

The tightness of substrate binding to the lipid is generally indicated by an equilibrium dissociation constant, K_d, which in fact represents several more primary constants. Experimentally, the K_d can be controlled by altering the composition of the lipid using mixtures of acidic lipids like phosphatidylserine, PS, and neutral lipids, such as phosphatidylcholine, PC (Zwaal, 1978; Hermens et al., 1988). For factor X binding to mixtures of 20% PS and 80% PC, the K_d has been estimated to be 520 nM (Forman and Nemerson, 1986) and, for slightly different lipid mixtures, has been estimated by several different methods to be 100–200 nM (Hermens et al., 1988).

In our simulation models, surface binding and dissociation are controlled by the probability of a molecule that diffuses to the surface will actually bind to the lipid, $P_{on.L}$, and the first-order off rate for lipid-bound molecules, $k_{off.L}$, which has been estimated to be 3.3 s^{-1} (Krishnaswamy et al., 1988). The probability of binding is difficult to measure because it is conditional on the a priori delivery of substrate to the surface. If the rate of collision is $CR_{s.l}$, then

$$K_d = k_{off.L}/(P_{on.L} * CR_{s.l}) .$$

The collision rate is controlled by the diffusion process and [S] in the fluid; it may be calculated empirically from simulation data recording actual surface collisions. For the V-model with a $D_f = 5 \times 10^{-7}$ cm^2 s^{-1}, the observed collision rate was 0.095 nM^{-1} s^{-1}. This gives a K_d of 52.7 nM when $P_{on.L} = 1$ and $k_{off.L} = 5$ s^{-1}. Alternatively, the apparent K_d can be estimated for a fixed lipid off rate and binding probability by a substrate titration series of simulations recording the fraction of lipid-binding sites occupied, F, and fitting the data to

$$F = F_{max}[S]/([S] + K_d) .$$

The fractional binding capacity of the surface, F_{max}, was ~1 for simulations with $D_f = 5 \times 10^{-7}$ cm^2 s^{-1}, $P_{on.L} = 1$ and $k_{off.L} = 5$ s^{-1} that resulted in $K_d = 57.6 \pm 2.82$ nM, whereas a similar series of simulations with $P_{on.L} = 0.1$, which should theoretically result in a K_d of about 527, resulted in a binding isotherm fit of K_d of 523.8 ± 16.7 nM.

To explore the influence of the lipid-binding parameter on the enzyme kinetics, simulations were run with different choices of $P_{on.L}$ and $k_{off.L}$ to simulate K_d values ~52, 520, and 5200 nM and infinity (i.e., with no binding to the lipid). For each K_d, a series of simulations were performed for at least 15 values of [S], ranging from 25 to 800 nM; because of the stochastic nature of the random walk algorithm, the observed initial velocity data exhibit the variability observed in laboratory experiments, as illustrated in Fig. 1. These initial velocities were fit to the Michaelis-Menten

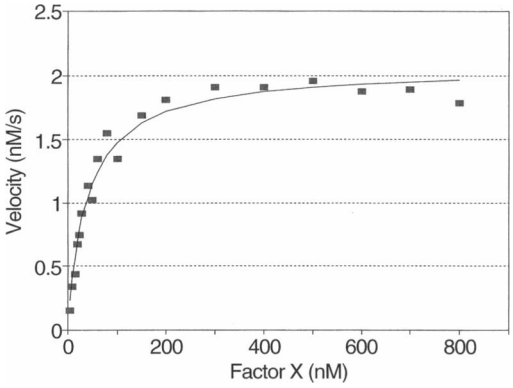

FIGURE 1 Representative K_m simulation for lipid vesicle; lipid K_D = 520 nM; D_f:D_l = 5000.

equation to estimate K_m and V_{max} (Table 1). The estimated maximum velocity is independent of K_d. The maximum K_m, observed with no surface binding, is only 12.7% higher, 44.61 vs. 39.59 nM, than that observed with K_d = 520 nM, which is approximately the dissociation constant of factor X observed under comparable conditions (Forman and Nemerson, 1986). Thus, variations in lipid binding do not account for the three orders of magnitude reduction in the K_m observed experimentally when lipid binding is introduced by changing the lipid composition (D. Repke, R. Gentry, and Y. Nemerson, unpublished observations).

