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ORIGINAL ARTICLE

The limits of simulation of the clotting system

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Summary. *Objective:* To investigate in how far successful simulation of a thrombin generation (TG) curve gives information about the underlying biochemical reaction mechanism. *Results:* The large majority of TG curves do not contain more information than can be expressed by four parameters. A limited kinetic mechanism of six reactions, comprising proteolytic activation of factor (F) X and FII, feedback activation of FV, a cofactor function of FVa and thrombin inactivation by antithrombin can simulate any TG curve in a number of different ways. The information content of a TG curve is thus much smaller than the information required to describe a physiologically realistic reaction scheme of TG Consequently, much of the input information is irrelevant for the output. FVIII deficiency or activation of protein C can, for example, be simulated by a reaction mechanism in which these factors do not occur. *Conclusion:* A model that comprises not more than six reactions can simulate the same TG curve in a number of possible ways. The possibilities increase exponentially as the model grows more realistic. Successful simulation of experimental data therefore does not validate the underlying assumptions. *A fortiori*, simulation that is not checked against experimental data lacks any probative force. Simulation can be of use, however, to detect mistaken hypotheses and for parameter estimation in systems with fewer than five free parameters.

Keywords: mathematical simulation, model discrimination, thrombin generation.

Introduction

One of the traditional approaches to the study of the coagulation system is monitoring the course of activated factors in clotting blood or plasma [1]. Notably the rise and fall of thrombin in clotting blood, that is, the thrombin generation (TG) curve is of renewed interest [2,3]. Computer simulation can be used to link observed time courses to the underlying reaction mechanism [4,5].

After developing suitable numerical methods [6], we introduced simulation as a tool in the analysis of the web of coagulation reactions in 1991. We obtained near-perfect similarities between simulated reaction mechanisms and the outcome of real experiments [4]. Nevertheless, we have published sparingly on this subject because we soon realized that it is possible to postulate a large variety of mechanisms *all* of which can explain the same experimental results. This is not surprising because the information required as an input for a plausible reaction mechanism of TG is much larger than the information that characterizes the output. Consequently, much information must be irrelevant in the process of simulation and variations in such irrelevant information cannot be seen in the outcome.

In enzyme kinetics (see e.g. [7]) even the most simple model ($E + S \leftrightarrow C \rightarrow E + P$) contains three reaction constants, whereas only two experimental parameters, K_m and k_{cat}, can be obtained from initial rate measurements. When simulating this system one can postulate any value for the backward constant (even zero) as long as the three constants together lead to the experimentally observed values of K_m and k_{cat}. It also is standard knowledge that, on basis of K_m and k_{cat}, one cannot distinguish between this simple model and a large variety of more complicated ones; that is, it makes no sense to postulate more reactions than necessary to explain the experimental findings.

In the case of the coagulation reactions this leads to the question: if we include the available pre-existing knowledge, do we postulate more reactions and parameters than necessary to explain the TG curve? If yes, the excess information does not contribute to the outcome and can be arbitrarily varied.

If a simulated curve does not mimic the experimental data within the limits of experimental error, one can be sure that the postulated mechanism and/or constants are not correct. The inverse is not true however; a fitting simulation does not validate the underlying assumptions. As soon as more information is entered in the simulation than required to define the TG curve it becomes possible that alternative

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mechanisms or constants could simulate the data equally well. The larger the number of postulated reaction constants compared to the number of parameters in the experimental output, the more degrees of freedom and the less probative force of a fitting simulation.

The recent increase in the use of numerical simulation ('experiments *in silico*', e.g. [8–18]) has led us to assess this global reasoning quantitatively. To this end, we first show that four independent parameters can be experimentally obtained from a TG curve. Then we show that a web of only six reactions simulate any TG curve. Any plasmatic clotting mechanism is much larger. Consequently unequivocal attribution of one set of plausible concentrations and reaction constants to one experimental TG curve obtained in plasma is impossible.

