A non-linear reaction scheme of blood coagulation

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A NON-LINEAR REACTION SCHEME OF BLOOD COAGULATION

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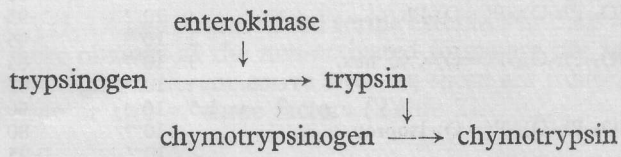
The basic mechanism of coagulation is an enzyme cascade as recognised by MACFARLANE and DAVIES and RATNOFF about 10 years ago (1, 15).

A model of a cascade can easily be constructed. When a small amount of enterokinase is added to a mixture of trypsinogen and chymotrypsinogen, the enterokinase will convert the trypsinogen into trypsin and the trypsin will convert the chymotrypsinogen into chymotrypsin:



There are few reasons why this example would not actually work in practice, but we will come to this later.

Originally it was thought that all coagulation factors were proenzymes, which would be converted into enzymes. This resulted in the well known classical cascade scheme:

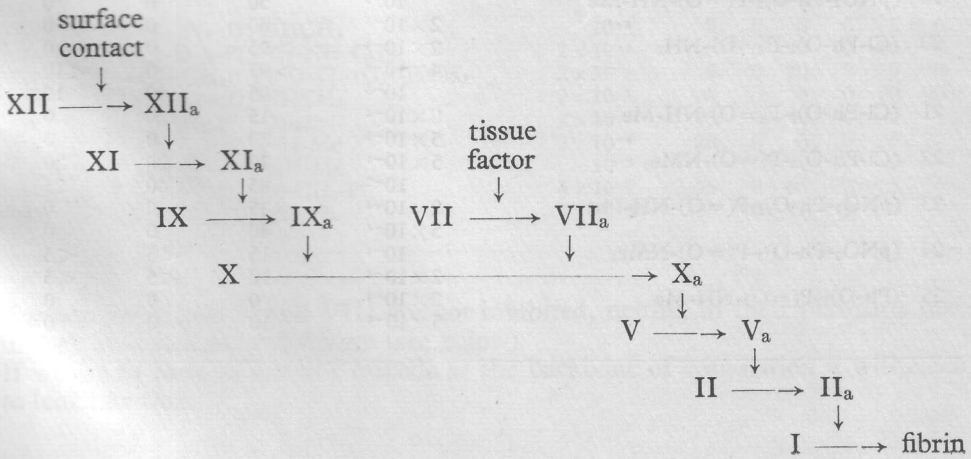


TABLE 1

Inhibition of coagulation factors IIa, VIIa and Xa by various serine esterase inhibitors (from ref. 13)