The effect of lipid size on K_m

Clearly, substrate binding to lipid far from the enzyme will not have an immediate effect on the enzyme's catalytic events; indeed, if it cannot reach the enzyme before closer substrate can, such binding will have no effect on the kinetics. To formalize this concept, we introduce the notion of a *region of influence* (discussed later) and the *effective capture radius* of an enzyme. On a surface, the *region of influence* is the neighborhood of the enzyme, indicated by its radius, in which substrate binding will influence the rate of reaction; substrate binding to the lipid further away from the enzyme will not enhance the observed reaction rate. The *radius of capture* depends on the mean time for the bound molecule to diffuse to the enzyme, the time between successive enzyme-substrate interactions from both fluid and surface source substrate, and the residence time of an enzyme complex. It depends on the apparent surface diffusion rate, which may be slower than the theoretical rate because of impaired movement when the surface-binding sites become saturated. It also depends on the fluid diffusion rate and the access of substrate to the enzyme, which affect the mean time between enzyme complex formations.

To explore these aspects, the P-model was used with different size lipid sheets, for which apparent Michaelis-Menten parameters were determined by simulation. The square lipid of size $(N\delta)^2$ has (N + 1) nodes per edge; N was even and varied from 0 to 24. The results in Fig. 2 show an initial drop in K_m as the lipid size increases to N = 6, with a subsequent rise in the K_m as the lipid size increases, suggesting that for these conditions an effective lipid-*radius of capture* for the enzyme may be at most 4 to 8 × δ/2 ≈ 32–64 nm. As the lipid size increases past the lipid-radius of capture, additional substrate binding to the outer portion of the lipid not only does not enhance the reaction rate but, in fact, lowers the fluid substrate concentration. Thus, as the lipid size increases beyond the lipid-radius of capture the lipid surface in effect acts as a competitive inhibitor, soaking up substrate and sequestering it from the enzyme. This effect is most pronounced at low substrate levels when lipid binding is tight and diffusion is very slow. This phenomena explains the increase observed in the apparent K_m as the lipid size is increased.

TABLE 1 Fitted K_m and V_{max} for different dissociation constants for substrate binding to lipid vesicles

K_d (nM)	K_m (nM)	V_{max} (nM s^{-1})
52	34.36 ± 3.29	2.090 ± 0.0551
520	39.59 ± 3.69	2.056 ± 0.0492
5200	43.62 ± 3.16	2.037 ± 0.0389
∞	44.61 ± 4.11	2.047 ± 0.0511

The kinetic contribution of a surface per se

To investigate further the kinetic role of surfaces, a series of four K_m simulations was run with the P-model in which the access of the substrate to the enzyme was controlled as well as binding to the lipid. The K_m and V_{max} calculated from simulations with a square lipid of size N = 10 are indicated in Table 2. The apparent K_m is greater when only bound substrate can form E-S complexes than when both fluid and lipid bound substrate can form complexes. This indicates that the mean vacancy time of an enzyme is greater without fluid source substrate. This restriction must then influence the lipid *radius of capture* of an enzyme. To demonstrate the

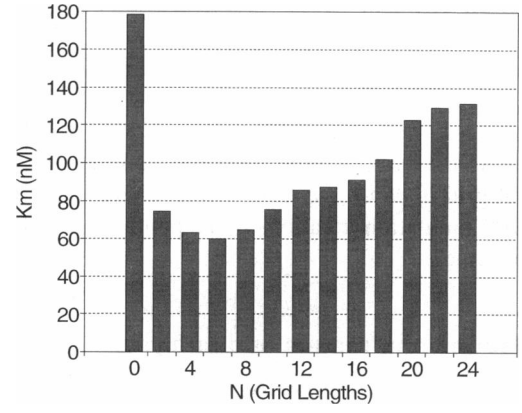

FIGURE 2 The apparent K_m as a function of the size, N^2, of the planar lipid sheet: grid Length 16.23 nm; relative diffusion ratio D_f:D_l = 1000.

TABLE 2 Simulations of lipid bilayer model with restricted access to the enzyme and with and without substrate binding to lipid

	Substrate		Source	
	Fluid and lipid	Lipid only	Fluid only with lipid binding	Fluid only without lipid binding
K_m (nM)	73	75	269	208
V_{max} (nM/s)	16.2	15.1	18.6	16.5

influence of fluid access on the capture radius, another set of simulations were run, varying the lipid size, but this time only permitting lipid bound substrate to bind with the enzyme. The results, indicated in Fig. 3, were similar to those with both pathways of substrate delivery; however, in this case the apparent *radius of capture* increased to 4 to 10 × $\delta/2$ or 32–80 nm. Thus, restricting enzyme access to only lipid-bound substrate molecules increases by 25% the upper bound for the lipid capture radius.