Materials and methods

Platelet poor pooled plasma (PPP) was prepared as described previously [19] from blood taken on 0.1 volume of 0.13 M sodium citrate from at least 12 apparently healthy donors. It was stored at −80 °C for < 3 months. As a source of recombinant tissue factor (rTF) we used Innovin (Dade-Behring, Marburg, Germany). Procoagulant phospholipids containing 60 mole% dioleoyl PC, 20 mole% dioleoyl PS and 20 mole% dioleoyl PE were prepared as described earlier [20]. Thrombomodulin (TM) was soluble recombinant TM, a gift of Asahi, Japan. Activated protein C (APC) was a gift of Regnault (INSERM unit 284, Nancy, France), prepared according to Regnault *et al.* [21]. The samples of hemophiliac plasma after infusion of a factor (F) VIII concentrate were donated by H. M. Van den Berg, Van Creveld kliniek, Utrecht, the Netherlands. TG curves were obtained via calibrated automated thrombinography as described in detail previously [22]. Briefly, the reaction mixtures (120 μL) contained in all cases citrated PPP diluted 2:3, 5 pM rTF, 4 μM PL, 16.7 mM $CaCl_2$, 417 μM Z-GGR-AMC and, where indicated, 6 nM APC or 6 nM TM. Each experiment was carried out in 16 replicates.

Average curves and limits of experimental accuracy

To obtain an average curve from a number of replicate curves, we first measured the lag time of each individual curve, arbitrarily defined as the moment that the thrombin concentration stayed above the 10 nM level, and determined the average lag-time (t_{lag}) and its standard deviation (SD). Then we lined up the curves at the average lag time and determined the average thrombin concentration (T_t) at each time point (t) and its SD. Around every experimental point of the average curve, the SDs along the time and the concentration axis were plotted. We considered a fit acceptable if the simulated thrombin concentration did not at any time-point differ more than 1 SD from the average T_t. To visualize the goodness of fit of simulated curves we plotted the difference between the observed and the calculated values (the residuals) and compared it to the SD (Figs 1 and 4, lower panels).

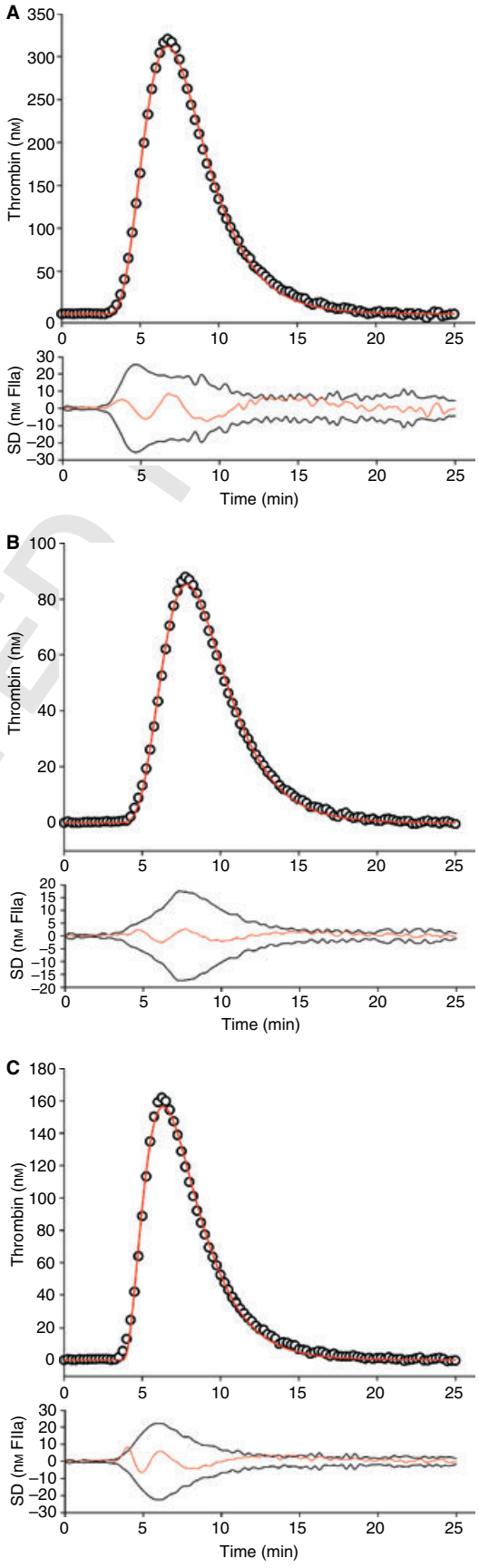

Fig. 1. Fitting of the W function to thrombin generation (TG) curves. Upper panels: the mean course of thrombin concentration (open circles, $n = 16$) and the ± 1 SD confidence area (black lines, see Materials and methods). Red line: fit of the experimental data by the *W*-function. Lower panels: $\pm$SD per time point (black lines) and the difference between the fitted and the experimental curves (red line). From left to right: NPP without any additions ($t_0 = 2.474$, $a = 1657$, $b = 0.478$ and $c = 7.69$); aPC added ($t_0 = 2.688$, $a = 465$, $b = 0.477$ $c = 11.6$); TM added ($t_0 = 3.740$, $a = 735$, $b = 0.497$ and $c = 3.631$).

