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THE ROLE OF BLOOD CLOTTING FACTOR V IN THE CONVERSION OF PROTHROMBIN AND A DECARBOXY PROTHROMBIN INTO THROMBIN

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Summary

Purified PIVKA-II * exhibits some factor II (prothrombin) activity in the one-stage coagulation assay and this factor II activity does not come from residual amounts of factor II but originates from PIVKA-II itself. It is shown that PIVKA-II is converted by a normal prothrombinase complex (factor V_a and factor X_a adsorbed onto a phospholipid interface) more readily than by phospholipids and factor X_a alone. This suggests that binding between PIVKA-II and factor V_a is an essential feature in the formation of the enzyme · substrate complex and from this we infer that a direct interaction between factor V_a and prothrombin plays a rôle in the prothrombinase · prothrombin complex.

Introduction

Activated factor V is known to be an essential component of the prothrombinase complex [2,3]. Although it has no known enzymatic properties of its own, it markedly accelerates the thrombin generation from factor II (prothrombin) by activated factor X. This acceleration is even more pronounced when the factors II, V_a and X_a are adsorbed to a phospholipid-water interface [3]. Ca^{2+} presumably bind to the γ-carboxy glutamic acid residues (γ-carboxy-Glu) of the factors II and X_a and thereby favor the binding of these factors to the phospholipids [4—6]. Factor V_a presumably binds to the phospholipid via a hydrophobic bond [7].

The mechanism of the accelerating role of factor V in the prothrombinase · prothrombin complex is unknown. It has been shown that a part of the pro-

Abbreviation: SP-Sephadex, sulpho-propyl Sephadex.

* PIVKA-II, a decarboxy prothrombin, is the protein analogous to prothrombin which is induced in human and bovine plasma by vitamin K absence or coumarin treatment [1].

thrombin molecule binds to factor V_a [2]. Possible roles of factor V are [8]: (a) inducing favorable conformational changes in the active site-bearing protein (factor X_a) and/or a similar role towards the substrate (factor II); (b) binding to factor X_a and/or factor II in order to fix these proteins in a juxtaposition favorable for their interaction.

In this article we report the importance of the γ-carboxy-Glu residues for the interaction between the three clotting factors on the phospholipid by comparing PIVKA-II and factor II. These γ-carboxy-Glu residues are present in the factors II and X_a, but they are absent in PIVKA-II. As a consequence the affinity of PIVKA-II towards phospholipids is strongly reduced [9]. The behaviour of PIVKA-II as a substrate in prothrombinase will therefore depend solely on protein-protein interactions in the enzyme · substrate complex.

Materials and Methods

Buffers. Buffer A: 0.02 M Tris · HCl, pH 7.2/0.1 M NaCl/0.01 M benzamidinium chloride. Buffer B: 0.02 M Tris · HCl, pH 7.2/0.25 M NaCl/0.01 M benzamidinium chloride. Buffer C: 0.0286 M sodium barbital, pH 7.4/0.0286 M sodium acetate/0.1164 M NaCl. Buffer D: 0.005 M phosphate buffer, pH 6.8/0.1 M KCl/0.01 M benzamidinium chloride. Buffer E: 0.2 M phosphate buffer, pH 6.8/0.1 M KCl/0.01 M benzamidinium chloride.

Coagulation tests. Factor II activity was assayed with the one-stage coagulation assay and PIVKA-II with the *Echis Carinatus* venom assay as described before [10]. For the two-stage detection of thrombin in a system containing purified clotting factors the bovine fibrinogen solution [10], which was devoid of factors II, VII and X, was freed from residual factor V by repeated precipitation with 2 M glycine and subsequent dialysis against buffer C.

From reaction mixtures in which thrombin was generated we took at various incubation times 0.2-ml aliquots which were mixed with 0.2 ml of the fibrinogen solution (2 mg/ml) at 37° C. The time required for clot formation in these samples was used to determine the amount of thrombin in the original reaction mixtures by comparison with clotting times obtained with known concentrations of thrombin.

Normal pool plasma, definition of units. Bovine reference plasma was prepared as described in ref. 10. The amount of each clotting factor present in this plasma was arbitrarily taken 100% or 1 unit/ml. In a similar way 100% (1 unit/ml) of PIVKA-II was defined as the amount of PIVKA-II which gives the same clotting time in the *E. Carinatus* assay as plasma containing 100% of factor II.