Enzyme complexes and mandatory lipid binding

A controversy that has persisted in the coagulation field is the nature of the enzyme-substrate complex for specific reactions. One school of thought is that lipid or surface binding of the substrate is an essential prerequisite to the formation of an enzyme complex (Mann et al., 1988), which implies that the complex is formed with the substrate still bound to the lipid, yielding a lipid-enzyme-substrate (L-E-S) complex. To elucidate the implications of such models, a series of simulations was run with two different P-models. One being the standard E-S model used previously, in which E-S complexes can be formed with various controls of substrate access to the enzyme and *no restrictions on the substrate leaving a complex.* The second, referred to as the lipid-complex or L-E-S model, has mandatory binding of substrate to the lipid before formation of a L-E-S complex. In this model, substrate remains adjacent to the enzyme on the lipid surface and consequently *product, upon formation, is also bound to the lipid.* Hence, after the dissociation of the L-E-S complex, the movement of the released substrate or product is governed by the diffusion rate on the lipid and the off rate from the lipid. In analyzing the initial velocities of these simulations, the calculated K_m is an "apparent" K_m as the observed k_{cat}, denoted by $k_{cat.obs}$, depends on the reaction environment rather than simply the true catalytic efficiency of the enzyme.

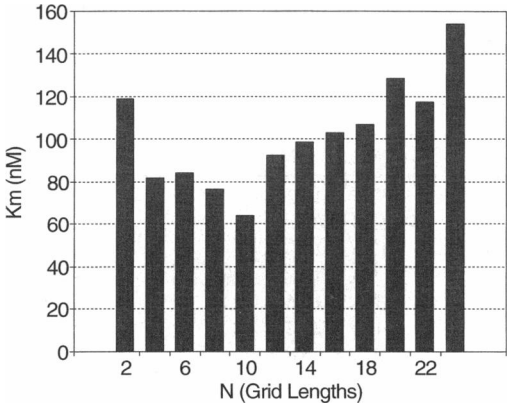

FIGURE 3 The apparent K_m as a function of the lipid size, N^2; conditions are as in Fig. 2, with substrate binding to the enzyme only permitted from the lipid surface.

Before considering simulations with different models of enzyme complex formation, we make a slight diversion to discuss the relationship between the observed and the theoretical values of k_{cat}. The maximum possible catalytic rate of the reaction is T_{res}, as

$$k_{cat} = P_{act}/T_{res}\,.$$

This equation assumes that enzyme is 100% occupied by substrate (and never product), which presumes instantaneous substrate access to the enzyme when [S] is very high. This is not the case when restrictive assumptions are made concerning the formation of enzyme complexes or the process by which dissociated substrate or product leaves an enzyme. In our models and, we conjecture in real reaction systems, restrictions on the leaving process effectively increase the probability of activation above the putative value of P_{act}. This occurs when the surface is saturated and unconverted substrate cannot leave an enzyme complex because of occupancy of adjacent sites; the prolonged residence time increases the substrate's chance of activation. A similar prolongation has been suggested in response to increased fluid viscosity (Somogyi et al., 1978; Somogyi and Damjanovich, 1975). The theoretical formula for k_{cat} must be modified for our simulations because an unconverted substrate that cannot leave the enzyme, because the adjacent lipid site is occupied, is given another chance to be converted to a product. An attempt to move the molecule is made on the next iteration, with the probability the same chance, P_{act}, of being activated. This process is repeated until the molecule leaves the enzyme. Thus, the modified theoretical bound for k_{cat} will be larger by a factor of $F = 1/(1 - P)$, where $P = (1 - P_{act})P_{occ}$ and P_{occ} is the probability of the destination site being occupied, which naturally increases with increasing substrate levels and is also a function of the lipid K_d.

For instance, in the basic P-model, considering the chance that a nonconverted substrate is designated to move to an occupied lipid site, gives this factor as $F = 1.168$ when lipid saturation is 90%, and $F = 1.19$ when the lipid is 100% occupied. The corresponding effective probabilities of activation at high substrate levels are then 74 and 76% compared with the theoretical 64% entered as the model parameter P_{act}. Table 3 indicates the impact of lipid saturation on the modified theoretical upper bounds for the observed k_{cat}.

