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Two Types of Prothrombin in Vitamin K Deficiency

H. C. Hemker, Annemarie D. Muller and E. A. Loeliger

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The mode of action of vitamin K and hence of vitamin K antagonists is essentially unknown although recognition and management of drug induced or spontaneous vitamin K deficiencies is of great medical importance.

In 1963 we postulated that vitamin K acts at a stage in coagulation factor synthesis after the synthesis of the polypeptide chain *per se* (1). This conclusion is supported by experiments conducted on this topic by other investigators (2, 3, 4, 5). We based our assumption on the observation that parallel with the decrease of the coagulation factors II, VII, IX and X the occurrence was observed of a *P*rotein *I*nduced by *V*itamin *K* *A*bsence or *A*ntagonists (PIVKA). This protein was discerned because it acted as a competitive inhibitor of factor X conversion. It was postulated that PIVKA was the product of polypeptide chain synthesis unmodified by the vitamin K dependant mechanism. PIVKA was originally called preprothrombin, but a more neutral name seems preferable. This communication is to report the finding of a slow generation of thrombin activity from PIVKA.

This slow generation seems to be at the basis of the discrepancy between the one- and two-stage estimations of prothrombin (factor II) in stable anticoagulation. Biggs et al. (6) claim the level of factor II to be 10–30% higher than that of factors VII, IX and X, whereas Loeliger et al. (7) found all four factors to be mutually equally lowered. Biggs et al. estimated factor II in a two-stage procedure, Loeliger et al. used a one-stage estimation method. The difference between these two methods lies in the fact that in the two-stage procedure all the material able to produce thrombin is converted into thrombin and measured as such, whereas the one-stage procedure estimates the prothrombin content by measuring the initial rate of the reaction in which prothrombin is rate-limiting (8), i.e.:

$$\text{prothrombin} \xrightarrow{\text{prothrombinase}} \text{thrombin}$$

A difficulty inherent to the two-stage procedure is that thrombin as it generates in the mixture is inactivated by antithrombin III. The velocity of inactivation is proportional to the concentrations of thrombin and antithrombin III (9). Hence the amount of thrombin found with this method is dependant upon the concentrations of antithrombin III as well as upon the concentrations of prothrombin.

To overcome this difficulty created by the presence of antithrombin III, we adopted a two-stage procedure after elimination of antithrombin III (see legend to Fig. 1). The thrombin generated then will not deteriorate to any extend for an appreciable time. The level of thrombin that eventually develops is taken as a measure of the prothrombin content in the samples tested.

Mixtures of pooled normal plasma (prepared from 30 individuals) and $Al(OH)_3$ adsorbed normal plasma were tested side by side with plasmas from patients with absolute or drug-induced vitamin K deficiency. The amounts of thrombin obtained with different dilutions of normal plasma were used to construct a reference curve. With normal plasma and dilutions thereof containing between 100 and 2% prothrombin, thrombin formation always was complete within 5 min. In striking contrast with this