Preparation of PIVKA-II concentrate. Normal healthy cows were anticoagulated with 500 mg of Marcoumar on the first day and 150 mg each following day. At the fifth day 5 l of blood from each cow were collected in oxalate and the plasma was separated from the blood cells by centrifugation for 20 min at 3000 × *g*. Normal clotting factors II, VII and X were removed by $Al(OH)_3$ adsorption (1.5 mg/ml) and PIVKA-X was removed by adsorption onto $BaSO_4$ (50 mg/ml). Subsequently PIVKA-II was isolated from the plasma by batchwise adsorption on QAE-Sephadex (20 ml slurry per l of plasma). After stirring for 1 h the slurry was brought into a column (2.5 × 100 cm) and washed with buffer A. PIVKA-II was eluted by a frontal elution with buffer B. The concen-

trated PIVKA-II was dialyzed against buffer A and frozen at −70° C or used immediately for further experiments.

Snake venoms. Echis carinatus venom and Russell's viper venom were obtained from Sigma. The two components in Russell's viper venom which selectively activate the factors V and X were separated as described by Schiffman et al. [11]. The activating components will be referred to as venom-V and venom-X, respectively.

Coagulation factors. Factor II was purified according to Owen et al. [12]. Factor V was purified according to Dombrose et al. [13] and factor X was purified according to Fujikawa et al. [14]. The factors V and X were activated with venom-V and venom-X, respectively, by addition of 1 μg of venom per unit of coagulation factor to an incubation mixture containing 20 mM $CaCl_2$, 0.1 M NaCl and 20 mM Tris · HCl, pH 7.4. The incubation was carried out for 5 min at 37° C and the activated factors were subsequently separated from the venom by Sephadex G-100 chromatography.

Phospholipids. A human brain phospholipid preparation was made according to Bell and Alton [15]. The final concentration was 0.5 mg/ml.

Quantitative determination of γ-carboxy-Glu residues in PIVKA-II. The determination of γ-carboxy-Glu residues was performed according to a modified procedure of Zytkovicz and Nelsestuen [16]. This procedure which will be presented in more detail elsewhere (Kop, J. and Hemker, H.C., unpublished results) is based on acetylation of the protein preparation followed by reduction with [^{3}H]diborane. The reduction was accomplished while stirring the solution at room temperature for 100 h. After this period of time the reduction was completed and subsequently amino acid analysis was performed on an LKB amino acid analyzer Model 3201. The amino acids were separated at a temperature of 30° C. Fractions of 0.8 ml were collected and counted. The retention times for 5,5-dihydroxyleucine, homoserine, 5-hydroxynorvaline and glutamic acid were 72, 86, 90 and 102 min, respectively. Homoserine and 5-hydroxynorvaline are obtained after reduction of aspartic acid and glutamic acid and are separated completely from the other amino acids. From the peak height (absorbance) and the radioactivity (dpm) we calculated the specific radioactivity, which is defined as dpm obtained when one amino acid per molecule (PIVKA-II or factor II) is reduced. This is necessary because 5,5-dihydroxyleucine (obtained after reduction of the γ-carboxy-Glu residues) did not separate from other (unlabelled) amino acids. When the specific radioactivity is known, the amount of γ-carboxy-Glu residues can easily be calculated from the total dpm at the position of 5,5-dihydroxyleucine.

Labelling of PIVKA-II and factor II. Samples containing either PIVKA-II alone or a mixture of PIVKA-II and factor II were labelled by reductive methylation according to the method of Gualerzi et al. [17]. The reaction was performed with 1-ml samples containing 2 mg/ml of protein. After dialysis against borax buffer (50 mM $Na_2B_4O_7$, 200 mM KCl, pH 9.0), 0.02 mCi [^{14}C]formaldehyde (15 Ci/mol) was added and the mixture was incubated for 30 s in ice. The reaction was repeated with 0.02 mCi $^{14}CH_2O$, followed by incubation for 45 s in ice. After addition of 20 μl $NaBH_4$ (2% in borax buffer), the reaction mixture was dialyzed extensively against buffer A without benzidinium chloride. This procedure gives radiolabelled clotting factors without observable loss

of clotting activity, with a specific activity of $2 \cdot 10^6$ dpm/mg.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was accomplished in gels containing 7% polyacrylamide in a buffer containing 5 mM Tris · HCl, pH 8.4, 38.4 mM glycine and 1.5 mM $CaCl_2$ as initially described by Davis [18].