No.	Structure	Molar concentration	Inhibition (%) of factor II_a	VII_a	X_a
1	*p*Cl-Ph-O-C(=O)-NH-Me	2×10^{-4}	17	0	20
2	Ph-O-C(=O)-NH-Me	2×10^{-4}	0	0	20
		5×10^{-4}	0	0	25
3	Ph-O-C(=O)-NH-Et	5×10^{-4}	5	0	15
		10^{-3}	30	20	20
4	Ph-O-C(=O)-NH-Pr	5×10^{-4}	10	0	10
		10^{-3}	20	10	20
5	*p*Cl-Ph-O-C(=O)-NH-Ph	10^{-4}	20	10	<5
		2×10^{-4}	30	15	<5
6	*p*Me-Ph-O-C(=O)-NH-Me	10^{-4}	20	5	<5
		2×10^{-4}	30	5	<5
7	$o(NO_2)$-Ph-O-C(=O)-N-Me_2	4×10^{-4}	0	0	0
8	$o(Me_3)$-N-Ph-O-C(=O)-NH-Me	2×10^{-4}	0	0	0
9	$m(NH_2)$-Ph-O-C(=O)-N$(CH_3)_2$	2×10^{-3}	35	0	0
10	$m(NO_2)$-Ph-O-C(=O)-N$(CH_3)_2$	4×10^{-4}	25	0	10
11	$m(Me_2$-CH$(CH_3))$ Ph-O-C(=O)NHCH_3	2×10^{-4}	5	0	0
12	Ph_2-N-C(=O)-O-Ph-$o(NO_2)p$COOH	2×10^{-4}	45	30	0
		4×10^{-4}	70	33	0
		10^{-3}	80	35	0
13	$(p(NO_2)$-Ph-O$)_2$-P(=O)-Ph	10^{-5}	>95	30	0
		2×10^{-5}	>95	55	15
14	$(p(NO_2)$-Ph-O$)_2$-P(=O)-Cyc. hex.	5×10^{-5}	65	0	0
		10^{-4}	65	0	0
		1.5×10^{-4}	90	0	5
15	$(p(NO_2)$-Ph-O$)_2$-P(=O)-Isoprop.	10^{-5}	80	<5	<10
		2×10^{-5}	>95	10	<10
		4×10^{-5}	>95	20	<10
16	$(p(NO_2)$-Ph-O$)_2$-P(=O)-eth.	10^{-5}	>95	25	0
		2×10^{-5}	>95	30	0
17	$(p(NO_2)$-Ph-O$)_2$-P(=O)-CH_2-Phe	2×10^{-5}	>95	>65	40
		5×10^{-5}	>95	>65	50
18	$(p(NO_2)$-Ph-O$)_2$-P(=O)-O-Ph	4×10^{-5}	>95	30	5
		10^{-4}	>95	40	<10
19	$(p(NO_2)$-Ph-O$)_2$-P(=O)-NH-Me	10^{-5}	50	0	0
		2×10^{-5}	60	0	0
20	(Cl-Ph-O$)_2$-P(=O)-NH_2	2×10^{-4}	>95	0	0
		4×10^{-4}	>95	0	<10
		10^{-3}	>95	40	10
21	(Cl-Ph-O$)_2$-P(=O)-NH-Me	2×10^{-4}	15	0	0
		5×10^{-4}	20	0	0
22	(Cl-Ph-O$)_2$-P(=O)-NMe_2	5×10^{-4}	70	20	20
		10^{-3}	85	40	25
23	$(p(NO_2)$-Ph-O$)_2$-P(=O)-NH-Phe	2×10^{-4}	35	0	0
		5×10^{-4}	40	0	0
24	$(p(NO_2)$-Ph-O$)_2$-P(=O)-NMe_2	10^{-4}	15	<5	<5
		2×10^{-4}	10	<5	<5
25	(Ph-O$)_2$-P(=O)-NH-Me	2×10^{-4}	0	0	0
		5×10^{-4}	10	0	0

Later it was recognised that the factors V and VIII probably are not proenzymes. These two factors have some properties in common, among them the fact that it was impossible to find any enzymatic action in them.

On the other hand the factors II, VII, IX and X also have many properties in common:

1. The physical properties are very much alike. This makes them difficult to separate. Until quite recently, it depended upon the geografic location whether one considered them as one protein or as four different ones.
2. All four are dependent upon vitamin K.
3. All four appear to be proenzymes of serine proteases. For factor II this has been proven beyond reasonable doubt by MAGNUSSON (2) who determined the primary structure of thrombin. For factor X it has been proven by ESNOUF and LEVESON (14) and TITANI et al. (21). For the factors VII and IX however, this is less certain, as no data on the primary structure of these factors exist.

In order to determine the (pro)serine-esterase character of these factors we took a large series (78) of serine-esterase inhibitors of different types, phosphate, carbamates etc. and determined what was their inhibiting action upon the different coagulation factors (13). The purified activated factors were incubated with the inhibition under standardised conditions, and the residual activity was determined after a certain time. Some results are shown in Table 1.

From these and similar experiments we drew the conclusions that:

— The four factors II, VII, IX and X are indeed serine esterases in their activated form. This is the more obvious as the non-activated forms are not inhibited.
— The four factors must have different active sites, as there are inhibitors that specifically inhibit one or two or three factors (Table 2).

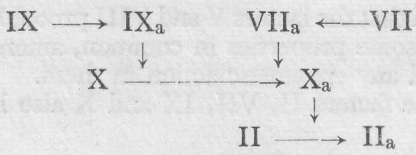
TABLE 2

Specific inhibition of various coagulation factors by selected serine esterase inhibitors (from ref. 13).

