The role of factors VIII and IX in blood coagulation

In: Current Topics in Haemophilia
(Ed R.T. Wensley) NOT Print, Manchester 1983
pp. 3-14

The role of factors VIII and IX in blood coagulation.

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The severity of the bleeding disorders Haemophilia A and B is a reflection of the essential role of factors VIII and IX in blood coagulation. The two proteins participate in the intrinsic activation of factor X , a reaction which further requires the presence of a membrane surface containing negatively charged phospholipids (see ref. 1 for a review). In vivo, blood platelets provide such a surface.

Methods to prepare highly purified factors IX and X are available, and the molecular changes in the zymogens IX and X when they are converted to the active serine proteases factors IXa and Xa are known (1,2). The structure of human factor VIII:C (coagulant), the molecule that corrects haemophilia A is not yet known (see ref 3 for a review). Studies on bovine factor VIII indicate a molecular weight of about 220.000 and the presence of three peptide chains in the activated factor VIII (4). Factor VIII:VWF is a molecule different from factor VIII:C. It is deficient in van Willebrands disease, and functions in the adhesion of blood platelets to damaged endothelium. Factor VIII:VWF is also designated as factor VIII:RAG (factor VIII related antigen) and as the ristocetin cofactor activity. In plasma and cryoprecipitate factor VIII:VWF and factor VIII:C are found associated in the so called factor VIII:complex (3). In our study we have used purified bovine factor VIII:C and factor VIII complex. Until now no differences in factor X activating activity between the preparations have been found. The phospholipids used in this work are unilamellar (single bilayer) vesicles obtained by sonication of lipid suspensions consisting of 25% phosphatidylserine and 75% phosphatidylcholine.

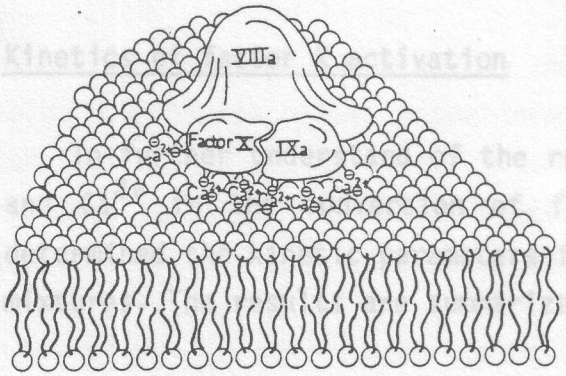

Fig. 1 Assembly of the components of the intrinsic factor X activating complex.

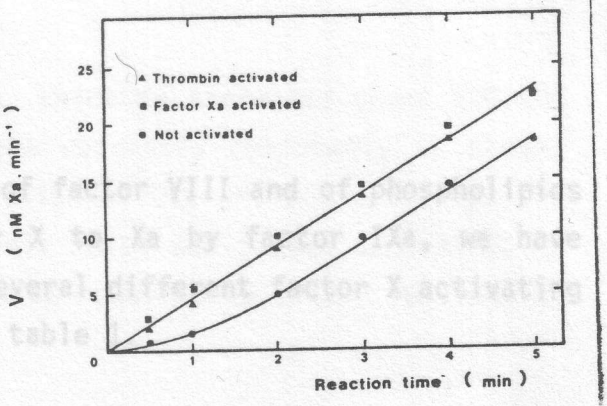

Fig. 2 Effect of preincubation of factor VIII:C with either thrombin or factor Xa on the time course of factor X activation.

It is thought that during activation the factors IXa VIIIa and X are bound at the phospholipid surface. Factors IXa and X are equipped with 10-12 modified glutamic acid residues (gammacarboxy glutamic acid residues), that enables these proteins to bind to the negatively charged phospholipids of the membrane surface via a Ca^{2+}-bridge (Fig. 1.) The interaction of factor VIII with lipids has not been investigated in detail.

Factor X activation can be followed on a spectrophotometer using the chromogenic substrate S2337. Factor Xa hydrolyses this substrate liberating paranitroaniline, that absorbs at 405 nm. The rate of S2337 conversion is proportional with the amount of factor Xa present.

Factor VIII must be activated before it can participate in factor X activation. In fig. 2 is shown the time course of factor X activation by factor IXa in the presence of PL, Ca^{2+} and factor VIII. With unactivated factor VIII (closed circles) a lag period is seen which most likely reflects the time required for factor VIII activation in the reaction mixture, since preincubation of factor VIII with factor Xa (1 nM, 5 min. 37^{o}) in presence of phospholipids and Ca^{2+} prior to addition of factors IXa and X abolished the lag period. The lag is also abolished after a 1 min incubation of factor VIII with 1.5 nM thrombin. In all further experiments we use thrombin activated factor VIII.