Results

The PIVKA-II concentrate obtained as described in Materials and Methods was applied to a QAE-Sephadex column (1 × 30 cm) in buffer A. The column was washed with the same buffer and eluted with a linear gradient (twice 250 ml) from buffer A to buffer B. The fractions were dialyzed against buffer A without benzamidinium chloride and assayed for PIVKA-II with the aid of the *E. Carinatus* assay and for factor II with the one-stage coagulation assay (Fig. 1). In this way we obtained two peaks: a first one which was active in the *E. Carinatus* assay but hardly in the one-stage coagulation assay, and a second one eluting at a higher salt concentration, which was equally active in both the *E. Carinatus* and the coagulation assay. Since we were able to remove both

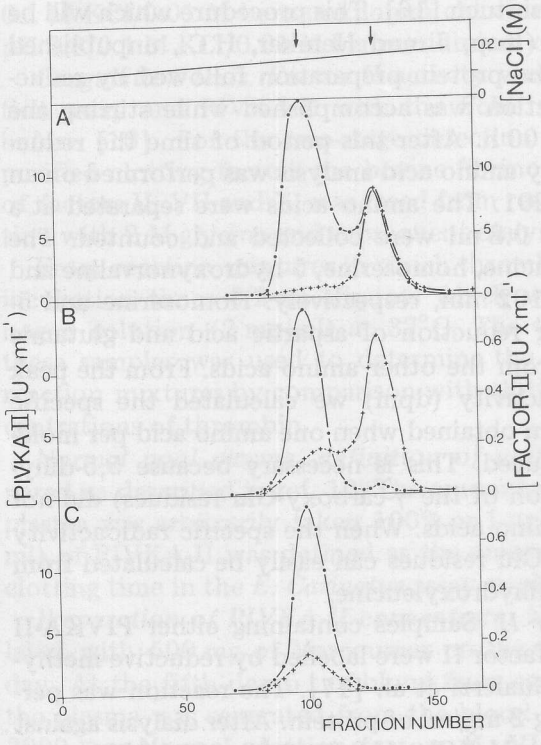

Fig. 1. Separation of PIVKA-II from prothrombin on QAE-Sephadex columns. Because of the high concentrations of benzamidinium chloride no absorbance pattern could be recorded. Fractions were diluted and tested with the *E. Carinatus* assay (●———●), and the one-stage coagulation assay (+- - - - -+). Mind the different right hand scale in A versus B and C. (A) The separation of a mixture containing 300 units of PIVKA-II and 100 units of factor II. (B) The separation of concentrated PIVKA-II (see Materials and Methods) obtained from 5 l of coumarin plasma. (C) The same fractions after dialysis against buffer A and $BaSO_4$ absorption (twice 100 mg/ml).

activities from the latter peak by $BaSO_4$ adsorption, we concluded that these fractions contained factor II.

On the contrary, the first peak did not adsorb onto $BaSO_4$ and we were concerned with the question: is the factor II activity in our PIVKA-II preparation due to a contamination of PIVKA-II with thrombin or prothrombin or does PIVKA-II exhibit a one-stage factor II activity on its own?

The presence of thrombin could be excluded because: (a) in the absence of $CaCl_2$ no clotting was observed; (b) before and after SP-Sephadex chromatography the clotting times remained unchanged (as reported by Esmon et al. [19] SP-Sephadex removes any thrombin present in factor II or PIVKA-II preparations); and (c) addition of 10 mM of the thrombin inhibitor diisopropylfluorophosphate and subsequent dialysis against buffer C did not prolong the clotting time.

It turned out to be more difficult to exclude the possibility that small amounts of prothrombin contaminated our PIVKA-II preparation. Therefore, we purified PIVKA-II to homogeneity and analyzed it with the aid of physicochemical, chemical and kinetic techniques. This is reported in some detail (sub A, B and C), because only when it is assured that no trace amounts of prothrombin contaminated our PIVKA-II preparation, legitimate conclusions can be drawn about the rôle of γ-carboxy-Glu residues in the action of prothrombinase (sub D).

(A) The purification of PIVKA-II and the physicochemical determination of contaminating prothrombin

PIVKA-II was further purified according to a slightly modified procedure of Stenflo and Ganrot [20]. The fractions of the first peak shown in Fig. 1 containing PIVKA-II and only very few factor II activity were pooled and dialyzed against buffer A. The solution was subsequently adsorbed three times with $BaSO_4$ (100 mg/ml). After each adsorption step the solution was centrifuged for 5 min at 3000 × *g* and finally the supernatant was centrifuged for 20 min at 20 000 × *g* and further purified on DEAE-Sephadex, gradient elution from buffer A to buffer B, hydroxyapatite, gradient elution from buffer D to buffer E, Sephadex G-150 and Sephadex G-100, both in buffer A. The latter column was recycled once before the fractions were collected. The benzamidinium chloride was removed by dialysis and the preparation was concentrated to 400% and filtered through an SP-Sephadex column in buffer A without benzamidinium chloride. This procedure yields PIVKA-II which is homogeneous when assayed in polyacrylamide gels with or without $CaCl_2$ in the presence or absence of sodium dodecyl sulfate (SDS) and in bidimensional immunoelectrophoresis. The amino acid composition was equal to that found by Magnusson et al. [21] to within 3%. This PIVKA-II preparation we used for all experiments described below and was called purified PIVKA-II.