TABLE 3 Upper bounds for the observed value of k_{cat}

T_{res} (s)	0.016	0.0008	0.00016
Theoretical k_{cat} (s^{-1}) no saturation effect	40	800	4000
Modified k_{cat} (s^{-1}) 90% lipid occupancy	46.7	934	4672
Modified k_{cat} (s^{-1}) 100% lipid occupancy	47.6	952	4760

Upper bounds for the observed value of k_{cat}

A similar modification of the theoretical upper bound for an observed k_{cat} can be made for the lipid-complex model. A significant feature of this model is that the substrate or product leaving the enzyme is attached to the lipid and, thus, remains near the enzyme for a short period. This means that substrate not converted to product may re-contact the enzyme at a greater rate than the statistical average for the E-S model with completely free egress of substrate/product from an enzyme complex. It also means that recently enzyme-associated products may present a physical barrier to enzyme access, thus lowering the apparent k_{cat}. Because the L-E-S simulation model restricts both enzyme access and egress to only four adjacent lipid binding sites, the theoretical k_{cat} for this model is given by the modified formula:

$$k_{cat} = P_{act}/\max\{T_{res}, LDT/4\}\,.$$

where LDT is the mean time between diffusion moves on the lipid surface.

The results of simulations of the L-E-S model with $P_{on.e} = 0.5$ and the off rate from the complex governed by the indicated T_{res} are indicated in Table 4. In these simulations, K_d was kept constant by varying $P_{on.L}$ and $k_{off.L}$ inversely. Simulations were run with $[S]$ ranging from 2 to 4000 nM and from 40 to 40,000 nM for low and high K_m values, respectively. In general, the fact that the $k_{cat.obs}$ are significantly below the values expected theoretically indicates that under certain conditions, namely, when both surface diffusion and the $k_{off.L}$ are slow, the reaction at infinite $[S]$ is governed by these parameters rather than by the intrinsic k_{cat}.

L-E-S lipid complex model T_{res}

To discern whether it is the limited access to the enzyme or the restricted egress that is the controlling factor, a similar set of simulations were run with the E-S model and enzyme access limited to surface-bound substrate. In this case, the product is allowed to leave directly to the fluid phase, which should partially relieve any product inhibition. The results of these simulations are shown in Table 5. Under these conditions, the $k_{cat.obs}$ is still limited by the diffusion rate on the surface when T_{res} is short. Note that in this model when the surface is blocked the product can escape to the fluid phase directly.

E-S complex planar model: only lipid-source substrate T_{res}

The more general E-S model, with no restriction on substrate access to the enzyme, was also examined. As indicated in Table 6, removing restrictions on enzyme access raises the $k_{cat.obs}$. When both lipid-bound and fluid phase substrate are allowed to form E-S complexes, the $k_{cat.obs}$ becomes essentially independent of the two-dimensional diffusion rate. However, the K_m varies inversely with the two-dimensional diffusion rate, with the change being most pronounced when the enzyme-substrate complex residence time is shortest. Thus, the effect of allowing fluid-source substrate direct access to the enzyme is to relieve the product inhibition and surface saturation effects seen when previous lipid binding is mandated for a substrate to form an E-S complex.

DISCUSSION

A central issue in the study of biochemical reactions that occur on cell or lipid surfaces is the extent to which surface diffusion influences the kinetics of the reactions. Some very important aspects of surface-mediated biochemical reac-

TABLE 4 L-E-S lipid complex model

T_{res} (s)	D_f/D_l	$P_{on.L}$	$K_{off.L}$ (s^{-1})	$K_m \pm$ SE (nM)	$k_{cat.obs} \pm$ SE (s^{-1})
0.016	1000	0.5	5	88.5 ± 12.2	35.1 ± 1.02
0.016	1000	1.0	10	67.8 ± 8.4	34.4 ± 0.84
0.016	10	0.5	5	12.6 ± 1.7	44.0 ± 1.13
0.016	10	1.0	10	9.3 ± 1.4	42.9 ± 1.16
0.0008	1000	0.5	5	161 ± 13.6	78.4 ± 1.60
0.0008	1000	1.0	10	164 ± 22.2	86.9 ± 2.85
0.0008	10	0.5	5	214 ± 26.8	639 ± 20.2
0.0008	10	1.0	10	199 ± 24.3	720 ± 21.8
0.00016	1000	0.5	5	171 ± 19.3	87.1 ± 2.39
0.00016	1000	1.0	10	181 ± 26.0	94.8 ± 3.34
0.00016	10	0.5	5	442 ± 31.8	1091 ± 23.4
0.00016	10	1.0	10	396 ± 57.1	1342 ± 55.7

