# Some Aspects of Kinetics of the First Stages of Blood Thromboplastin Formation

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In 1953 Biggs and Douglas showed that a powerful thromboplastic activity is generated in a mixture of adsorbed plasma, serum, and platelets, together with Ca ions. The substance formed, which they called blood thromboplastin, is able to convert prothrombin into thrombin within a few seconds (4).

It is now generally admitted that the following clotting factors participate in the formation of blood thromboplastin:

1) Plasma Thromboplastin Antecedent (PTA) and Hageman factor present in $BaSO_4$ adsorbed plasma and in serum (10, 11).
2) Factor V and VIII in $BaSO_4$ treated plasma.
3) Factor IX (Christmas factor, PTC), Stuart-Prower factor and Prephase accelerator (PPA) in serum (6).
4) Platelet factor 3 and $Ca^{++}$.

The whole formation is a sequence of reactions where some factors act as substrates and the others as enzymes. In 1955 Bergsagel (2) was able to demonstrate the existence of intermediate phases during the whole process, for which Hougie (8) gave the following scheme (table 1).

Tab. 1

1. F. VIII + F. IX + Stuart-Prower F. + Ca → Intermediate Product I
2. Intermediate Product I + Platelets ——→ Intermediate Product II
3. Intermediate Product II + F. V ——→ Blood Thromboplastin

Bergsagel (3) and Hougie (8) proved experimentally the existence of reactions 2 and 3. Until now, the mechanism of intermediate product I formation still remains unknown. It was the purpose of the experiments reported in this paper to elucidate the rôle of factors VIII, IX, PPA and Stuart-Prower in the formation of blood thromboplastin.

## Methods

*Intermediate product I formation.* A modification of Bergsagel and Biggs method was used

0.2 ml $BaSO_4$ treated plasma, undiluted.
0.2 ml serum, undiluted.
0.1 ml $CaCl_2$ 1/20 m.
0.5 ml Veronal-Acetate Buffer, pH 7,35.

The mixture is incubated at 37° C and aliquots (0.1 ml) are tested on Ca-free plasma (5) (Dowex-50 plasma, 0.1 ml) at 4 min. intervals. When pathological sera are used, the blood (10 ml) is collected into tubes containing human brain thromboplastin (0.1 ml), the prothrombin consumption is normalized.

*Veronal Buffer:* Veronal-Acetate Solution: 9.714 g sodium acetate. ($3H_2O$) and 14.714 g diethyl-barbituric Na (Veronal) in 500 ml distilled water. Buffer solution: 250 ml Veronal-Acetate solution, 200 ml 4.25% NaCl, 217 ml HCl 0.1 n and 683 ml distilled water.

*Paper electrophoresis:* Method of Fisch (6). The electrophoresis was conducted on Whatman paper n° 120 (7 × 40 cm) for 12 hours under the following conditions: 4° C, veronal buffer pH 9, $\mu = 0.06$, 130 Volts and 11 mA.

*Prothrombin* and *factor VII complex* assays according to Koller, Loeliger and Duckert (9).

*Stuart-Prower factor assay:* Method of Bachmann, Duckert and Koller (1).

*Factor VIII and IX assays,* according to Geiger, Duckert and Koller (7).

*Thromboplastin generation test* according to the modification of Duckert, Flückiger, Isenschmid, Matter, Vogel-Meng and Koller (5).

## Results

1) *Intermediate product I formation in normal conditions.*

To study the influence of the different factors it was very important to choose conditions comparable to the physiological ones. In order to maintain the quantitative relationship between plasma and serum factors as exactly as possible in the incubation mixture, we used undiluted $BaSO_4$ treated plasma and undiluted serum. Platelets were absent. Factor V was neglected because it intervenes later in the whole process (table 1) (8).