In order to find out what is the lowest amount of factor II which is detectable in PIVKA-II we added small amounts of factor II to our PIVKA-II preparation and examined these mixtures with the aid of several physicochemical techniques. The conventional way of separating PIVKA-II and factor II is crossed immunoelectrophoresis [20]. Less than 5% of factor II (w/w) could not be detected in PIVKA-II, however. A better resolution was obtained with poly-

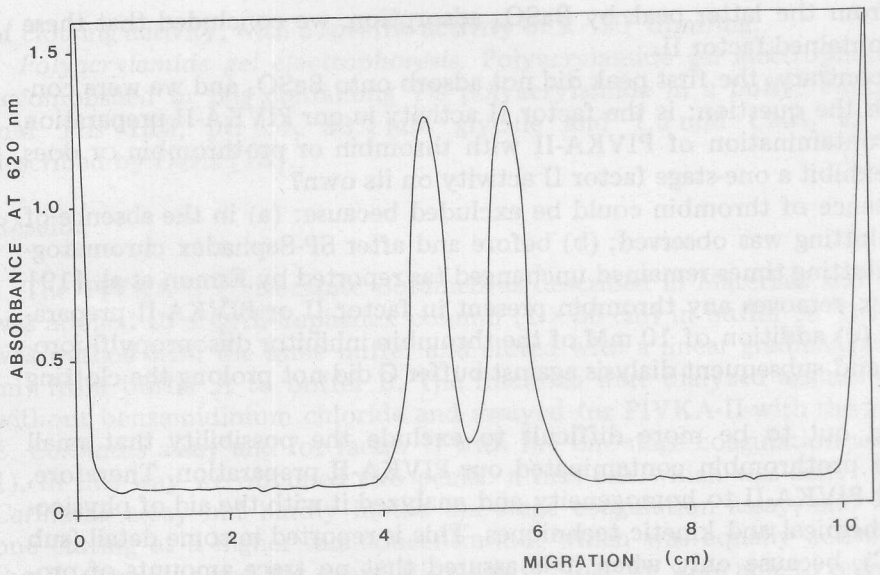

Fig. 2. Separation profile of PIVKA-II and factor II on polyacrylamide gel. The gels were run for 4 h at 5 mA/gel in a buffer containing 1.5 mM $CaCl_2$. Thereafter, they were stained with amido black and scanned at 620 nm. Equal amounts (50 μg) of each clotting factor were applied to the gel.

acrylamide gel electrophoresis in the presence of 1.5 mM $CaCl_2$ (see Materials and Methods). Factor II and PIVKA-II are completely separated with this technique (Fig. 2). Radiolabelling the samples with [^{14}C]formaldehyde prior to electrophoresis and subsequent slicing and counting the gels appeared to be the most sensitive method for protein detection. Amounts of 0.5% (w/w) of added prothrombin could be easily detected in this way. No prothrombin was found in our PIVKA-II preparation, however.

(B) Chemical determination of contaminating prothrombin in PIVKA-II

With the method initially developed by Zytkovicz and Nelsestuen [16] and modified in our laboratory (see Materials and Methods) it was possible to quantitate the amount of γ-carboxy-Glu residues in prothrombin. In mixtures containing prothrombin and PIVKA-II a mean value of the amount of γ-carboxy-Glu residues per molecule was obtained, e.g. a contamination of 10% prothrombin in PIVKA-II gave one residue per molecule (prothrombin contains 10 residues per molecule). Analysis of our purified PIVKA-II preparation revealed that less than 0.05 residue per molecule was present (0.05 residue per molecule was the lower limit of detection). This means that also with this method the contamination of PIVKA-II with prothrombin is estimated to be less than 0.5%.

(C) Kinetic evidence for the absence of factor II in PIVKA-II

Purified PIVKA-II and factor II were tested at various dilutions with the one-stage coagulation assay and with the *E. Carinatus* assay. When we plot the one-stage clotting time (t_c) against the reciprocal concentration of PIVKA-II and factor II, straight lines are obtained (Fig. 3). As described by Hemker et al. [23, 24], from these drawings the Michaelis constant, K_m, and the minimal coagula-