tions cannot be easily investigated experimentally, such as the kinetic contributions of surfaces per se or the implications of saturable surface-binding capacity for alternative models of enzyme-substrate complex assembly. To study such phenomena, we developed a flexible computer simulation model that incorporates both surface and fluid diffusion with a microscopic kinetics model. It extends existing methodology because it does not impose a sparse substrate restriction and allows fine control of substrate (or product) access to or egress from a surface-bound enzyme, binding to and dissociation from the lipid surface, diffusion rates in the fluid and on the surface, and the kinetics of activation. Consequently, by systematic simulations, with variations in specific parameters, the model can be used to explore the contributions of each factor to the apparent reaction kinetics.

Experimental evidence suggests that the rate of factor X activation depends critically on the composition of the lipid. The activation of factor X using a 30:70 PS/PC mixture is held generally to be much faster than that observed with a pure PC lipid that does not bind factor X. The reason that the introduction of lipid-binding increases the efficiency of the reaction is that it reduces the K_m. Variations in lipid composition can be simulated by varying the effective dissociation constant for the binding of substrate and product to the surface by changing the probability of binding and the off rate from the lipid. The estimates of Michaelis-Menten parameters presented in Table 1 have SEs of ~10%, reflecting the stochastic nature of the simulations. The observed K_m decreased monotonically as the lipid binding became tighter, suggesting that two-dimensional diffusion, even when assumed to be slower by a factor of 5000, creates a more efficient mechanism for supplying substrate to the enzymatic site. However, the observed increase in the K_m between relative tight binding, K_d = 52 nM, and no lipid binding, $K_d = \infty$, is too small to account for the increase observed experimentally. One set of negative simulations does not in any way prove or disprove what is actually the cause of the experimental observations. It does suggest that there is more to the incorporation of acidic phospholipids than simply introducing surface binding of the substrate.

The effective surface-radius of capture was investigated by a series of simulations using the planar model in which the extent of the lipid surface was increased, allowing more substrate to bind to the lipid but progressively at greater distances from the enzyme. The observed two-thirds decrease and subsequent increase in K_m as the lipid became larger (Fig. 2) occurred with no significant change in V_{max}. These data suggest that the *radius of capture* of an enzyme on the lipid surface, under the simulated conditions, is in the range of 41–74 nm; substrate binding beyond this distance in fact increased the K_m. When access to the enzyme was limited to only surface-bound substrate, the upper bound for the *radius of capture* increased to ~90 nm, reflecting the increased vacancy time of enzymes because of reduction in substrate collisions with the enzyme when $[S] \approx K_m$.

The kinetic contribution of a surface per se

To explore the effect of surfaces per se on activations by surface-bound enzymes, a set of simulations was run with various limitations on the access of substrate to the enzyme. The full access model was compared with models with access limited to fluid phase substrate, both with and without concurrent binding to the lipid surface, and to a model with access limited to surface-bound substrate. As might be expected, the data of Table 2 indicate that restriction to lipid-bound substrate increased the apparent K_m by ~20%. However, restriction to fluid-phase substrate resulted in a threefold increase in the apparent K_m, reflecting the nonproductive sequestering of substrate on the lipid surface.

In a pure reaction system with a 100% efficient enzyme, the apparent K_m indicates the substrate concentration at which the average vacancy time of the enzyme is equal to the average residence time of an enzyme-substrate complex (Gentry et al., 1995). The increase in $K_{m\text{-app}}$ that arises when only lipid-source substrate is permitted to bind to enzyme indicates greater enzyme vacancy times for low substrate reactions than occur when both fluid and lipid source substrate are permitted to bind. Consequently, one would expect the capture radius for the enzyme to increase under the restricted conditions. The data shown in Fig. 3, for simula-