The intermediate product I represents the first detectable substance in the chain of enzymatic reactions leading to the blood thromboplastin formation. Its synthesis must involve one or more enzyme-substrate reactions, at least one enzyme and one substrate. The enzyme (E) acts as catalyst converting the substrate (S) into product (P) according to the equation:

$$E + S \rightleftharpoons ES \rightleftharpoons P + E$$

In this reaction the substrate is consumed and the resulting product P can be used either as an enzyme or a substrate in the next reactions. The development of such an enzymatic reaction is known, and it is expressed by a first grade exponential curve. The normal formation curve of intermediate product I (Ordinate, int. prod. I in %. Abscissa, inc. time in min.) showed a S shape. After an incubation period the activity reaches rapidly the maximum, then it

decreases slowly. This curve indicates that, without any doubt, more than one enzyme-substrate reaction took place. The following three phases can be distinguished (Fig. 1)

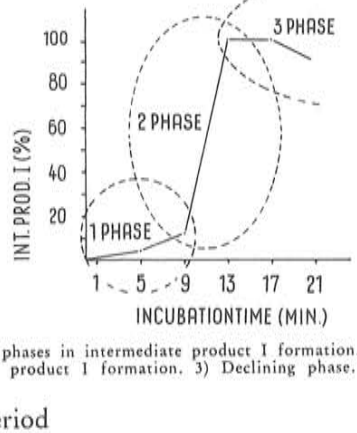


Fig. 1: The three different phases in intermediate product I formation. 1) Lag period. 2) Intermediate product I formation. 3) Declining phase.

1. phase: Lag period
2. phase: Intermediate product I formation phase
3. phase: Declining phase.

The number of reactions taking place during the lag period is still unpredictable. The second phase is a typical example of an enzyme-substrate reaction. In the third phase a decline of intermediate product I activity may be due to an active destruction or to a simple disintegration.

The delinitation of each phase should be accurately defined. The transition from the first to the second phase is not always well marked, it is therefore necessary to separate both phases artificially. The curve shows that after a period of very slow and insignificant generation, the activity rises suddenly, reaching rapidly the maximal rate of intermediate product I formation. The point where the slope of the curve is abruptly changed was chosen as the limit between the first and the second phase. It corresponds to a formation of 20% of intermediate product I activity. The lag period is defined as the time elapsing between the beginning of incubation and the moment when 20% of activity is reached. We consider that, at this point the first phase terminates and the second phase commences. This 20% value chosen is convenient because it is measured more precisely than the lower values. This procedure presents naturally some disadvantages. If the concentration of the second phase factors is diminished, the first phase being absolutely normal, the maximal rate of formation decreases so that the lag period seems to be prolonged. This prolongation is apparent and only due to the slowing down of the reaction in the second phase. It is expressed by the declining slope of the curve of intermediate product I formation. The

actual time consumed by the first phase is the difference between the time obtained at the 20% value of intermediate product I, and the time obtained by taking into consideration, the slope of the second phase curve. The example given in figure 2 illustrates our determination method. We chose two curves obtained by dilution of normal $BaSO_4$ treated plasma, curve 1 gives the normal formation, curve 1/8 shows a delay in both first and second phases.

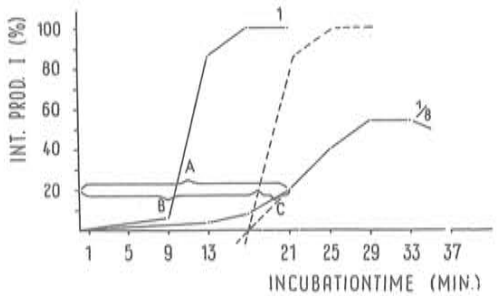


Fig. 2: Delimitation of the first and second phases. A) Total formation time at the 20% value of intermediate product I. B) Actual time consumed by the first phase. C) Prolongation due only to the slowing down of the second phase.

The second phase (intermediate product I formation phase) is characterized by the formation rate. In an enzymatic reaction the maximal rate is attained at the beginning of the reaction. At this point the substrate is in excess, and the whole system far from being in equilibrium the reaction progresses only in the forward direction. Later the measurement becomes fictitious because the reaction reaches its equilibrium and the declining phase begins. In our particular case the maximal rate is generally reached at the 20% value of intermediate product I, so that we again chose this point for measuring the rate expressed by the amount of activity formed during the time unit (min).

The third or declining phase already influencing the second phase controls, together with the substrate concentration the total amount of intermediate product I. Therefore, in order to characterize exactly the generation of intermediate product I (second phase) we used only its formation rate.

2) *Influence of plasma and serum on the intermediate product I formation*

a) *Influence of $BaSO_4$-treated plasma factors.* In this system, both normal $BaSO_4$ plasma and serum are used. The serum concentration is kept constant, and a series of plasma dilution are tested. All plasma factors are diluted simultaneously and to the same extent (fig. 3). The dilution of these factors modifies both the first and the second phases. This dilution also controls the final yield of intermediate product I. Figure 4 gives a graphic representation of

the action of the plasma dilutions on the first phase. A decrease of $BaSO_4$ adsorbed plasma concentration distinctly prolongs the lag period.