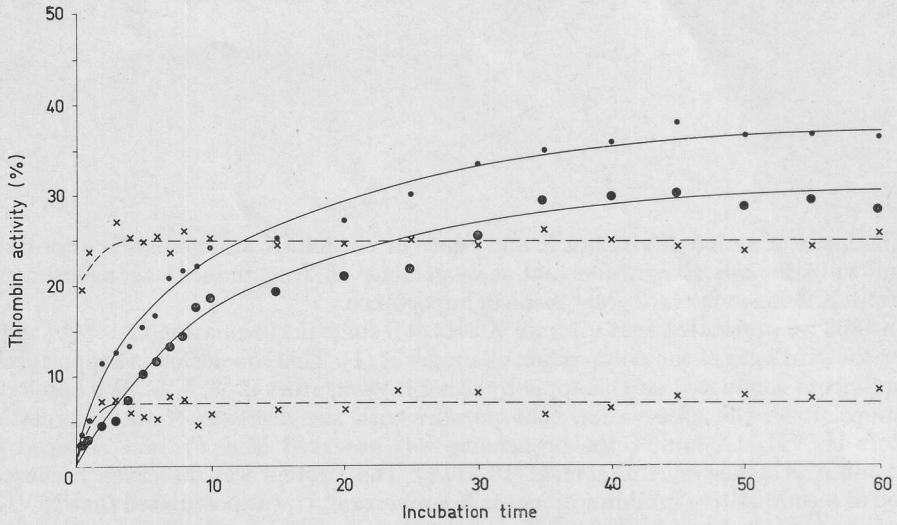

Fig. 1. *Thrombin generation in an antithrombin III free medium.* From the samples to be tested we prepared a euglobulin fraction, which contains a fixed fraction (approx. 50%) of the procoagulants of the original plasma and no antithrombin III (9). The euglobulin precipitate of 0.5 ml plasma is dissoluted in 2.5 ml citrated Michaëlisbuffer pH 7.4 (conc. of citrate 22 mM), and thrombin is generated in an mixture of equal amounts of euglobulin solution, $CaCl_2$ (10 mM final concentration), human brain thromboplastin (prepared according to Owren and Aas, final dilution 1:30) and a preparation containing 50% factor X und 76% factor VII and no other coagulation factors, prepared from normal serum according to Prou-Wartelle (1, 2). ●——● a) lower curve: thrombin generated from plasma from a patient patient with severe vitamin K-deficiency (one-stage factor II <2%); b) upper curve: thrombin generated in the same plasma with 6% normal plasma added; x——x c) upper curve: thrombin generated from normal plasma diluted 1 in 4; d) lower curve: subtraction of the curves a and b.

(and completely unexpectedly) in preparations obtained from plasma from severely vitamin K deficient patients (prothrombin level as assessed by the one-stage procedure $< 2\%$ of normal) a slow thrombin generation was observed which in 60 min reached levels of 20–30% of normal (Fig. 1). This slow rise could not be attributed to deficiencies of the factors VII or X, as these factors were added in excess to the reaction medium. Neither is this slow generation likely to be caused by an inhibition of the conversion of essentially normal prothrombin. In the first place because the anticoagulant circulating in vitamin K deficiency (c.q. PIVKA) does not inhibit the prothrombin ⟶ thrombin reaction (11). Secondly the thrombin generation curve obtained with a mixture of normal plasma and plasma from a severely vitamin K deficient patient cannot be distinguished from the sum of the slowly rising curve obtained with the latter plasma alone and the normal quick rising curve as obtained with a small amount of normal plasma present (Fig. 1).

It thus seems that in this medium normal prothrombin is activated normally and that the slow formation of thrombin results from the activation of an abnormal prothrombin.

Fig. 2 shows that a discrepancy between the one-stage and two-stage procedures is found in vitamin K deficiency (spontaneous as well as coumarin induced), but not in dilutions of normal plasma and disturbances of factor II level due to hepato-cellular

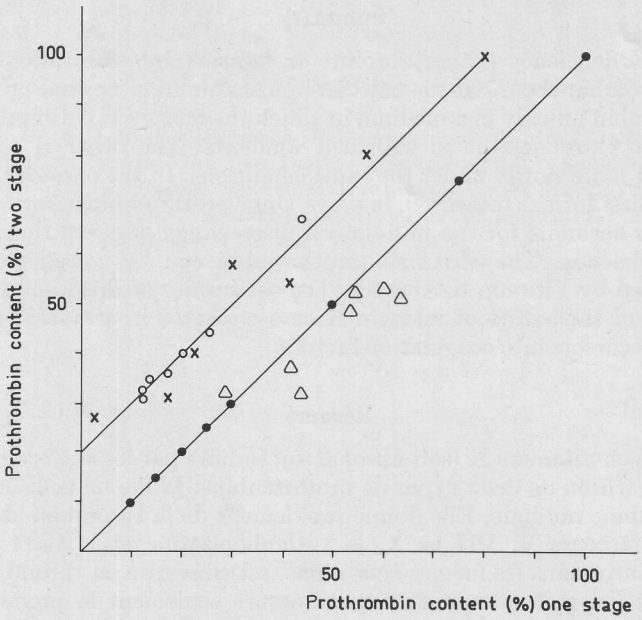

Fig. 2. *Comparison between one-stage and two-stage estimations of prothrombin.* ● dilutions of normal plasma (note: these are used as a reference in both methods, the points therefore do not deviate from a straight line through the origin indicating a 1 to 1 relationship); ○ pooled plasmas from 30 patients anticoagulated to a different degree by phenprocoumon intake; x plasmas from individual patients with vitamin K deficiency, proven clinically afterwards by favourable reaction to administration of vitamin K_1; △ plasmas from individual patients with parenchymatous hepatic disease but without vitamin K deficiency.

damage. The amount of excess thrombin in the two-stage test appears to be independant of the level of anticoagulation c. q. severity of vitamin K deficiency; it approximately equals 20% of the normal factor II level. These results could be explained by the hypothesis that the one-stage determination of prothrombin was systematically underestimated in plasma from vitamin K deficient patients e. g. by the occurrence of a specific inhibitor of this determination. In previous experiments no evidence of such an inhibitor has been found, however (11).