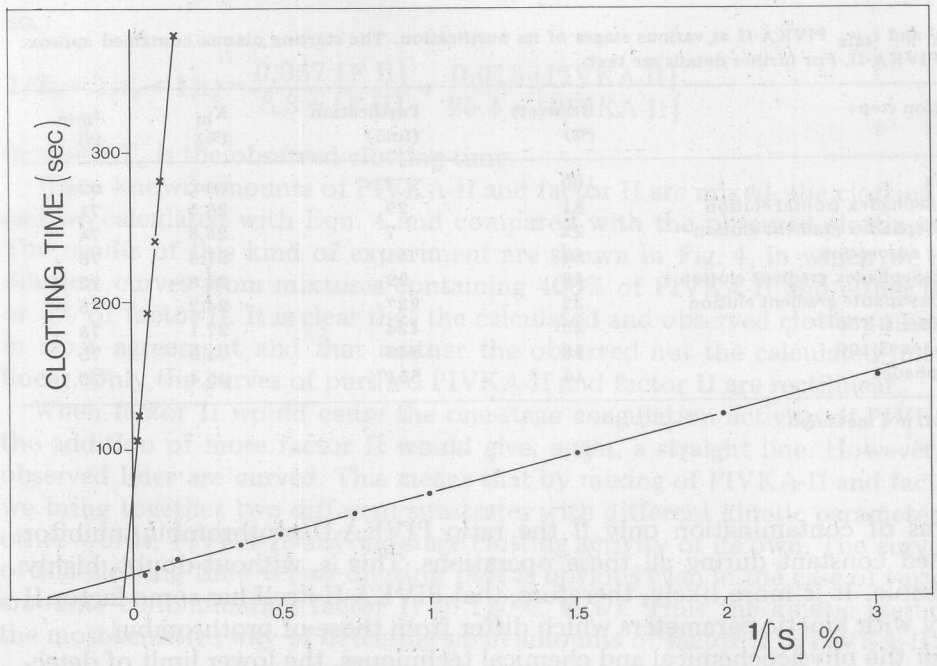

Fig. 3. t vs. D plot of purified factor II and purified PIVKA-II. The clotting times of factor II (●——●) and PIVKA-II (X——X) were clotting against the reciprocal substrate concentration. The clotting times were obtained with the one-stage coagulation assay. The concentration of PIVKA-II was assessed with the *Echis Carinatus* assay.

tion time, t_{min}, (proportional to the inverse of maximal clotting velocity V) are calculated. The values for factor II were $K_m = 3.3\%$ and $t_{min} = 15$ s. For PIVKA-II they were $K^*_m = 25.4\%$ and $t^*_{min} = 75$ s.

If the one-stage clotting activity of our PIVKA-II preparation is caused by contaminating prothrombin only, the different K_m can be readily explained. A contamination of $(3.3/25.4) \times 100\% = 13\%$ (w/w) of prothrombin would bring about this effect. This amount of prothrombin would be detected easily with our chemical and physicochemical techniques. Moreover, when this was the case, evidently t_{min} would not differ. Because t_{min} does differ we have to assume that either some modificator of factor II activity is present in the preparation or that PIVKA-II itself is a substrate in the one-stage factor II assay (see also Discussion).

Several data argue against the first possibility, namely that our PIVKA-II preparation contains both factor II and a factor II inhibitor. Firstly, the amino acid composition of PIVKA-II was found to be similar to that of purified factor II. An inhibitor in PIVKA-II would be detected because the total amino acid composition would differ from that of factor II. Secondly, the ratio between the activities in the one-stage clotting assay and the *E. Carinatus* assay remained constant at all stages of the purification of PIVKA-II. Also the K_m and t_{min} values remained constant (Table I). Even when the purified preparation was adsorbed with excessive amounts of $BaSO_4$, which is known to be a highly selective absorbent for factor II, the K_m and t_{min} did not change, even when 96% of PIVKA-II had been adsorbed from the solution. This can be explained

TABLE I

The K_m and t_{min} PIVKA-II at various stages of its purification. The starting plasma contained approx. 50% of PIVKA-II. For further details see text.

Purification step	Recovery (%)	Purification (fold)	K_m (%)	t_{min} (s)
1 Plasma	100	1	n.m.	n.m.
2 QAE-Sephadex frontal elution	81	29	26.5	71
3 QAE-Sephadex gradient elution	72	7	25.8	74
4 $BaSO_4$ adsorption	68	51	27.3	76
5 DEAE-Sephadex gradient elution	49	99	24.8	72
6 Hydroxyapatite gradient elution	32	237	24.2	75
7 Sephadex G-150	25	424	27.1	73
8 Sephadex G-100	18	543	24.9	75
9 SP-Sephadex	16	551	25.4	75

n.m., not measured.

in terms of contamination only if the ratio PIVKA-II/prothrombin/inhibitor remained constant during all these operations. This is, without doubt, highly improbable. It is more likely, therefore, that PIVKA-II itself has some factor II activity with kinetic parameters which differ from those of prothrombin.

As in the physicochemical and chemical techniques, the lower limit of detection of factor II in a PIVKA-II preparation can be determined by kinetic means by measuring the effect of small amounts of added prothrombin on the clotting time, the K_m and t_{min} values.