Mathematical procedures

In the experimental section we define the W-function, an explicit four-parameter function that fits to experimental TG curves. The procedure for fitting a function to experimental data is incorporated in a number of commercially available programs. We used that available from SIGMAPLOT or self-written software. The mathematical background of the fitting [23–25] is given in the annexe. There we also show the mathematical method to determine the maximal number of parameters that can be reliably computed from a set of experimental data. If more than this maximal number of parameters is used for optimization, the model starts to fit to arbitrary experimental noise. The additional freedom introduced by adding an extra parameter cannot be used to decrease the discrepancy between model and experiments below the level that is inevitably determined by the experimental error. In fact, the additional freedom would introduce manifold possible models of indistinguishable validity.

Results and discussion

The information content of the TG curve; the W-function

We determined TG curves in 16 replicates for normal PPP in the presence and absence of APC or TM, and calculated the average curve and the SEM in every point (Fig. 1, upper panels). These curves could be fitted to an explicit function with four parameters:

$$T = W(a, b, c, t_0, t) = abc \times e^{-bc(t-t0)} \times (e^{b(t-t0)} - 1)^{(c-1)}$$

(where T = thrombin concentration, t is time and t_0 a, b, c are constants [> 0]).

This function, called the W-function, could fit any experimental TG curve that we tested within the limits of experimental error. Not only the curves of Fig. 1 but also a series of curves in which one of FII, FV, FVII, FX and FXI was present in limiting concentration [26] (results not shown) and curves obtained at 10 time points after injection of a dose of 50 IU kg^{-1} of FVIII in a hemophilic patient (see further [27]). In Fig. 2, four of these curves are shown, the other time points, and a similar series in another hemophilic patient showed similar results.

The W-function does not reflect an underlying model of chemical reaction, as does, for example, the hyperbola of classical enzyme kinetics. It remains perfectly possible that TG curves exist that cannot be fitted to the W-function. We found such curves, for example, when measuring TG in platelet rich plasma. They could always be fitted to the sum of two W-functions, that is, with eight parameters (results not shown). For the development of our argument, it does not matter whether four or eight parameters describe the TG curve, as both figures are considerably smaller than the number of parameters (concentrations and constants) that describe the chemistry of TG.

Computation of the sensitivity matrix according to Golub and Ortega, and Walter and Pronzato [24,25], applied to the data of Figs 1 and 2, showed that the singular values of the four parameters varied by three orders of magnitude (e.g. 8054, 543, 182, and 21). The first value is 400-fold as big as the last, which indicates that a random deviation of the data that is so small as to cause a 0.1% variation in the best determined parameter can

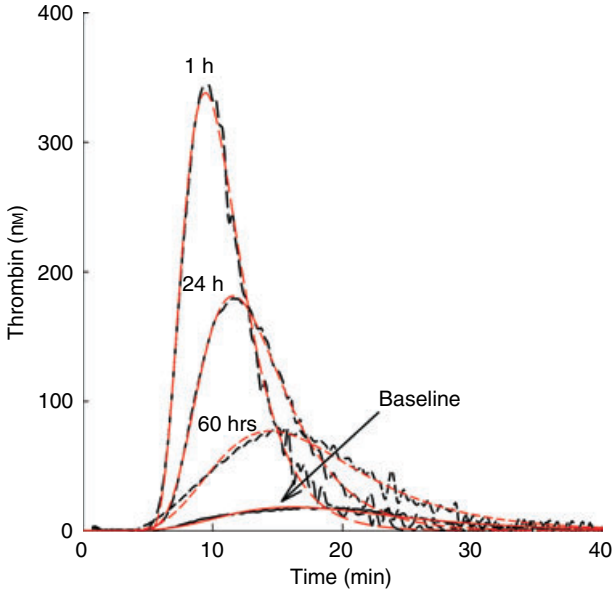

Fig. 2. Fitting of the *W*-function to TG curves in hemophilia. 50 IU of factor VIII (FVIII) per kg body weight were injected into a severe hemophilic patient [see 27] and TG curves were determined at $t = 0$, 0.25, 0.5, 1, 3, 5, 7, 12, 24, 30, 48 and 60 h. For clarity, only the $t = 0$, 1, 24 and 60 h curves are shown. Black lines: measured data, red lines fitted curves. Similar fits were obtained for the curves not shown.