Recent methodological investigations have shown that from the thrombin generation curve a reasonable estimate of the thrombin content can equally well be obtained in the presence of antithrombin III. This is done by linear extrapolation to zero time of a plot of the logarithm of thrombin concentration against incubation time (10).

We think the present experiments suggest the presence of two populations of thrombin generating zymogens in vitamin K deficiency. Besides the normal zymogen an abnormal, slowly activating species seems to be present. These observations fit well with the observations of Josso and Nilehn & Ganroth that in vitamin K deficiency two types of electrophoretically different species can be distinguished that both show a precipitation reaction with antibodies against human prothrombin. Assuming that PIVKA has antigenic properties in common with prothrombin and that it is a zymogen of thrombin as well, the concept of a two-step synthesis of the vitamin K dependant coagulation factors holds well as an explanation for these observations.

Summary

In vitamin K deficiency (either absolute or induced by oral anticoagulants) two types of prothrombin occur. One is not distinguishable from normal prothrombin. It generates thrombin quickly in a medium in which the factors V, VII and X, thromboplastin and Ca^{++} are present in sufficient amounts. The other is converted into thrombin much more slowly under the same conditions. In the onestage prothrombin assay only the first form is measured, in a two-stage prothrombin assay both forms are estimated. This accounts for the well-known discrepancy between these two tests in vitamin K deficiency. The abnormal prothrombin can be considered one of the Proteins Induced by Vitamin K Absence. The occurrence of this kind of proteins fits in the concept of the action of vitamin K as a co-factor in a system that converts polypeptide-precursors into coagulation factors.

Résumé

La déficience en vitamine K (soit absolue soit induite par les anticoagulants oraux) provoque l'apparition de deux types de prothrombine. L'une ne peut être distinguée de la prothrombine normale. Elle donne rapidement de la thrombine dans un milieu contenant les facteurs V, VII et X, la thromboplastine et le Ca^{++} en quantités suffisantes. L'autre dans les mêmes conditions, est convertie en thrombine beaucoup plus lentement. La méthode en un temps mesure seulement la première forme, la méthode en deux temps enregistre l'activité des deux formes. Cela rend compte de la différence bien connue entre ces deux systèmes de dosage, dans les cas de déficience en vitamine K. La prothrombine anormale peut être considérée comme une des « Protéines Induites, la Vitamine K étant Absente ». L'occurence de cette sorte de protéines convient bien à la théorie selon la quelle la vitamine K agit comme co-facteur dans le système qui convertit des polypeptides précurseurs en facteurs de coagulation.

Zusammenfassung

Bei Vitamin K-Mangel (entweder absolut oder durch orale Antikoagulantien induziert) finden sich zwei Typen von Prothrombin. Eines kann nicht von normalem Prothrombin unterschieden werden. Es bildet schnell Thrombin in einem Medium, in dem die Faktoren V, VII und X, Thromboplastin und Ca^{++} in ausreichenden Mengen vorhanden sind. Das andere wird unter denselben Bedingungen viel langsamer in Thrombin umgewandelt. In der Einstufen-Prothrombin-Bestimmungsmethode wird nur die erste Form gemessen, in einer Zweistufen-Methode werden beide Formen bestimmt. Dies erklärt die wohl bekannte Diskrepanz zwischen diesen beiden Testen bei Vitamin K-Mangel. Das abnorme Prothrombin kann als einer der Eiweißkörper, die durch Vitamin K-Mangel induziert werden, aufgefaßt werden. Das Vorkommen dieser Art von Eiweißkörpern paßt in das Konzept der Wirkung des Vitamin K als eines Kofaktors in dem System, das Polypeptidvorstufen in Gerinnungsfaktoren umwandelt.

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