TABLE 5 E-S complex planar model: only lipid-source substrate

T_{res} (s)	D_f/D_l	$P_{on.L}$	$K_{off.L}$ (s^{-1})	$K_m \pm$ SE (nM)	$k_{cat.obs} \pm$ SE (s^{-1})
0.016	1000	0.5	5	82.0 ± 10.4	38.2 ± 1.00
0.016	1000	1.0	10	95.1 ± 8.3	42.9 ± 0.80
0.016	10	0.5	5	13.4 ± 1.9	45.3 ± 1.24
0.016	10	1.0	10	17.0 ± 1.7	48.5 ± 0.95
0.0008	1000	0.5	5	490 ± 50.9	168.0 ± 5.34
0.0008	1000	1.0	10	435 ± 68.3	170.0 ± 7.95
0.0008	10	0.5	5	338 ± 29.8	864.3 ± 21.2
0.0008	10	1.0	10	295 ± 22.8	961.1 ± 20.0
0.00016	1000	0.5	5	498 ± 74.1	183.1 ± 6.43
0.00016	1000	1.0	10	559 ± 63.5	181.4 ± 5.06
0.00016	10	0.5	5	2206 ± 85.8	3669 ± 37.0
0.00016	10	1.0	10	1999 ± 97.0	3811 ± 46.3

TABLE 6 E-S complex planar model: fluid and lipid-source substrate

T_{res} (s)	D_f/D_l	$P_{on.L}$	$K_{off.L}$ (s^{-1})	$K_m \pm$ SE (nM)	$k_{cat.obs} \pm$ SE (s^{-1})
0.016	1000	0.5	5	67.2 ± 10.5	42.9 ± 1.31
0.016	1000	1.0	10	95.7 ± 9.0	47.8 ± 0.97
0.016	10	0.5	5	16.8 ± 3.1	46.6 ± 0.95
0.016	10	1.0	10	17.4 ± 3.6	48.9 ± 1.13
0.0008	1000	0.5	5	3741 ± 297	856 ± 21.5
0.0008	1000	1.0	10	3689 ± 350	921 ± 27.6
0.0008	10	0.5	5	339 ± 30.8	934 ± 23.5
0.0008	10	1.0	10	326 ± 17.5	966 ± 14.4
0.00016	1000	0.5	5	22800 ± 1617	4350 ± 177.3
0.00016	1000	1.0	10	22176 ± 1151	4526 ± 133.7
0.00016	10	0.5	5	1817 ± 44.6	3572 ± 40.7
0.00016	10	1.0	10	1770 ± 72.4	3917 ± 73.7

tions with enzyme binding restricted to lipid-bound substrate, indicate an increase in the apparent *radius of capture* to the range 73–89 nm. Curiously, the minimal apparent K_m is ~70 nM for both scenarios (Figs. 2 and 3), suggesting that the increased capture radius compensates, at least to some extent, for the lack of fluid-phase substrate accessibility to the enzyme.

The difference between observed k_{cat} values and the corresponding theoretical bounds (Tables 3 and 4) when enzyme access is limited to bound substrate is a consequence of the saturability of the surface. In this situation, the true V_{max} is not attained because the underlying assumption of infinite substrate concentration, implying an infinite collision frequency between E and S, is not fulfilled. This phenomenon is clearly present in the lipid-complex model simulations (Table 4). To summarize these results, when the k_{cat} is fast, it is not the rate-limiting step, which is the overall leaving rate of product from the vicinity of the enzyme. Conversely, if the true k_{cat} is slow compared with the leaving rate, the observed and calculated values for this parameter agree. Thus, for example, with the experimentally suggested ratio of diffusion rates, $D_f/D_l = 10^3$, when $T_{res} < 6.5 \times 10^{-4}$ s, the reaction is diffusion-limited, because the leaving rate is $LDT/4 \approx 0.65 \times 10^{-6} * D_f/D_l$, and the modified theoretical $k_{cat} \approx 985$ is about 10 times the $k_{cat.obs}$ when $T_{res} = 0.00016$ s. For the lipid-complex model, $k_{cat.obs}$ is also influenced by the off rate of substrate and product from the lipid surface, as observed in the consistently lower values when $K_{off.L}$ is 5 vs. 10 s^{-1}. Differences between the simulation based parameters and the theoretical bounds also arise because the ideal assumptions of the theoretical analysis are not satisfied. For example, one of the reported simulations with $[S] = 4000$ nM had ~12% product-enzyme complexes, which clearly would reduce the $k_{cat.obs}$. An additional factor is the vacancy of lipid-binding sites adjacent to the enzyme, which occurs even when the substrate concentrations are very high and in turn results in enzyme vacancy, which was observed to be ~8% of the time at $[S] = 4000$ nM. The $k_{cat.obs}$ with the E-S model (Tables 5 and 6) agree relatively well with the theoretical or modified theoretical values when the reaction is slower ($T_{res} = 0.016$ s) and when the lipid diffusion is fast ($D_f/D_l = 10$). For only lipid source substrate E-S simulations (Table 5), the modified theoretical k_{cat} is not achieved for all residence times when the diffusion ratio, D_f/D_l, is 1000; the corresponding estimates are considerably higher when the enzyme is accessible to both fluid and lipid source substrate. Even with fluid substrate enzyme accessibility, very fast reactions cannot easily reach the theoretical k_{cat} when $D_f/D_l = 1000$, with $k_{cat.obs}$ less than half the theoretical maximum (Table 6). It is interesting to note that the basic E-S model Michaelis-Menten parameters change with T_{res} when surface diffusion is relatively very fast, but not for the diffusion ratio that is probably closer to the physiological state.