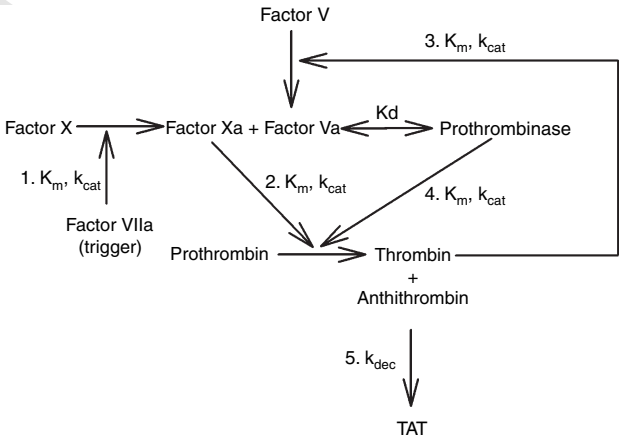

Fig. 3. A minimal model for TG in plasma.

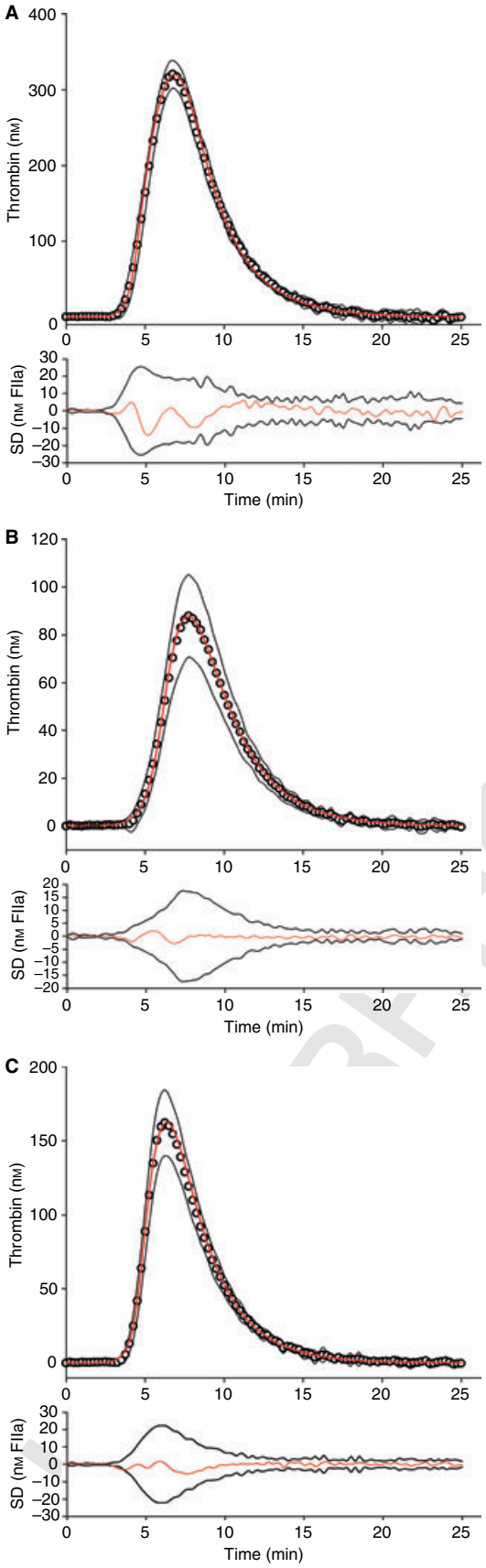

Fig. 4. Simulation of TG-curves with the minimal reaction mechanism. Upper panels: the mean course of thrombin concentration (open circles, $n = 16$) and the ± 1 SD confidence area (black lines, see Materials and methods). Red line: fit of the experimental data by the *W*-function. Lower panels: $\pm$SD per time point (black lines) and the difference between the fitted and the experimental curves (red line). From left to right: NPP without any additions ($t_0 = 2.474$, $a = 1657$, $b = 0.478$ and $c = 7.69$); aPC added ($t_0 = 2.688$, $a = 465$, $b = 0.477$ $c = 11.6$); TM added ($t_0 = 3.740$, $a = 735$, $b = 0.497$ and $c = 3.631$). Three sets of parameters were used to obtain the fit. The fixed parameters were: Trigger: 15 pM; Factor X (FX) activation by trigger: $S = 30$ nM, $K_m = 10$ nM, $k_{cat} = 4.5$ s^{-1}; Prothrombin activation by Xa: $K_m = 5$ nM; Factor V (FV) activation by thrombin: $K_m = 40$ nM; K_d of prothrombinase complex (PTase): 60 pM; Prothrombin activation by PTase: K_m: 450 nM; Thrombin inactivation by AT: AT = 2.5 μM. Variable reaction parameters:

	Left	Middle	Right
FV (μM)	4	5.5	5
Prothrombin (μM)	1.25	0.276	0.5
k_{cat} FII by Xa (s^{-1})	0.002	0.0023	0.0014
k_{cat} FV by FIIa (s^{-1})	0.018	0.005	0.04
k_{cat} FII by PTase (s^{-1})	6.2	8	6.2
k_{cat} FIIa inact. by AT $(M.s)^{-1}$	14260	7843	9270

Because in practice the fluorogenic substrate Z-GGR-AMC is present, its effect on the TG is taken into account. The used K_m was 440 μM and the k_{cat} 1.33 s^{-1}.