No.	Structure	Molar conc.	II_a	V	VII_a	VIII	IX_a	X_a
21	$(Cl\text{-}Ph\text{-}O)_2\text{-}P(=O)\text{-}NHCH_3$	10^{-4}	0	0	0	0	3	0
19	$(pNO_2\text{-}Ph\text{-}O\text{-})_2\text{-}P(=O)\text{-}NH\text{-}CH_3$	2×10^{-5}	60	0	0	0	10	0
11	$m(Me_2\text{-}CH(CH_3)).Ph\text{-}O\text{-}C(=O)NHCH_3$	2×10^{-4}	5	0	20	0	0	0
26	$CH_3\text{-}Ph\text{-}O\text{-}C(=O)\text{-}NHCH_3$	2×10^{-4}	0	0	0	0	20	20
16	$(pNO_2\text{-}Ph\text{-}O)_2\text{-}P(=O)\text{-eth}$	2×10^{-5}	95	0	40	0	0	5
1	$p\text{-}Cl\text{-}Ph\text{-}O\text{-}C(=O)\text{-}NH\text{-}Me$	2×10^{-4}	20	0	0	0	0	20
2	$Ph\text{-}O\text{-}C(=O)\text{-}NH\text{-}Me$	10^{-3}	0	0	30	0	0	25
17	$(pNO_2\text{-}Ph\text{-}O)_2\text{-}P(=O)\text{-}CH_2\text{-}Ph$	4×10^{-5}	95	0	65	0	<5	50
27	$CH_3\text{-}Ph\text{-}O\text{-}C(=O)\text{-}NH\text{-}Ph$	4×10^{-4}	30	0	45	0	<5	0

Further the factors V and VIII are not inhibited, neither in their plasmatic nor in their so-called activated form (see below).

If we are to have an enzyme cascade as the backbone of coagulation it will have to look like this:



The first reactions are triggered by activated contact product for the intrinsic and by tissue thromboplastin for the extrinsic pathway. Activated contact product and tissue thromboplastin will not be discussed further here, as this would lead us off the main line of reasoning in this discussion. Essentially, the idea of a cascade is maintained but the number of steps is reduced.

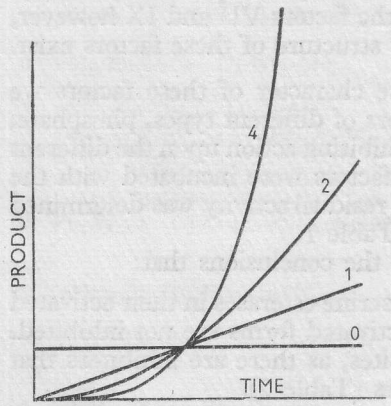


Fig. 1. Product formation in an enzyme cascade.
0 — concentration of triggering enzyme,
1 — concentration of the first product,
2 — concentration of the product of a two-step cascade,
4 — concentration of the product of a four-step cascade
(it is assumed that simple first order kinetics apply).

The kinetic behaviour can be accurately calculated (6). It can also be seen intuitively. When we return to our first example concerning enterokinase, trypsin and chymotrypsin, and we assume zero order kinetics, then the constant amount of enterokinase will cause a constant conversion rate of trypsinogen. This will cause an increase in the trypsin concentration that is linear in time. Hence the velocity of chymotrypsin conversion will increase linearly in time and the amount of chymotrypsin will increase as a second order function. In a n-step cascade the last product will arise as a n-power function (Fig. 1).

Such kinetic behaviour can account for two characteristic features of the coagulation process viz. a) the "magnifying" effect that causes that a few molecules of activated contact product can bring about a massive formation of fibrin and b) the "avalanche" effect. This indicates that the small triggering which stimulates the formation of activated contact product, eventually has large results, but that it requires time to achieve them, because the development of the reactions goes through a latent phase of barely visible production of the end product.

Such a cascade does not, however, account for two equally specific features of coagulation, namely:

a) The extraordinary specificity, that results in a fact that four factors that are so much alike still act in an ordered sequence without a lot of side reactions going on (it is in this respect for instance that our first example of a simple cascade would go wrong).

b) The self-limiting character, which makes that coagulation *in vivo* does not

end in a complete thrombosis. It is here that the factors V and VIII enter into the scene.

It has been found that for factor V_a to be able to convert factor II it has to be adsorbed together with factor V at a phospholipid-water interphase. The evidence for this is of three kinds:

a) It has been proven impossible to find prothrombinase activity in preparations that did not contain factor X_a, Ca^{2+}-ions and phospholipids together. The same holds for Xase and factor IX_a and VIII.

b) It has been proven that both factor X^a and factor V adsorb onto phospholipid. The binding of X_a is enhanced by Ca^{2+}-ions. The binding of V is inhibited by Ca^{2+}-ions. The optimal Ca is where about equimolar quantities of X_a and V are bound. Again the same holds for factors IX_a and VIII (2).

c) The kinetics of prothrombinase formation are not in accordance with the enzymatic action of one of the factors upon the other, but lend support to the scheme:

$$\text{phospholipid} + \text{V} \overset{k_1}{\leftrightharpoons} \text{phospholipid} - \text{V}$$

$$\text{phospholipid} + \text{X}_a \overset{k_2}{\leftrightharpoons} \text{phospholipid} - \text{X}_a$$

$$\text{V} - \text{phospholipid} + \text{X}_a \overset{k_2'}{\leftrightharpoons} \text{II-ase}$$

$$\text{X}_a - \text{phospholipid} + \text{V} \overset{k_1'}{\leftrightharpoons} \text{II-ase}$$

It is interesting to note that we found no evidence of k_1 and k_1' to be very different. This suggests that the primary action in the formation of these complexes is between the phospholipid and the protein rather than between one protein and another factor adsorbed at the phospholipid (5).