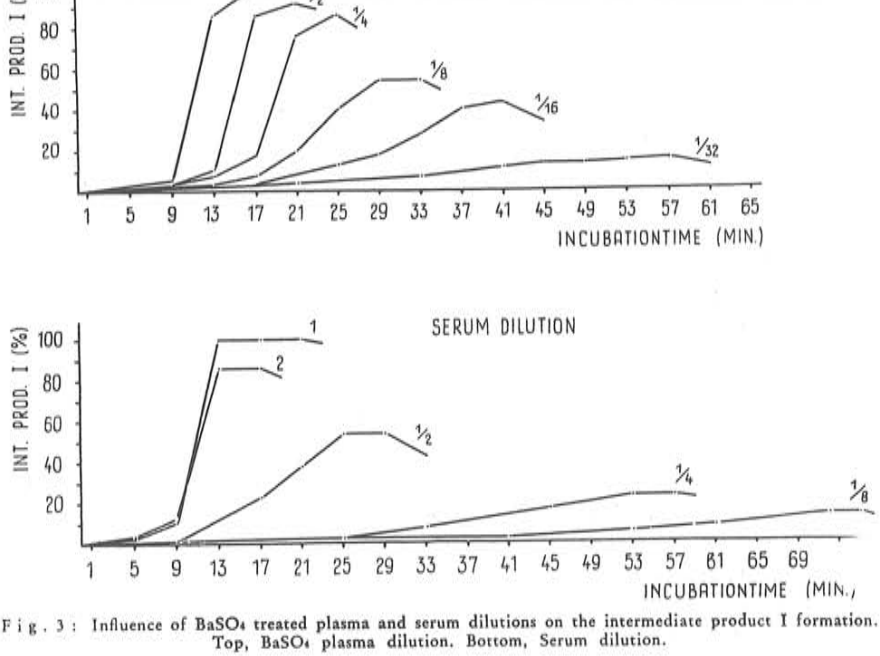


Fig. 3: Influence of $BaSO_4$ treated plasma and serum dilutions on the intermediate product I formation. Top, $BaSO_4$ plasma dilution. Bottom, Serum dilution.

The second phase is also disturbed by a decrease in plasma concentration (fig. 4). The curve represents the formation rate of intermediate product I in function of the plasma dilutions. Dilution slows down the formation rate, and the slope of the curve is typical for a substrate decrease (hyperbolic type). The progressive reduction of the yield corroborates this finding, indicating that when plasma is diluted, a substrate is also being diluted.

b) *Influence of serum factors.* In this experiment the $BaSO_4$ treated plasma concentration is kept constant, while the serum concentration varies (fig. 3). All serum factors are equally diluted. A decrease of serum concentration greatly prolongs the lag period (fig. 4), beyond the 1/4 dilution this period becomes unmeasurable. The formation rate of intermediate product I diminishes very rapidly. The course of the curve is neither typical for an enzyme nor for a substrate dilution (fig. 4). Since serum contains many factors it is likely that more than one serum factor participates in the development of the second

phase. The total yield is reduced in proportion to the dilution, therefore one of the serum factors may act as substrate. It is of interest to note that an increase of the serum concentration over the normal range reduced both the reaction rate and the final yield (fig. 3).

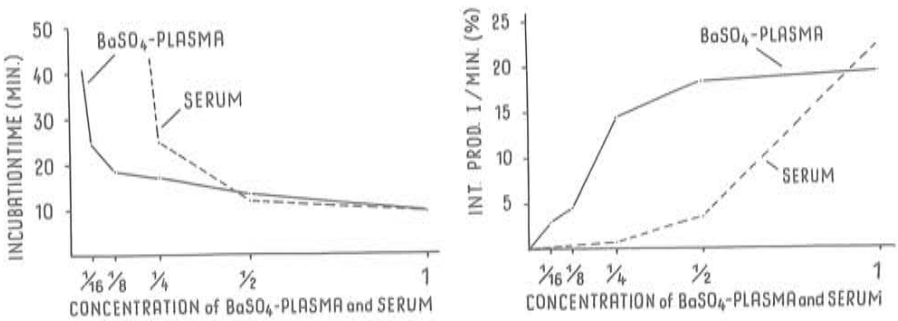


Fig. 4: Influence of $BaSO_4$ plasma and serum dilutions on the first phase, incubation time (left). Influence of $BaSO_4$ plasma and serum dilutions on the second phase, intermediate product I formation (right).