also be accommodated by a 40% change in the least well determined one.

The minimal mechanism

The link between the experimental curves and a reaction mechanism cannot be calculated analytically and must be approached by numerical simulation. As discussed in the introduction, the limits of meaningful simulation are set by the smallest chemical mechanism that can fit a TG curve. This appeared to be a set of six reactions (Fig. 3), quantitatively defined by the initial concentration of five proteins, eight kinetic constants and a decay constant, that is, by 14 parameters. Upon simulation, the reaction scheme of Fig. 3 can fit the TG curves of Figs 1 and 2 as well as any other TG curve that we tried (Figs 4 and 5). Readers can find a program at http://www.thrombin.com to try this for themselves. Fig. 4 shows that the effects of added APC and TM could be simulated without introducing APC- or TM-dependent reactions. In Fig. 5 it is seen that variation of FVIII is readily simulated by a reaction scheme, in which FVIII does not play a role (Fig. 5). Minor adaptations in the reaction constants of the FV activation reaction and the k_{cat} of the prothrombinase reaction, together with large changes in the prothrombin concentration, are sufficient to fit the experimental curves. It should be noted that we purposely changed the prothrombin concentration to values that we know to be false, in order to stress the fact that near to perfect simulation still does not prove the underlying assumptions to be correct.

Not only known reactants such as FVIII, FIX and TFPI are missing from this scheme, but also phospholipid-protein interactions are not included. It is simply assumed that FXa