CONCLUSIONS

Our simulation model provides a complement to laboratory experiments designed to elucidate the mechanisms of specific surface-mediated enzymatic reactions. In general, our simulations confirm the observations of Axelrod and Wang (1994) that surface contributions to reactions are complicated and depend on many parameters. In particular, our simulations provide insight to the kinetics of factor X activation for two possible mechanisms for the assembly of the enzyme-substrate complex. We have shown that the models of unrestricted assembly of E-S complexes and of the formation of lipid-enzyme-substrate complexes requiring previous lipid surface binding of substrate are difficult to distinguish when the enzyme's intrinsic catalytic rate is not too fast. As the reaction becomes faster, the lipid-complex system does not reach the true k_{cat} of the enzyme as $[S]$ increases to "infinity." Instead, the maximum velocity is limited by the diffusion rate on the lipid and the rate at which product can leave the enzyme. These factors are mitigated by the saturation of surface-binding sites for substrate and product. This suggests that one way to distinguish the two models experimentally would be to decrease the lipid diffusion rate, or the substrate's affinity for the membrane, by changing the lipid composition or its ionic strength. Under these conditions, we would expect that the lipid-complex model have much lower $k_{cat.obs}$ and K_m than would be observed for the general E-S model. Other simu-

lations using a vesicle model showed that simply increasing the binding of substrate to the vesicle can decrease the apparent K_m of the reaction by only one-fourth, and thus cannot account for the magnitude of change observed experimentally for factor X when acidic phospholipids are used instead of neutral lipids. This may be a function of the fact that the *radius of capture* of an enzyme, under the conditions simulated in this paper, is apparently 60 nm and increases to only 90 nm when only bound substrate can form E-S complexes. Thus, it would appear that lipid binding, with its inherently more efficient diffusion, is not as important kinetically as other surface effects, such as orientation of the substrate or restriction of the rotational diffusion of the enzyme.

This work supported in part by grant OPG0009100, Natural Science and Engineering Research Council, Canada, and grant HL 29019 from National Institutes of Health.

REFERENCES

Adam, G., and M. Delbrück. 1968. Reduction of Dimensionality in Biological Diffusion Processes. *In Structural Chemistry and Molecular Biology.* A. Rich, and N. Davidson, Editors. Freeman, San Francisco, CA. 198–215.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1983. *Molecular Biology of the Cell.* Garland Publishing Co., New York. 281 pp.

Axelrod, D. and M. D. Wang. 1994. Reduction-of-Dimensionality Kinetics at Reaction-Limited Cell Surface Receptors. *Biophys. J.* 66:588–600.

Berg, H. C. 1983. Random Walks in Biology. Princeton University Press, Princeton, New Jersey. 142 pp.

Berg, O. G., and P. H. von Hippel. 1985. Diffusion-Controlled Macromolecular Interactions. *Ann. Rev. Biophys. Chem.* 14:131–160.

Britton, N. F. 1986. Reaction-Diffusion Equations and Their Applications to Biology. Academic Press Inc., Orlando, Florida. 277 pp.

Fife, P. C. 1979. Mathematical Aspects of Reacting and Diffusing Systems. (Series Editor: S. Levin. Lecture Notes in Biomathematics, Vol. 28.) Springer-Verlag, Berlin. 185 pp.

Forman, S. D. 1986. A Unique Role for Acidic Phospholipids in the Tissue Factor Pathway of Blood Coagulation. Ph. D. *Thesis*, The City University of New York. 101 pp.

Forman, S. D., and Y. Nemerson. 1986. Membrane-Dependent Coagulation Reaction Is Independent of the Concentration of Phospholipid-Bound Substrate: Fluid phase factor X regulates the extrinsic system. *Proc. Natl. Sci. USA. Biochemistry.* 83:4675–4679.