As shown by Hemker et al. [22—24] the coagulation reactions of clotting factors obey the rules of general enzyme kinetics, so we may express the reaction velocity $v(= 1/t_c)$ as follows:

$$V = \frac{V \cdot [S]}{K_m + [S]} \tag{1}$$

in which $[S]$ is the substrate concentration (factor II or PIVKA-II). When v and V are expressed as the reciprocal clotting time (t_c) and the reciprocal minimal clotting time (t_{min}) and the observed values are substituted in Eqn. 1 we obtain for factor II:

$$v = 1/t_c = \frac{1/t_{min} \cdot [\text{F II}]}{K_m + [\text{F II}]} = \frac{0.067\ [\text{F II}]}{3.3 + [\text{F II}]} \tag{2}$$

and for PIVKA-II:

$$v^* = 1/t_c^* = \frac{1/t_{min}^*\ [\text{PIVKA-II}]}{K_m^* + [\text{PIVKA-II}]} = \frac{0.013\ [\text{PIVKA-II}]}{25.4 + [\text{PIVKA-II}]} \tag{3}$$

When factor II and PIVKA-II are both present in the same reaction mixtures, the rate of thrombin generation may be thought to be the sum of the individual reaction rates. From Eqns. 2 and 3 we get:

$$V_{total} = V + V^*$$

so

$$1/T_c = 1/t_c + 1/t_c^* = \frac{0.067\ [\text{F II}]}{3.3 + [\text{F II}]} + \frac{0.013\ [\text{PIVKA-II}]}{25.4 + [\text{PIVKA-II}]} \quad (4)$$

in which T_c is the observed clotting time.

When known amounts of PIVKA-II and factor II are mixed, the clotting time can be calculated with Eqn. 4 and compared with the observed clotting time. The results of this kind of experiment are shown in Fig. 4, in which we made dilution curves from mixtures containing 400% of PIVKA-II and either 0.5, 2 or 5% of factor II. It is clear that the calculated and observed clotting times are in close agreement and that neither the observed not the calculated lines are linear. Only the curves of purified PIVKA-II and factor II are rectilinear.

When factor II would cause the one-stage coagulation activity of PIVKA-II, the addition of more factor II would give, again, a straight line. However, the observed lines are curved. This means that by mixing of PIVKA-II and factor II we bring together two different substrates with different kinetic parameters. In other words, PIVKA-II has one-stage clotting activity of its own. The curvature of the clotting time versus dilution plot is obvious even in the case of very low amounts contaminating factor II (0.125%, w/w). Thus the kinetic method is the most sensitive way of detecting small amounts of factor II in PIVKA-II and,

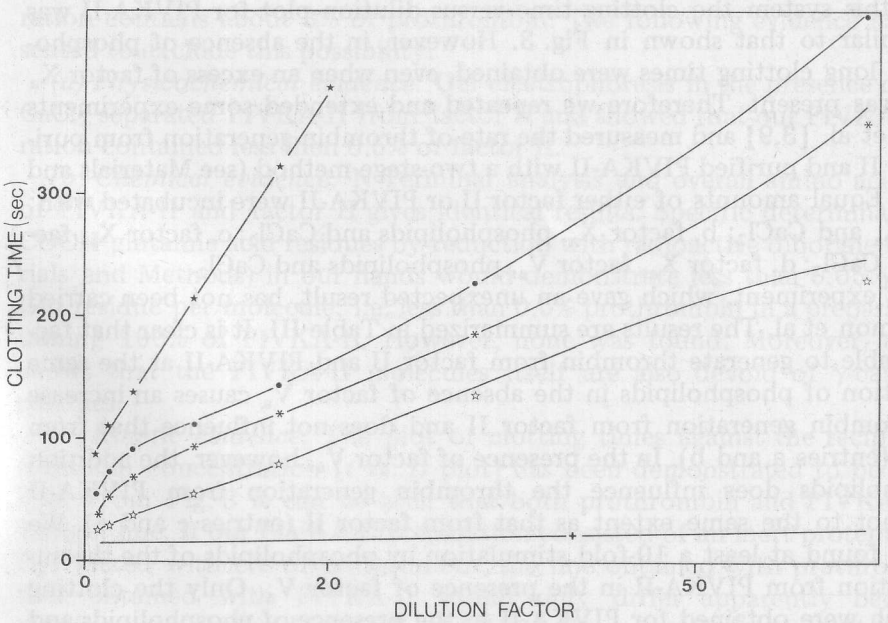

Fig. 4. t vs. D plots of mixtures containing purified PIVKA-II and purified factor II. With the aid of the known values of the K_m and t_{min} of PIVKA-II and factor II the clotting times of these mixtures were calculated (see text). The following samples were examined: ★———★, 4 units/ml of PIVKA-II; ●———●, 4 units/ml of PIVKA-II, containing 0.125% (w/w) of factor II; *———*, 4 units/ml of PIVKA-II, containing 0.5% (w/w) of factor II; ☆———☆, 4 units/ml of PIVKA-II, containing 1.25% (w/w) of factor II; +———+, 4 units/ml of factor II. In the case of the mixtures the solid line is the calculated curve and the markings represent the observed values.