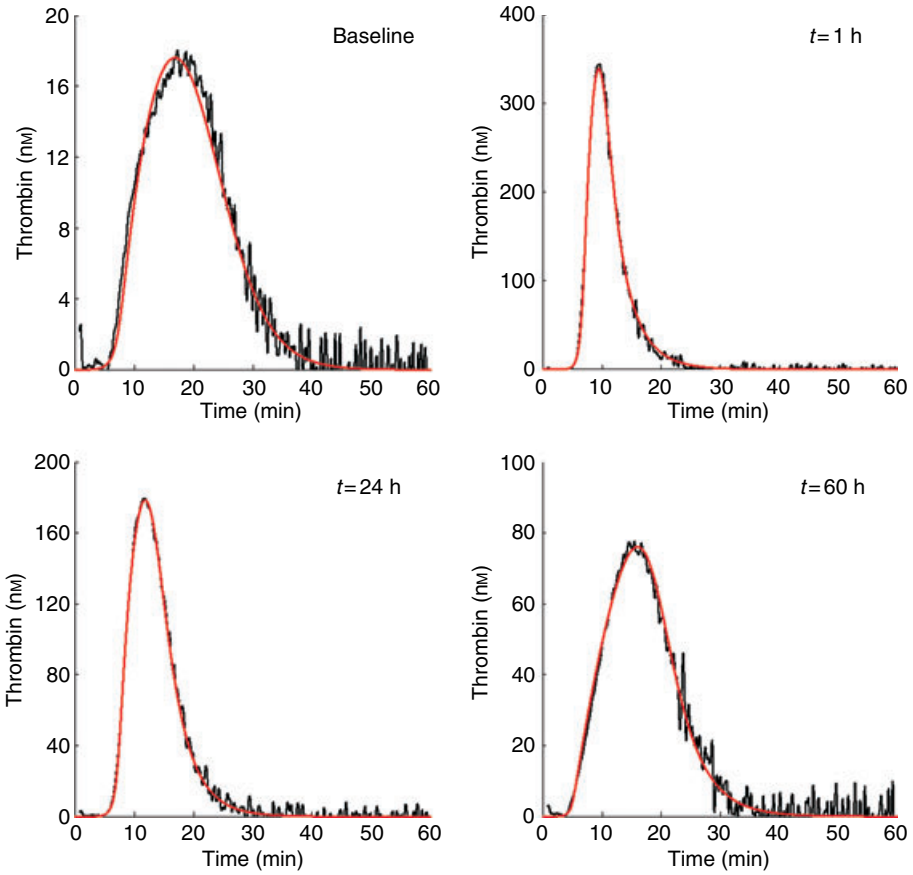

Fig. 5. Simulation of TG curves in severe hemophilia by the minimal reaction mechanism. 50 IU of FVIII per kg body weight were injected into a severe hemophilic patient [see 27] and TG curves were determined at t = 0, 0.25, 0.5, 1, 3, 5, 7, 12, 24, 30, 48, and 60 h. For clarity, only the t = 0, 1, 24 and 60 h curves are shown. Black lines: measured data, red lines fitted curves. Similar fits were obtained for the curves not shown. The fixed parameters were: Trigger: 10 pM; FX activation by trigger: S = 10.5 nM, K_m = 10 nM, k_{cat} = 4.5 s^{-1}; Prothrombin activation by Xa: S: K_m = 5 nM, k_{cat} = 0.0005 s^{-1}; K_d of prothrombinase complex = 60 pM; FV activation by thrombin: S: 5 nM, K_m = 40 nM. The variable parameters are given in the table below. Also in this case the effect of the fluorogenic substrate was taken into account.
Variable reaction parameters:

	Top left	Top right	Bottom left	Bottom right
Prothrombin (μM)	0.12	1.37	0.85	0.42
Antithrombin (μM)	1.8	2.2	1.8	1.8
k_{cat} Factor (F) Va by FIIa (s^{-1})	0.12	0.0055	0.012	0.09
K_m PTase (nM)	95	450	300	95
k_{cat} PTase (s^{-1})	0.2	10	3.1	0.5
k_{cat} FIIa inact. by AT (s^{-1})	7490	14260	12840	7490

and FVa form a prothrombinase complex (K_d 60 pM) that has a lower K_m and a higher k_{cat} than FXa alone. This shows that in a realistic reaction scheme there is much more information than needed to simulate a TG curve. In reality, the number of reactions in the mechanism is at least 24, and needs at least 54 parameters (concentrations and kinetic constants) [14].

Does the minimal reaction mechanism allow one or more solutions?

The next question is whether the fits obtained with the oversimplified reaction scheme are unique. Figure 6 shows two curves that are indistinguishable from each other and yet are obtained by different sets of parameters. A multiplicity of such sets is easily found. It suffices to replace one of the parameters by an arbitrary value of the same order of magnitude and adapt the others always to obtain a fitting curve. This shows that a curve, indistinguishable from an experimental result, can be obtained in a variety of ways, and that even the simplest possible reaction scheme could not be used to obtain an unequivocal simulation of a real TG curve.

We conclude that the technique of simulation is unable to distinguish between a large number of different possibilities.