Since in these experiments, the whole plasma or the whole serum are diluted the results cannot be used to characterize the rôle of a given factor in the intermediate product I generation. Therefore in another series of experiments clotting systems were investigated in which only one factor was varied at the same time.

### 3) *Influence of isolated clotting factors*

a) *Factor VIII.* When a series of factor VIII dilutions are made by mixing both $BaSO_4$ treated haemophilic and normal plasma, PTA and Hageman factor, which are present in normal concentrations in both plasmas, are being kept constant automatically. The influence of factor VIII is indicated in figure 5. All the variations are due to factor VIII only. The first phase (fig. 6) is almost unaffected by changes in factor VIII concentration. The lag period remains constant at dilutions up to 1/4 and increases very little at higher dilutions. One can safely come to a conclusion that the influence exerted on the lag period by factor VIII is negligible.

On the contrary, the formation phase (second phase) is delayed. The rate variation expressed as function of factor VIII dilution gave a curve of the hyperbolic type (fig. 6). This indicates that factor VIII acts as a substrate in the intermediate product I formation. The total yield is reduced by decreasing the factor VIII concentration.

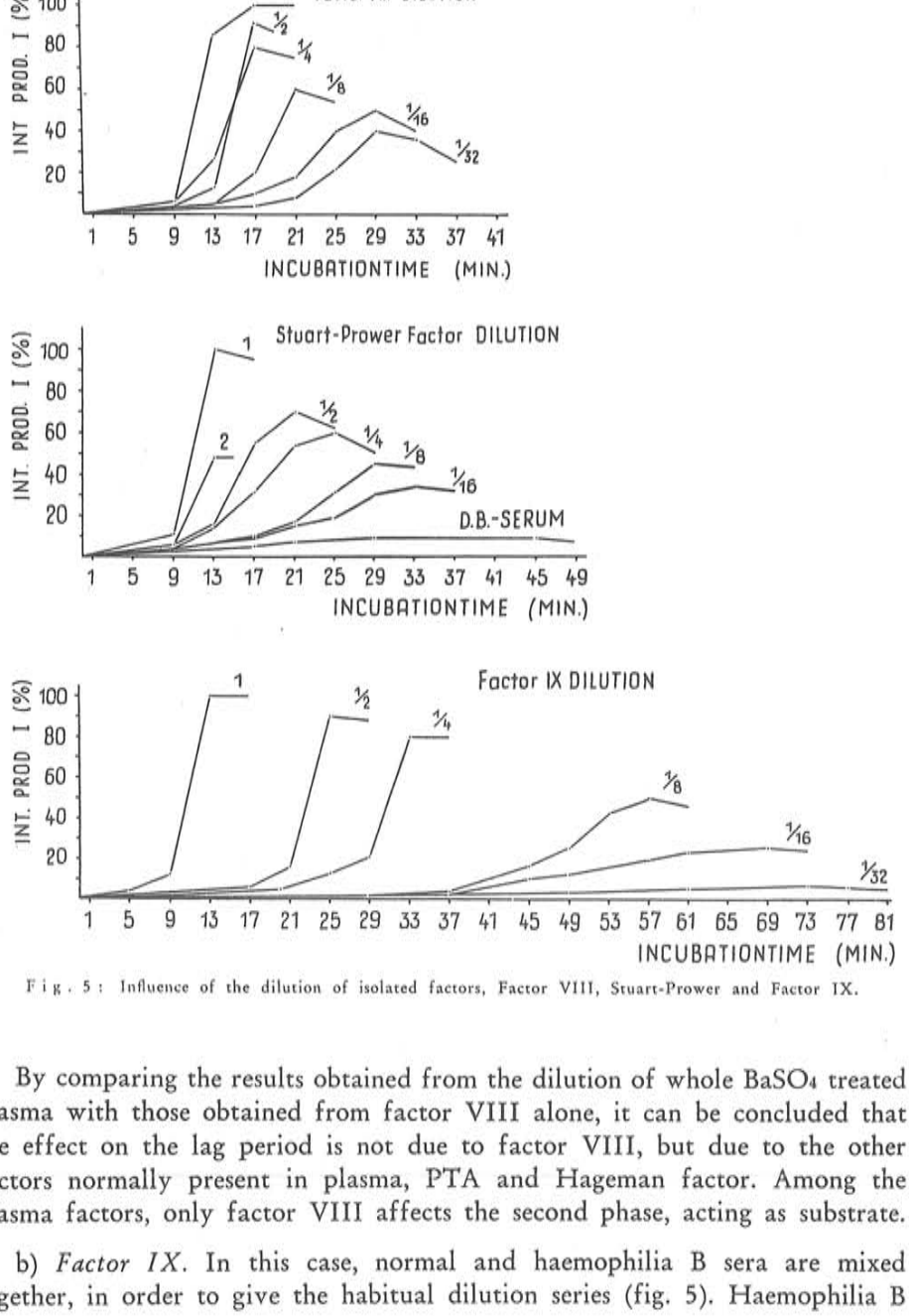