The fact that factor X_a binds via Ca^{2+}-ions suggests that an ionic site of the protein interacts with an ionic site at the phospholipid surface.

We did a set of experiments where we substituted Ba-stearate for phospholipid. It turned out that factor V binds irreversibly to such a hydrophobic powder, but that it keeps at least part of its activity when adsorbed. This makes us think that hydrophobic interactions are involved in the binding of factor V (11).

For a phospholipid to have thromboplastic properties, it must present a mosaic or hydrophilic and hydrophobic attachment site. Whether the attachment sites are preexistant or induced remains open (Fig. 2).

As no enzymatic activity of factor V alone is known, what then can be the function of factor V?

There are two possibilities:

a) Factor V could change the conformation of factor X_a so that its active site is able to work on prothrombin, or

b) Factor V could provide accessory binding sites necessary to hold factor II in a position appropriate for attack by factor X_a.

In order to investigate this we did experiments with synthetic substrates. We took tosylarginine methylester (TAMe) which is a poor substrate for X_a just as prothrombin. It showed that it was impossible to improve the TAMe splitting by adding factor V or phospholipid (Table 3).

The splitting could be inhibited by anti-factor-X-antibody which makes the possibility of a non-specific esterase faking these results rather less likely.

We think it more likely than not that factor V acts by providing accessory binding sites for the prothrombin. It also seems that part of the prothrombin molecule has a specific affinity for factor V (8). To illustrate this I will briefly review the work of MANN, HANAHAN, JACKSON and others (a. o. 4) on the activation of prothrombin.

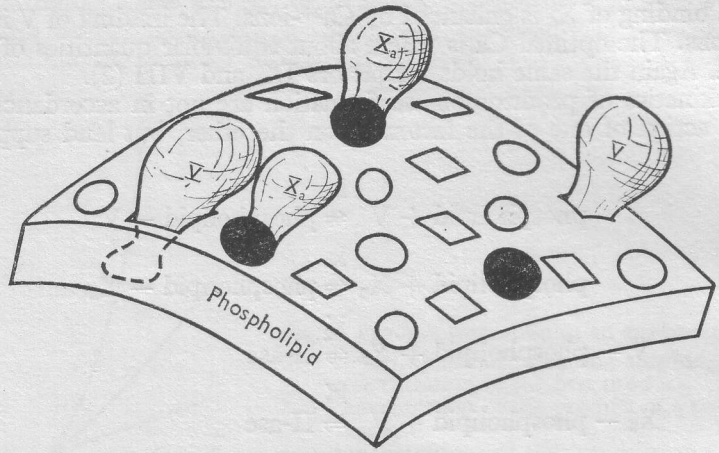


Fig. 2. Schematic view of prothrombinase. The hydrophobic binding sites are given as squares, the hydrophilic ones as circles. The black circles represent Ca-ions.

TABLE 3

The influence of factor V, Ca^{2+} and phospholipid in the TAMe esterase activity of factor X

Xa (units/ml)	Ca (mM)	V (units/ml)	ph. lip. (mg/ml)	relative velocity
250	0.0	0.0	0.0	1.00
250	0.17	0.0	0.0	0.85
250	0.17	4.0	0.0	0.75
250	0.17	4.0	7.5	0.70
250	0.17	24.0	7.5	0.50
250	0.17	24.0	15.0	0.30
375	0.17	24.0	22.5	0.50
375	1.0	24.0	22.5	0.45
500	1.0	24.0	22.5	0.85
500	1.0	24.0	30.0	0.80
625	1.0	24.0	37.5	1.00

The experiments were carried out at pH 8.0 in 10mM TAMe at pH 8.0

Prothrombin is a single polypeptide chain protein which in the course of its activation undergoes three cleavages. The first step renders fragment I (FI) and intermediate I (I1). This step can be brought about by either thrombin or activated factor X. The second step splits off fragment 2 and leaves behind intermediate 2. In a third step intermediate 2 is split in the α- and β-chains of

thrombin. These remain attached by a disulfide bond. The last two steps can be brought about by factor X_a only.