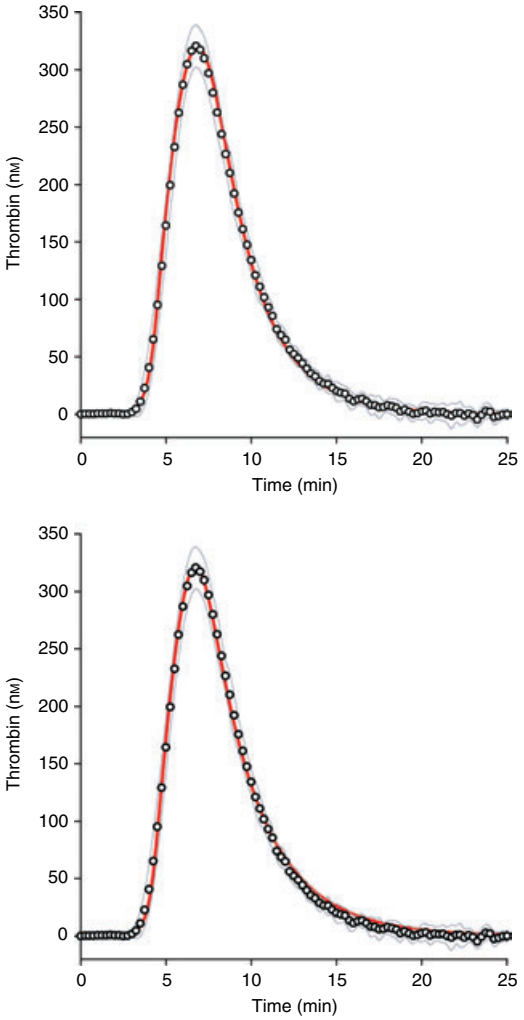

Fig. 6. Simulation of the TG curve with the minimal reaction mechanism and two sets of constants. To the experimental data of the mean TG curve of Fig. 1, first panel, two simulated TG curves were fitted using the minimal model with two different sets of constants. Fixed reaction parameters: FX activation by trigger: $S = 30$ nM, $K_m = 10$ nM, $k_{cat} = 4.5$ s^{-1}; Prothrombin activation by Xa: $K_m = 5$ nM, $k_{cat} = 0.002$ s^{-1}; FV activation by thrombin: $K_m = 40$ nM.
Variable reaction parameters:

	Left	Right
Trigger (nM)	0.015	0.0085
Factor (F) V (nM)	5	80
k_{cat} FV activation by FIIa (s^{-1})	0.018	0.0011
Prothrombin (μM)	1.35	0.95
K_m PTase (nM)	450	250
k_{cat}PTase (s^{-1})	6.2	8.0
Antithrombin (μM)	2.5	2
k_{dec} thrombin inactivation by AT $(M.s)^{-1}$	14260	16830

Among these there will be many, such as our minimal scheme, that can be discarded on the basis of existing scientific evidence. If, however, among the realistic possibilities there are two or more that are equally likely, simulation will not be able to identify the correct one.

The introduction of parameters from other experiments

One can decrease the number of possible interpretations by introducing parameters that are independently obtained ('external parameters'). The initial concentrations of, for example, all the clotting factors can be readily determined. In experiments where a reaction sequence is reconstituted from isolated factors one can determine the reaction constants in independent experiments under conditions closely similar to those of the TG experiment [28]. Such systems have the additional advantage that one can be sure about the reaction mechanism and that no fibrin is formed, so that diffusional transport will not play a role (see below).

In plasma the decay constant of thrombin can be estimated directly [29] but others, such as, for example, the kinetic constants of prothrombinase cannot be determined under the conditions prevailing in plasma. Values obtained in purified systems [20,30–32] differ up to 100-fold and it remains unknown which, if any, apply in plasma, because kinetic 'constants' are strongly dependent upon the reaction conditions.

In plasma, not only the reaction constants but even the reaction mechanism itself is not known with certainty. The anticoagulant role of FV and the direct inhibitory role of protein S or the role of protein Z are examples of complications that went unrecognized until quite recently [33,34]. The precise role of 'minor players' such as β_2-glycoprotein 1 [35], annexin V [36,37] and other phospholipid binding proteins remains open, and it seems probable that among the 1175 known plasma proteins [38] one or more could play an as yet unrecognized role. A further complication is that in the presence of fibrin, the reaction velocity is co-determined by diffusion and therefore not adequately described by chemical processes alone [39].