Fig. 5: Influence of the dilution of isolated factors, Factor VIII, Stuart-Prower and Factor IX.

By comparing the results obtained from the dilution of whole $BaSO_4$ treated plasma with those obtained from factor VIII alone, it can be concluded that the effect on the lag period is not due to factor VIII, but due to the other factors normally present in plasma, PTA and Hageman factor. Among the plasma factors, only factor VIII affects the second phase, acting as substrate.

b) *Factor IX*. In this case, normal and haemophilia B sera are mixed together, in order to give the habitual dilution series (fig. 5). Haemophilia B

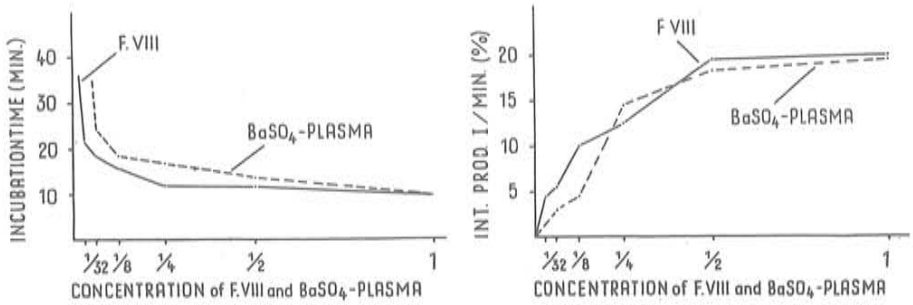


Fig. 6: Comparison of the influence of whole $BaSO_4$ plasma and Factor VIII dilutions. Left: first phase. Right: second phase.

serum is deprived of prothrombin by adding brain thromboplastin at the time of blood collection. At the 1/2 dilution stage, the lag period is already being delayed considerably by factor IX (fig. 7). In the second phase, factor IX acts exactly in the same way as factor VIII (fig. 7). The decrease of the formation rate gave an hyperbolic curve, a characteristic example for a substrate dilution. The effect of factor IX on the yield is similar to that of factor VIII.

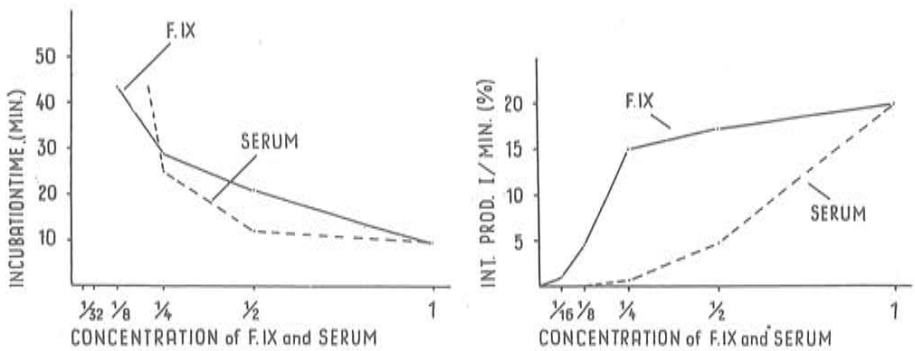


Fig. 7: Comparison of the influence of whole serum and Factor IX dilutions. Left: first phase. Right: second phase.