Kinetics of factor X activation

To further understand of the roles of factor VIII and of phospholipids and Ca^{2+} in the conversion of factor X to Xa by factor IXa, we have determined the kinetic parameters for several different factor X activating mixtures. The results are summarized in table 1.

Composition of factor X activating mixture	Km^{app}	Vmax
	μM	mol Xa.min^{-1}.mol IXa^{-1}
IXa	299	0.0022
IXa, $CaCl_2$	181	0.0105
IXa, $CaCl_2$,PL* (10 μM)	0.058	0.00247
IXa, $CaCl_2$, PL (10 μM), VIIIa(11 units/ml)+	0.063	500

* PL, Phospholipid, + VIIIa, factor VIII complex activated with thrombin.

Table 1: Effect of the accessory components (phospholipids, $CaCl_2$ and factor VIIIa) on the kinetic parameters of factor X activation. (data from: van Dieijen et al 1981; ref. 5)

Both phospholipid and factor VIIIa cause important changes of the kinetic parameters of factor X activation. In the presence of phospholipids the Km drops from 181 μM to 0.058 μM, with little change of Vmax. The

effect of factor VIIIa is mainly on Vmax, which is increased about 200.000 fold. The physiological importance of the accessory components is clear, considering the plasma factor X concentration of 0.2 μM. Phospholipids are required to bring the Km for factor X well below the plasma concentration and factor VIII, which increases the Vmax is required to obtain substantial levels of factor Xa.

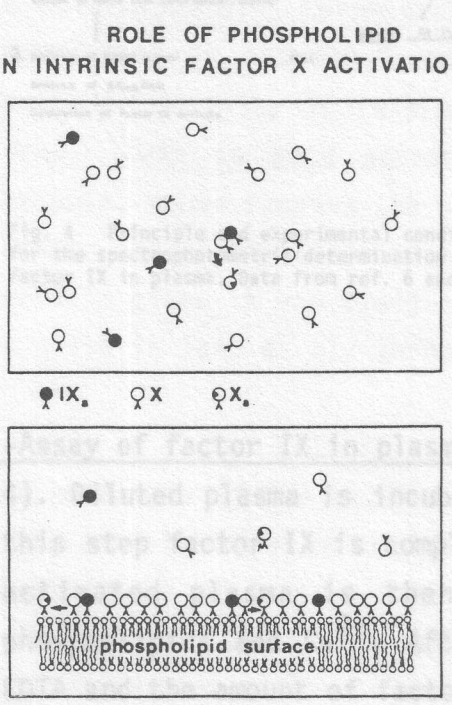

Fig. 3 Model to explain the lowering of Km in presence of a negatively charged phospholipid surface.

Fig. 3. provides an explanation why the Km for factor X is lowered in presence of a phospholipid surface. In solution (upper panel) a high concentration of factor X is required to saturate the enzyme factor Xa. When lipids and Ca^{2+} are present (lower panel) both factor IXa and factor X bind to the lipid surface. Hence, the local factor X concentration is highly increased. Since Km is expressed in terms of added factor X, it is clear that in presence of lipids much lower amounts of factor X are required to obtain half maximal rates of factor X activation.

Assays based on the intrinsic factor X activating system

The knowledge of the kinetic properties of the factor X activating system has enabled us to design assays for phospholipids, factor IXa, and factor VIIIa. In the assays experimental conditions were selected such that the rate of factor X activation is proportional with the component to be assayed.

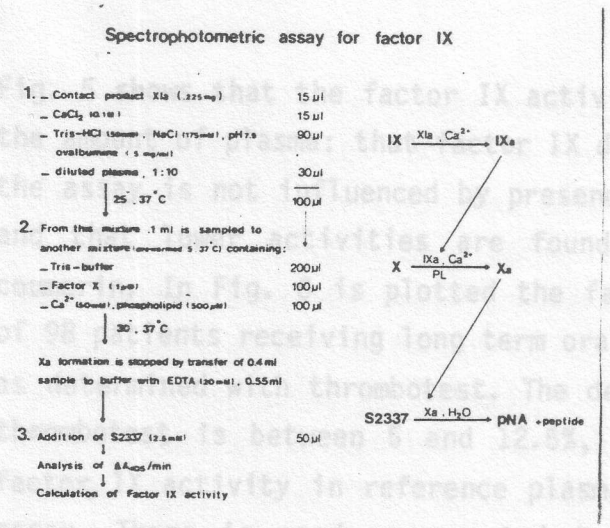

Fig. 4 Principle and experimental conditions for the spectrophotometric determination of factor IX in plasma. Data from ref. 6 and 7.