In view of these uncertainties concerning: (i) the reaction constants; (ii) the reaction mechanism; and (iii) the role of diffusional transport, one cannot claim *a priori*, the validity of the assumptions on which a simulation is based. Neither can one prove it *a posteriori* because, as we have seen above, a successful simulation can easily result from incorrect assumptions. The trap to be avoided here is the circular argument in which the acceptability of the simulated curve is thought to follow from the correctness of the assumed reaction mechanism and parameters, and the correctness of the assumptions from the acceptability of the fit.

In the present state of knowledge no simulation is better than the conjectures about the mechanism and the parameters [9,10]. In view of the reasonable doubt about the one as well as about the other, it seems wiser not to use simulation for clinical diagnosis and epidemiology but rather rely on measurement of TG [3,40].

Simulation of a system as complicated as plasmatic thrombin formation cannot be proven to be correct but can be proven to be false when it does not to fit to experimental data. In the literature (e.g. [8–18]) one occasionally encounters simulations

that are not quantitatively compared to experimental data. Such simulations can neither be verified nor disproved ('falsified') and therefore do not allow scientifically valid conclusions [41]. When the restraint of comparison to experimental observations is abandoned, simulation degenerates into an exercise with the reality content of every other computer game.

Requirements for successful use of simulation techniques

To escape the ambiguities of the simulation of large systems in plasma, one has to reduce the number of unknown parameters to a minimum without introducing parameters from incomparable experiments. In the first place one can restrict the reaction mechanism to a size that is amenable to simulation with a minimal degree of uncertainty; for example by triggering TG in plasma with the FX activating enzyme of Russell's viper venom [42]. The initial clotting factor concentrations can be determined independently as well as the kinetic constants of thrombin decay [29]. This reduces the number of free parameters to nine. With the aid of parameter fitting programs the full variety of solutions can be found [43]. Then experiments can be repeated with varying concentrations of one clotting factor (e.g. prothrombin) and from the possible solutions those can be selected in which the reaction constants do not vary as the factor concentration changes. Once such a small system is quantitatively well defined, a stepwise increase of complexity may in the end lead to exact simulation of the complete system. We recall, however, that our conclusions pertain to homogeneous closed systems, that is, plasma in a test tube. Open, non-homogeneous systems such as TG *in vivo* are much more complicated.

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Appendix: mathematical procedures

The mathematical technique behind the fitting procedure is the following: One starts with an arbitrary guess of the best parameters (the vector $\mathbf{P}_0$,), that is, in our case the four parameters of the W function (see below). Then a correction vector is calculated by solution of the linear system of equations $(\mathbf{J}(\mathbf{p})^T\mathbf{J}(\mathbf{p}))\Delta\mathbf{P} = \mathbf{J}(\mathbf{p})^T\ \mathbf{r}$, where $\mathbf{r}$ is the residue, that is, the difference between the measured and the calculated value at each time point. The new and better estimate for the parameters then is $\mathbf{P}_0 + \Delta\mathbf{P}$. This procedure is repeated until convergence. The goodness of fit is expressed by

$$s = \mathrm{sqr}(_{t=1}\sum^{N}((r_i(p))^2)/N).$$

This value should be of the order of the experimental error. If it is larger, the fit is not good enough, if it is much smaller, the fit determines parameters that cannot be attributed a meaning and thus are unnecessary.

To understand the computation of the minimal number of meaningful parameters, some knowledge of multivariate statistics is needed, as can be found e.g. in Anderson [23]. The technique itself is based on References [24] and [25].

Denoting the model by W ($\mathbf{p}$, t), where t (time) is the independent variable and the vector $\mathbf{p}$ contains the M available parameters, the sensitivity matrix $\mathbf{J}(\mathbf{p})$ is defined as

$$J(p) = (\mathrm{J(p)})_{\mathrm{i,j}} = (\delta W(p_j, t_i)/\delta p_j).$$

Here t_1, t_2, ……, t_N are the values of the independent variable for which the measurements were made. Clearly **J(p)** is an $N \times$ M-matrix with $N > M$, because more measurements should be available than free parameters in the model.

The size of the singular values of the matrix **J(p)** determines the number of free parameters that can be computed from the available measurements. The singular values $\sigma_1 \geq \sigma_2 \geq \ \ldots \geq 0$ are the square roots of the eigenvalues of the $M \times$ M-matrix $\mathbf{J(p)}^T\mathbf{J(p)}$.

The number of parameters that can be determined from the available data is equal to the number of singular values that is larger than $\sigma_1\tau$, where 100 τ is the percentage of the experimental error in the measurements.

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