FI contains 90 % of the carbohydrates, and is the part of the molecule that binds Ca, and via Ca binds to phospholipid. The Ca-binding property is endowed in the vitamin K-dependent step of the prothrombin synthesis (3, 10, 17).

Intermediate 1 can be converted into the normal thrombin, but much more slowly than factor II, just as the abnormal prothrombin (PIVKA-II) synthetized in the absence of functional factor II. F2 seems to be bound to factor V.

If you asked me to speculate on the most likely course of events in prothrombin activation, I think that all three factors X_a, V, and II bind to phospholipid, that X_a first splits off F1, so that prothrombin would no longer attach to the complex, if factor V would not fix it via fragment 2. Then factor X_a splits intermediate 1 at other sites. Much less is known on the factors IX_a and VIII, but what we know argues in favour of a complete parallelism with X_a and V. Factors V and VIII thus belong to a class of proteins that have to sit besides active enzymes in order to specifically modify their activity. We have therefore called them *para-enzymes* (7).

It may be that para-enzymes occur much more often than we now think. Enzyme-paraenzyme complexes at an interphase occur in coagulation and also in the action of the complement system. The situation in these complexes may be thought of as half-membranes. It may well be that at membrane-water interphases the close cooperation between an enzyme and a non-enzymatic protein occurs fairly often. This might in part account for the well known difficulty of separating and reconstituting functional units in membranes.

I think there is at least one specific example of this. In trying to separate the enzymes responsible for oxidative phosphorylation in mitochondria RACKER and coworkers could isolate a particle that had oligomycin-sensitive ATPase activity. This particle could be further separated in phospholipid, ATPase (oligomycin--insensitive) and factor 0 which in itself was inactive. These three could be reconstituted to give oligomycin-sensitive ATP-ase (18).

We see that factors V and VII may well account for the extraordinary specificity in the coagulation system by providing accessory binding sites. We think they also play a role in the determination of the self-limiting character of coagulation.

We know that factor V and factor VIII must be "activated" before they can take part in the coagulation process. This "activation" is not a proenzyme-enzyme conversion; it probably is a polymer-monomer transition. This process is partly spontaneous, but can be greatly enhanced by thrombin (or RVV-V for V) (12).

The thrombin activated factor V is much more labile than the original product. This causes a transient burst of activity of factor V in the presence of thrombin, compared to a steady low activity in its absence. At the moment, we favour the following type of reaction to explain this phenomenon:

$$\text{V (tetramer)} \rightleftarrows 4 \cdot \text{V (monomer)} \xrightleftarrows{\text{thrombin}} 4 \cdot \text{V (monomer)} \rightleftarrows 2 \cdot \text{V (dimer, inactive)}$$

The inactivation of factor V follows second order kinetics. This not only holds for V_a in solution, but also for V_a when it forms part of prothrombinase. Active factor V therefore inactivates prothrombinase. It thus appears that during thrombin generation several reactions develop that counteract thrombin generation.

a) Thrombin inactivates V eventually.
b) Thrombin converts factor II into intermediate I, which is less reactive.

c) At least one of the "fragments" has a coagulation inhibiting action.
d) An excess of V_a inhibits prothrombinase forming a phospholipid-X_a-V_a-V_a -complex.

We are trying to calculate the kinetics of this process. This is not at all finished, but one thing is clear: the kinetics bear a strongly non-linear character.

Now non-linear kinetics recently have been shown to account for several interesting phenomena, such as oscillations, multiple steady states and threshold phenomena (20). These phenomena apply to concentration changes in time in perfectly stirred vials. In unstirred samples diffusion has to be taken into account as well.

A. M. TURING has shown already in 1952 (21) that non-linear kinetics together with diffusion can account for concentration changes along the space coordinate in previously homogeneous mixtures. This can be applied to blood coagulation, too. The simplest model would be of the threshold type. A local source (e. g. a wound) could then generate a disturbance that only within a finite distance would be big enough to be above the threshold necessary to generate a transition to the coagulated state.

This would account for the limited explosion so characteristic of blood coagulation *in vivo*.

SUMMARY

A review is given of the reaction scheme of blood coagulation. Crucial are the concepts of a) the cascade-wise interaction of coagulation enzymes that accounts for the enhancement of enzymatic activity and the triggering effects observed, and b) the occurrence of para-enzymes, i. e. the factors V and VIII. These moieties are shown to be responsible for the specificity in blood coagulation by providing additional binding sites in the enzyme-substrate complexes. It is argued that recognition of the non-linear character of prothrombin conversion is essential in explaining the self-limiting character of blood coagulation.

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