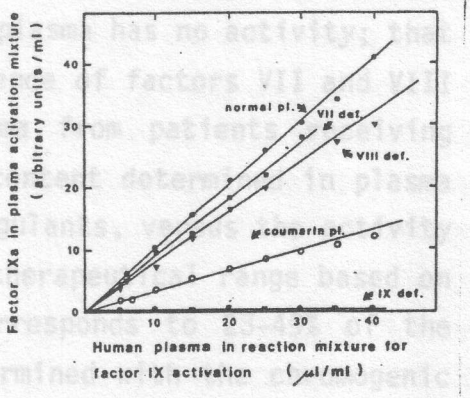

Fig. 5 Calibration curves obtained with various amounts of reference plasma, coumarin plasma and factor IX, VII, and VIII deficient plasmas in the spectrophotometric assay for factor IX.

-Assay of factor IX in plasma. The method consists of three steps. (Fig. 4). Diluted plasma is incubated with contact product (factor XIa), during this step factor IX is completely converted to factor IXa. A sample of the activated plasma is then incubated with factor X in presence of phospholipids and Ca^{2+}. After 30 min factor X activation is stopped with EDTA and the amount of factor Xa present is determined with the chromogenic subsrate S2337. From a calibration curve made with known amount of active site titrated factor IXa, the amount of factor IXa in the plasma can be calculated.

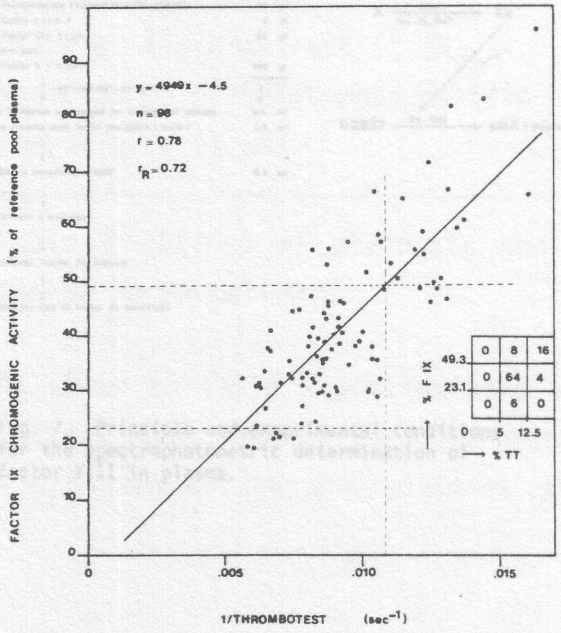

Fig. 6 Relationship between the factor IX activity determined with the chromogenic method and the activity in thrombotest, in plasma of 98 long-term anticoagulated human subjects.

Fig. 5 shows that the factor IX activity in the assay is proportional with the amount of plasma: that factor IX deficient plasma has no activity; that the assay is not influenced by presence or absence of factors VII and VIII and that lower activities are found in plasma from patients receiving coumarin. In Fig. 6 is plotted the factor IX content determined in plasma of 98 patients receiving long term oral anticoagulants, versus the activity as determined with thrombotest. The desirable therapeutical range based on thrombotest is between 5 and 12.5%, which corresponds to 23-49% of the factor IX activity in reference plasma as determined with the chromogenic assay. There is good agreement between the two methods, 80 out of 98 patients, would receive the same instructions with regards to dosage. These results and a comparison with spectrophotometric methods for factor II, VII and X is given in van Dieijen-Visser et al (7). The principle of the method is given in Tans et al. Thrombos. and Haemostas. In press (6).

- Assay of factor VIII in plasma

The principle of the assay is outlined in Fig. 7. Diluted plasma is incubated with thrombin to activate all factor VIII to VIIIa. a sample is added to a mixture of factors IXa, X and phospholipids plus $CaCl_2$. Factor X activation is stopped with EDTA, and the amount of factor Xa formed is determined with S2337.