The comparison of the curves obtained from the dilution of whole serum and factor IX respectively, shows that, the prolongation of the lag period is due to factor IX, while one or several other factors besides factor IX control the second phase. According to these results, factor IX should act in phases 1 and 2. In an earlier publication (6) we described the isolation by means of paper electrophoresis, of a factor which is able to correct only the first phase

when mixed with haemophilia B serum, i.e. this factor acts as accelerator in the lag phase. It was therefore called Prephase Accelerator or PPA. The genuine factor IX added to haemophilia B normalizes the second phase only (formation rate) and also the total amount of intermediate product I.

We have come to the conclusion that in haemophilia B serum, the activity of two factors is lacking: PPA and genuine factor IX, the former influencing the lag period and the latter the second phase.

c) *Stuart-Prower factor.* To an incubation mixture of $BaSO_4$ treated plasma and Stuart-Prower deficient serum, an electrophoretic fraction containing almost pure Stuart-Prower factor was added in order to get the same dilution curve as in the previous experiments (fig. 5).

The analysis of the dilution curves showed that the first phase remains unaffected by Stuart-Prower factor variations (fig. 8). On the other hand, the reaction velocity decreases proportionaly to the Stuart-Prower concentration, the rate expressed in function of dilution gave nearly a straight line (fig. 8). The shape of the curve is typical for an enzyme action. This finding is supported by the fact that Stuart-Prower factor remains unchanged during blood coagulation.

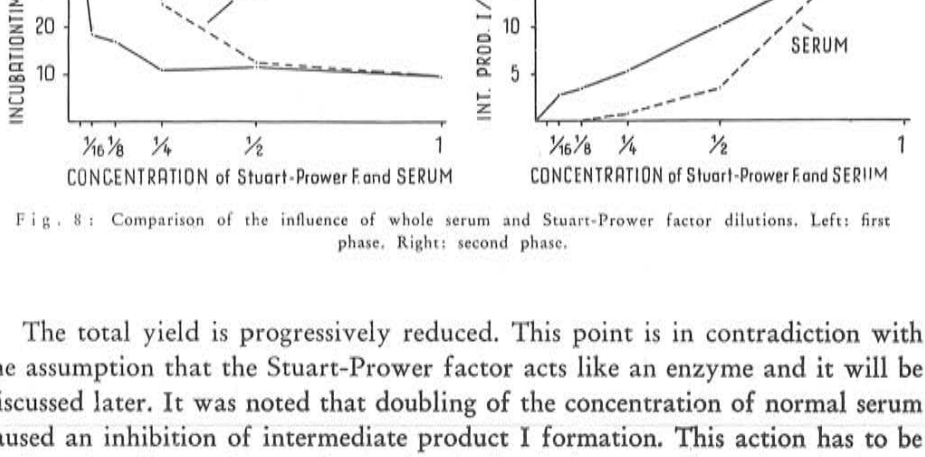


Fig. 8: Comparison of the influence of whole serum and Stuart-Prower factor dilutions. Left: first phase. Right: second phase.

The total yield is progressively reduced. This point is in contradiction with the assumption that the Stuart-Prower factor acts like an enzyme and it will be discussed later. It was noted that doubling of the concentration of normal serum caused an inhibition of intermediate product I formation. This action has to be attributed to Stuart-Prower factor (see in fig. 5 the curve 2) so that the hypothesis of an inhibitor can be discarded. It is furthermore known that the excess of one component can inhibit an enzymatic system.

## Discussion

The intermediate product I formation is an enzymatic process. Our experimental work was an attempt to analyse its kinetics. For this purpose we have distinguished three different phases during the intermediate product I generation (fig. 1). We found that the following factors, PTA and Hageman factor (in $BaSO_4$ plasma) and PPA (in serum) take part in the normal course of the first phase which we named the lag period. At this stage it is quite impossible to say how these different factors act. Factor VIII, genuine Factor IX and Stuart-Prower Factor are necessary for the second phase. Factor VIII and genuine Factor IX turned out to be substrates and Stuart-Prower Factor an enzyme. The declining (third) phase, although it is not directly a part of the intermediate product I formation is very important. This phase begins with the intermediate product I formation and controls the final yield. Its action is more pronounced in all systems where the initial rate is slow. The slowing down can be indifferently produced by decreases in either substrate or enzyme. Therefore the intermediate product I yield is no more proportional to the substrate concentration alone. It also depends on the enzyme activity. It is therefore understandable why the enzyme (Stuart-Prower factor) caused a decrease of the final yield.