TABLE II

The K_m and V of PIVKA-II after $BaSO_4$ adsorption. 3 ml of purified PIVKA-II were supplemented with $BaSO_4$ and shaken on a Vortex mixer for 2 min. After centrifugation for 20 min at 20 000 × g the PIVKA-II concentration was measured by the *E. Carinatus* assay. Similar amounts of purified factor II were adsorbed completely by less than 2 mg/ml of $BaSO_4$.

$BaSO_4$ added (mg/ml)	PIVKA-II (%) *E. Carinatus* assay	K_m (%)	t_{min} (s)
0	115	25.4	75
20	11	26.8	71
40	4.5	24.9	74

moreover, it gives the direct proof that PIVKA-II is active in the one-stage coagulation assay.

(D) The reaction mechanism of thrombin generation from PIVKA-II

Once we knew that out PIVKA-II preparation did not contain factor II or thrombin, we were interested in the question of how is PIVKA-II converted into thrombin in the one-stage coagulation assay? In other words: can the presence of only factor X_a explain the conversion of PIVKA-II or are other components (factor V, phospholipid) required in this reaction? We therefore modified our one-stage coagulation assay in such a way that the factors VII and X were replaced * by factor X_a while thromboplastin was replaced by phospholipids. In this system the clotting time versus dilution plot for PIVKA-II was almost similar to that shown in Fig. 3. However, in the absence of phospholipids very long clotting times were obtained, even when an excess of factor X_a (1000%) was present. Therefore we repeated and extended some experiments of Esmon et al. [3,9] and measured the rate of thrombin generation from purified factor II and purified PIVKA-II with a two-stage method (see Materials and Methods). Equal amounts of either factor II or PIVKA-II were incubated with: a, factor X_a and $CaCl_2$; b, factor X_a, phospholipids and $CaCl_2$; c, factor X_a, factor V_a and $CaCl_2$; d, factor X_a, factor V_a, phospholipids and $CaCl_2$.

The last experiment, which gave an unexpected result, has not been carried out by Esmon et al. The results are summarized in Table III. It is clear that factor X_a is able to generate thrombin from factor II and PIVKA-II at the same rate. Addition of phospholipids in the absence of factor V_a causes an increase of the thrombin generation from factor II and does not influence that from PIVKA-II (entries a and b). In the presence of factor V_a, however, the addition of phospholipids does influence the thrombin generation from PIVKA-II although not to the same extent as that from factor II (entries c and d). We repeatedly found at least a 10-fold stimulation by phospholipids of the thrombin generation from PIVKA-II in the presence of factor V_a. Only the clotting times which were obtained for PIVKA-II in the presence of phospholipids and factor V_a were in good agreement with those obtained in the one-stage coagulation assay.

* The factors VII and X were removed by $BaSO_4$ adsorption and a small volume of highly concentrated (6000 units/ml) factor X_a was added to the reagent.

TABLE III

The rate of thrombin formation from factor II and PIVKA-II in different activation mixtures. Factor II (50%) or PIVKA-II (50%) were incubated at 37°C with different combinations of activators. The incubations were carried out in reaction mixtures containing 20 mM Tris · HCl, pH 7.4, 8 mM $CaCl_2$ and 0.1 M NaCl. The concentrations of other components (if added) were: factor X_a, 14%; factor V_a, 14%; and phospholipids, 50 μg/ml. The activation rate is given in N.I.H. units of thrombin formed per min per ml. The relative rate of entry a was arbitrarily chosen to be l.

Activators	Factor II		PIVKA-II	
	Activation rate	Relative rate	Activation rate	Relative rate
(a) X_a, Ca^{2+}	0.04	1	0.04	1
(b) X_a, Ca^{2+}, PL	0.3	7.5	0.04	1
(c) X_a, Ca^{2+}, V_a	4.0	100	1.0	25
(d) X_a, Ca^{2+}, V_a, PL	440	11 000	12	300

Discussion

PIVKA-II preparations always show thrombin generation in a one-stage prothrombin assay. The clotting time obtained cannot be explained solely by the action of factor X_a in solution. Therefore, we wish to prove that the generation of thrombin from PIVKA-II by factor X_a is accelerated by phospholipids and factor V. This phenomenon is shown in the experiments reported in Table III. A trivial explanation for these observations would be that our PIVKA-II preparation contains about 3% of prothrombin. The following evidence can be presented to exclude this possibility.