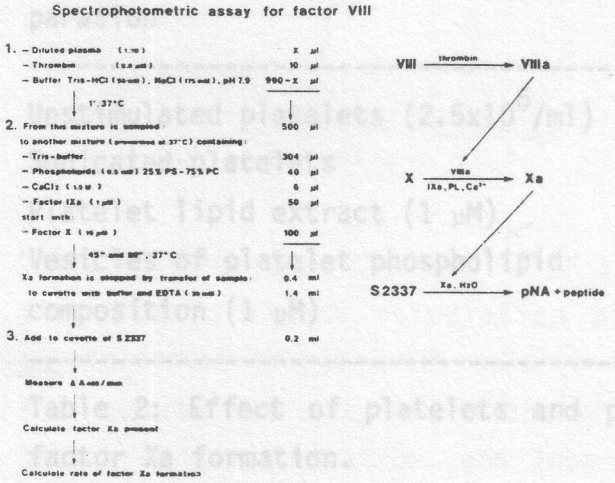

Fig. 7 Principle and experimental conditions for the spectrophotometric determination of factor VIII in plasma.

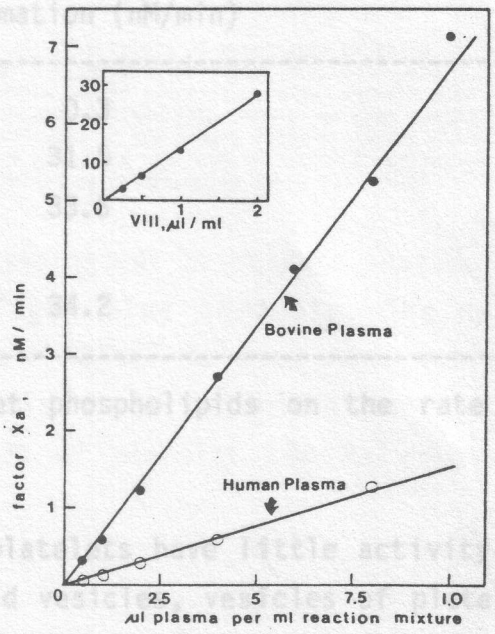

Fig. 8 Calibration curves obtained with various amounts of bovine and human plasma, and with highly purified bovine factor VIII:C, in the spectrophotometric assay for factor VIII.

As shown in fig. 8 a linear relationship is found between the amount of plasma and the rate of factor Xa formation in the test. Bovine plasma gives 5 times higher activities than human plasma. Bovine plasma may contain 5 times more factor VIII, or alternatively the bovine factor VIII molecule is more active than its human counterpart. The insert is the activity of a highly purified bovine factor VIII:C preparation containing 29 nM of factor VIII:C (Method to be published).
This allowed us to conclude that the factor VIII concentration in bovine plasma is 1.1 nM or 0.24 mg/l. When the molecular weight of factor VIII is 220.000 this would result in a specific clotting activity of 4132 U/mg. This is identical to the specific activity reported by Vehar and Davie (4).

Role of blood platelets in intrinsic factor X activation

The activity of platelets in factor X activation was studied using washed human platelets, that were stimulated for various time periods with various agonists. A mixture of factors IXa, VIIIa, X and Ca^{2+} was then added and the rate of factor Xa formation was determined with a chromogenic substrate.

Platelet or phospholipid preparation	Rate of fXa formation (nM/min)
Unstimulated platelets (2.5×10^6/ml)	0.3
Sonicated platelets	31.5
Platelet lipid extract (1 μM)	33.6
Vesicles of platelet phospholipid composition (1 μM)	34.2

Table 2: Effect of platelets and platelet phospholipids on the rate of factor Xa formation.

As shown in table 2 unstimulated washed platelets have little activity in factor X activation. Purified phospholipid vesicles, vesicles of platelet lipid composition and platelet phospholipid extract are highly active.

Platelets become active after disruption by sonication. This result is explained by the fact that the platelet phospholipids are asymmetrically distributed over the inner and outer leaflets of the platelet membrane phospholipid bilayer (Fig. 9). The negatively charged phospholipids, phosphatidylserine and phosphatidylinosithol are almost all located at the cytoplasmic side of the platelet membrane and therefore inaccessible for interaction with the clotting factors present in plasma (see ref. 8 from review). However, when platelets are stimulated by the combined action of collagen plus thrombin, activity appears in factor X activation. Thrombin alone is not an efficient stimulant (Fig. 10).