Gentry, R. D., L. Ye, and Y. Nemerson. 1995. A Microscopic Model of Enzyme Kinetics. *Biophys. J.* Companion paper submitted for publication MS# B50071.

Giesen, P. L. A., G. M. Willems, and W. T. Hermens. 1991. Production of Thrombin by the Prothrombinase Complex Is Regulated by Membrane-mediated Transport of Prothrombin. *J. Biol. Chem.* 266:1379–1382.

Goldstein, B. 1989. Diffusion Limited Effects of Receptor Clustering. *Comments Theoretical Biology* 1:109–127.

Hardt, S. L. 1979. Rates of Diffusion Controlled Reactions in One, Two and Three Dimensions. *Biophys. Chem.* 10:239–243.

Hermens, W. T., J. M. M. Kop, and G. M. Willems. 1988. Adsorption of Coagulation Factors II, V, and X at Phospholipid Membranes. Chap. 4, *In Coagulation and Lipids.* R. F. A. Zwaal, Editor. CRC Press, Inc., Boca Raton, Florida. 73–97.

Higgins, D. L., P. J. Callahan, F. G. Prendergast, M. E. Nesheim, and K. G. Mann. 1985. Lipid Mobility in the Assembly and Expression of the Activity of the Prothrombinase Complex. *J. Biol. Chem.* 260: 3604–3612.

Karlin, S. 1975. A First Course in Stochastic Processes. Second ed. Academic Press, New York.

Krieg, U. C., B. S. Isaacs, S. S. Yemul, C. T. Esmon, H. Bayley, and A. E. Johnson. 1987. Interaction of Blood Coagulation Factor Va with Phospholipid Vesicles Examined by Using Lipophilic Photoreagents. *Biochemistry.* 26:103–109.

Krishnaswamy, S., K. C. Jones, and K. G. Mann. 1988. Prothrombinase Complex Assembly; Kinetic Mechanism of Enzyme Assembly on Phospholipid Vesicles. *J. Biol. Chem.* 263:3823–3834.

Loeb, A. L. 1976. Space Structures: Their Harmony and Counterpoint. Addison-Wesley Publishing Company, Inc., Advanced Book Program, Reading, Massachusetts. 169 pp.

Mann, K. G., R. J. Jenny, and S. Krishnaswamy. 1988. Cofactor Protein in the Assembly and Expression of Blood Clotting Enzyme Complexes. *Ann. Rev. Biochem.* 57:915–956.

Murray, J. D. 1977. Reduction of Dimensionality in Diffusion Processes: Antenna Receptors of Moths. Chap. 3, In Lectures on Nonlinear-Differential-Equation Models in Biology. J. D. Murray, Editor. Clarendon Press, Oxford. 83–127.

Nemerson, Y. 1988. Tissue Factor and Hemostasis. *Blood* 71 (1):1–8.

Nemerson, Y. and R. Gentry. 1986. An Ordered Addition, Essential Activation Model of the Tissue Factor Pathway of Coagulation: Evidence for a Conformational Cage. *Biochemistry* 25(14):4020–4033.

Nesheim, M. E., R. P. Tracy, and K. G. Mann. 1984. "Clotspeed," a Mathematical Simulation of the Functional Properties of Prothrombinase. *J. Biol. Chem.* 259:1447–1453.

Somogyi, B., and S. Damjanovich. 1975. Relationship Between the Lifetime of an Enzyme-Substrate Complex and the Properties of the Molecular Environment. *J. Theor. Biol.* 51:393–401.

Somogyi, B., F. E. Karasz, L. Tron, and P. R. Couchman. 1978. The Effect of Viscosity on the Apparent Decomposition Rate of Enzyme-Ligand Complexes. *J. Theor. Biol.* 74:209–216.

Wang, D., S-Y. Gou, and D. Axelrod. 1992. Reaction rate enhancement by surface diffusion of adsorbates. *Biophys. Chem.* 43:117–137.

Waxman, E., J. B. Ross, T. M. Laue, A. Guha, S. V. Thiruvikraman, T. C. Lin, W. H. Konigsberg, and Y. Nemerson. 1992. Tissue factor and its extracellular soluble domain: the relationship between intermolecular association with factor VIIa and enzymatic activity of the complex. *Biochemistry.* 31(16): 3998–4003.

Zwaal, R. F. A. 1978. Membrane and lipid involvement in blood coagulation. *Biochim. Biophys. Acta.* 515:163–205.