The formation rate controls the maximal yield of intermediate product I, and blood thromboplastin. Therefore, a deficiency of any factor taking part directly in this generation, leads to haemorrhagic diseases.

In an earlier publication, it was pointed out that haemophilia B serum two clotting factors were deficient (PPA and genuine IX). The present experiments demonstrate that dilution of normal serum with haemophilia B serum influences independently the first and the second phases. The second phase remains almost unaffected by dilutions up to 1/4, whereas the lag period is triplicated. These and the previous results support our assumption of a double deficiency in Haemophilia B serum.

In a plasma collected with carefully siliconized needles and tubes, practically no factor IX activity can be detected. After coagulation, a high factor IX activity can be measured in the serum. An important part of this activity has to be attributed to PPA (6). To explain the problem of factor IX-PPA, we offer the following hypothesis: It is more probable that, as in most congenital haemorrhagic diseases, only one factor is lacking in haemophilia B. This factor exists in normal plasma. It functions as precursor of both PPA and genuine factor IX. Both factors participate in the intermediate product I formation, the former as accelerator of the first phase, and the latter as substrate in the second phase. The conversion of the precursor to PPA + genuine factor IX

needs the presence of Ca ions, plasma thromboplastin antecedent, Hageman factor and surface contact according to the following scheme:

$$1)\ \text{precursor} \xrightarrow{Ca^{\cdot\cdot}, \text{PTA, Hageman, Contact}} \text{PPA} + \text{genuine IX}$$

$$2)\ \text{genuine IX} + \text{VIII} \xrightarrow{\text{Stuart-Prower}} \text{intermediate product I}$$

In 1) the course of the reaction(s) is hypothetical. The development of 2) has been experimentally demonstrated in this work. In contradiction to an earlier hypothesis (2), factor VIII does not intervene in the activation process of factor IX precursor. Like genuine factor IX it takes part as substrate in the formation of intermediate product I. The similarity of their kinetics is in accordance with the indistinguishable clinical picture of the deficiencies of both factors.*)

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## Summary

The first stages of blood thromboplastin generation were investigated. Plasma Thromboplastin Antecedent, Prephase Accelerator, and Hageman factor are involved in the reactions taking place during the lag period. This phase is followed by the formation of intermediate product I. Studying the kinetics of this particular reaction it was possible to attribute to factors VIII and IX the rôle of substrates and to Stuart-Prower factor the rôle of an enzyme. It appears that in haemophilia B serum two distinct factors are lacking, the genuine factor IX, and the prephase accelerator (PPA). An hypothesis explaining tentatively the activation of a precursor to PPA and genuine factor IX is offered.

## Résumé

Les premiers stades de la formation de la thromboplastine sanguine sont étudiés. Les facteurs PTA, PPA et Hageman prennent part aux réactions d'une première phase ou période de latence (lag period). Cette phase est suivie par

*) The prephase accelerator, PPA, plays the most important rôle in the Factor X phenomenon. Stuart-Prower factor and prothrombin to a lesser degree are also involved in this phenomenon.

une phase dite phase de formation du produit intermédiaire I. L'étude de la cinétique de cette formation permet d'attribuer aux facteurs VIII et IX le rôle de substrats et au facteur Stuart-Prower celui d'enzyme. Il est à noter que dans le sérum d'une hémophilie B deux facteurs distincts sont déficients, le facteur IX vrai (substrat) et un accélérateur de la première phase (PPA). Une hypothèse rendant compte de l'activation d'un éventuel précurseur en facteur IX actif et PPA est avancée.

## Zusammenfassung

Die ersten Stufen der Blutthromboplastinbildung wurden untersucht. PTA, PPA und der Hageman-Faktor sind an den Reaktionen beteiligt, die während der Latenzperiode ablaufen. Diese wiederum ist gefolgt von der Zwischenprodukt-I-Bildung (2. Phase). Aus den Untersuchungen der Kinetik dieser Reaktion ergab sich, daß Faktor VIII und IX als Substrate, der Stuart-Prower-Faktor als Enzym wirken. Es zeigt sich, daß im Hämophilie-B-Serum zwei bestimmte Faktoren fehlen: der echte Faktor IX und der Vorphasen-Akzelerator. Es wird die Hypothese aufgestellt, daß eine gemeinsame Vorstufe zu echtem Faktor IX und PPA aktiviert wird.

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