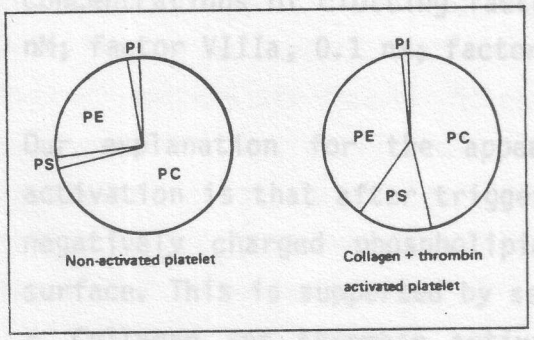

Fig. 9 Glycero-phospholipid composition of the outer monolayer of the platelet plasma membrane. PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, Phosphatidylinosithol. Data from Zwaal and Hemker ref. 8.

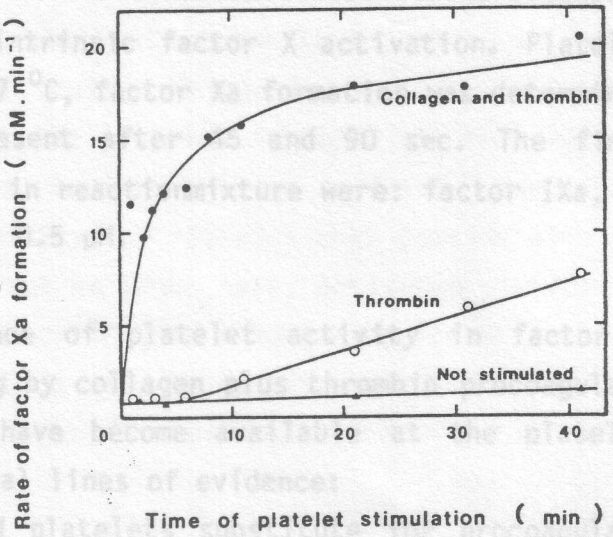

Fig. 10 Time course of appearance of platelet factor X activating activity. Platelets were activated, while stirring, without agonist with IIa or with Collagen/IIa. After the time period as indicated in the figure factors IXa VIIIa and X were added and the rate of factor X activation determined as described in table 3.

Table 3 summarizes the results of all physiological stimulants tested. We find the strongest stimulation by collagen plus thrombin. The non-physiological trigger, the calcium ionophore A23187 is even more active. The results can not be attributed to lysis since the platelets remain intact during activation, and less than 3% of platelet LDH is released during stimulation.

Platelet stimulator	Rate of fXa formation (nM/min)
Unstimulated	0.4
Thrombin (1.3 nM)	0.8
Collagen (10 µg/ml)	2.5
Thrombin plus collagen	6.0
A23187 (1 µM)	19.4
Thrombin plus collagen followed by incubation with phospholipase A2	0.1

Table 3: Effect of platelets on intrinsic factor X activation. Platelet activation was for 10 minutes at 37 ^{o}C, factor Xa formation was determined from the amount of factor Xa present after 45 and 90 sec. The final concentrations of clotting factors in reactionmixture were: factor IXa, 50 nM; factor VIIIa, 0.1 nM; factor X, 0.5 µM.

Our explanation for the appearance of platelet activity in factor X activation is that after triggering by collagen plus thrombin procoagulant negatively charged phospholipids have become available at the platelet surface. This is supported by several lines of evidence:

a. Collagen and thrombin activated platelets substitute for procoagulant phospholipid vesicles in factor X activation.
b. The activity of activated platelets is abolished after phospholipase A2 digestion (without platelet lysis)(table 3).
c. Direct analysis of the platelet outer surface by non-lytic degradation with phospholipase A2 shows that 25% of the platelet phosphatidylserine has become available at the platelet outer surface. (Bevers et al ref. 9).

We propose the following model for appearance of platelet procoagulant activity (Fig.11).

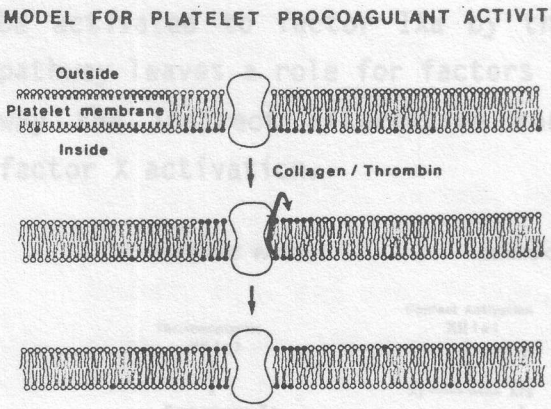

Fig. 11 Model for the appearance of platelet procoagulant activity. Schematically shown is the platelet plasma membrane lipid bilayer. The filled circles in the bilayer denote negatively charged polar head groups of procoagulant phospholipid, e.g. phosphatidylserine (PS), that interact with clotting factors. Platelet stimulation with Collagen and IIa results in PS exposure, resulting from PS transport from the inside to the platelet outside (flip-flop hypothesis; for details see ref. 8.

In the unstimulated platelet the procoagulant phospholipid, phosphatidylserine PS is located at the platelet inside. Triggering by collagen plus thrombin leads to appearance of PS at the platelet outside. the translayer transport of PS (flip-flop) may be facilitated by proteins. Membrane proteins may be present that have affinity for PS, such that domains with a high PS content are formed. As could not be shown here, activated platelets also stimulate the activation of prothrombin into thrombin by factor Xa and Va. Bevers et al (9). A detailed study on the activity of platelets in factor X and prothrombin activation is forthcoming. (Van Rijn et al. Manuscript in preparation).

Importance of intrinsic factor X activation in clotting pathways.

Intrinsic factor X activation must be a physiological important mechanism considering the seriousness of factors VIII and IX deficiencies. However the intrinsic and extrinsic clotting pathways do not readily explain this prominence (Fig. 12).

The extrinsic pathway of factor X activation bypasses the intrinsic route and does not explain why haemophilia is a disorder. The intrinsic pathway explains the importance of factors IX and VIII, but leaves no role for thromboplastin release as initiator of clotting. The importance of the contactsystem for the initiation of clotting is further complicated by the fact that deficiencies in factor XII, high molecular weight kininogen, and prekallikrein are not major bleeding disorders. The alternative pathway was discovered by Østerud and Rapaport in 1977, who showed that factor IX can

be activated to factor IXa by thromboplastin and factor VIIa (10). This pathway leaves a role for factors IX and VIII, however it must be explained why the indirect alternative route should be advantageous over direct factor X activation.

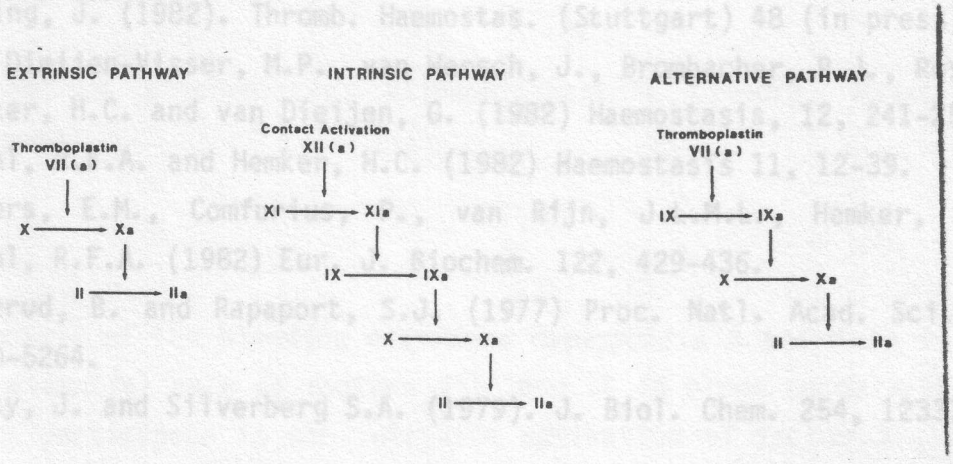

Fig. 12 Major clotting pathways in plasma.

The answer may be amplification. Jesty and Silverberg (11) have determined that with a given amount of thromboplastin, factor Xa is formed at 6-fold higher rates as factor IXa. However, our results (table 1) indicate that under optimal conditions, each molecule of factor IXa produces 500 molecules of factor Xa per minute. Therefore, the alternative pathway may produce factor Xa at 100 fold higher rates as the direct extrinsic pathway.

Acknowledgement: We thank G. Tans and M.P. van Dieijen-Visser for permission to reproduce data; KABI AB for donating part of the chromogenic substrates that were used in our work; T. Janssen-Claessen, J. Govers-Riemslag, J. Franssen and P. Comfurius for help in protein purification and expert technical assistance.

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