(a) Physicochemical evidence. Gel electrophoresis in the presence of 1.5 mM $CaCl_2$ separated PIVKA-II from factor II and showed that our PIVKA-II preparation contained less then 0.5% of factor II.

(b) Chemical evidence. N-Terminal analysis and overall amino acid analysis of PIVKA-II and factor II gives identical results. Specific determination of γ-COOH glutamic acid residues by reduction with radioactive diborane (see Materials and Methods) in our hands would demonstrate less than 0.05 γ-carboxy-Glu residue per molecule, i.e. less than 0.5% prothrombin in a preparation containing 100% of PIVKA-II. However, none was found. Moreover, this result shows that the PIVKA-II molecules itself are also devoid of γ-carboxy-Glu residues.

(c) Kinetic evidence. The plot of clotting times against the reciprocal prothrombin concentration (t vs. D plot) has been demonstrated to be a straight line. From Fig. 3 it can be seen that both prothrombin and PIVKA-II give a straight line. If our PIVKA-II preparation consisted of an inert protein (PIVKA-II) mixed with c% of prothrombin, the line obtained with prothrombin and that obtained with PIVKA-II would only differ apparently because the clotting time obtained with p% of PIVKA-II is entered at a point at the abcissa corresponding to $1/p$ whereas it should be entered at $100/c \times 1/p$. The effect would obviously be that the PIVKA-II line is the prothrombin line represented with an abcissa which is 'shrunk' by a factor $100/c$. The intercept with the ordinate would remain the same, but the intercept with the abcissa would be shifted right by the factor $c/100$, i.e. t_{min} would be identical to t^*_{min} and the

K_m^* should be $100/c \times K_m$. From Fig. 3 it is seen that (a) t_{min} is not identical and (b) $K_m^* = 8\ K_m$. As is argued above, (a) cannot be explained by contamination and (b) would be explained only by a 12.5% contamination of factor II in PIVKA-II. These amounts of prothrombin would have been readily detected by our chemical and physicochemical methods.

A theoretical possibility remains that our PIVKA-II preparation contains both normal prothrombin and a competitive inhibitor of normal prothrombin conversion and that this would explain our results. Three reasons argue against this explanation: (1) It would not explain the curved t vs. D plots upon mixing prothrombin and PIVKA-II (Fig. 4). (2) Adsorption onto $BaSO_4$ (as well as the PIVKA-II purification procedure) would shift the ratio prothrombin/inhibitor and thereby cause other apparent K_m and t_{min} values. It does not in the actual experiment. (3) The overall amino acid composition of PIVKA-II is similar to that found by Magnusson et al. [21] for factor II. A competitive inhibitor would change the amino acid composition.

It is possible to calculate the combined effect of prothrombin in a PIVKA-II preparation on basis of the t_{min} and K_m read from Fig. 3. In general this will result in curved t vs. D plots. The curvature of these plots is obvious even when factor II is present in PIVKA-II at a ratio of 1 : 800 (Fig. 4).

If the one-stage coagulation activity of PIVKA-II were due to the presence of contaminating factor II, straight lines with coinciding t_{min} would be expected upon addition of more factor II. The fact that we observed curved lines thus proves that PIVKA-II itself has clotting activity in the one-stage coagulation assay.

This conclusion is important for the interpretation of the results shown in Table III. In this table we show that in the absence of factor V_a the thrombin generation from PIVKA-II by factor X_a is not accelerated by the addition of phospholipids. On the contrary, in the presence of factor V_a, phospholipids stimulate this thrombin generation about 10-fold.

The only way to interpret these results is by assuming that by its affinity to factor V_a, PIVKA-II is able to bind indirectly to phospholipids, thus becoming more accessible to the proteolytic activity of factor X_a. Because Ca^{2+} were required for effective action of factor X_a, we were not able to investigate whether factor V_a needs Ca^{2+} in its bridging function between phospholipids and PIVKA-II.

Between factor II and PIVKA-II there are no differences in the fragment-2 region, which is the factor V_a-binding part [2,9]. Therefore we may expect that also during the conversion of prothrombin into thrombin, factor V_a binds to prothrombin and that this binding is essential for the accelerating effect of factor V in this reaction. This indicates that factor II is bound to the phospholipids in two ways: directly via γ-carboxy-Glu residues and indirectly via factor V_a. As is shown in Table III, there is a difference (about 4-fold) between the rate of thrombin generation from PIVKA-II and factor II in the presence of only the factors V_a and X_a. This is a variance with the results of Esmon et al. [9]. We cannot offer an explanation at this moment. Possible explanations are (a) trace amounts of phospholipid in our factor V preparation, but not in Esmon's; (b) trace amounts of prothrombin in Esmon's PIVKA-II preparation; (c) differences due to slightly different experimental conditions (e.g. concentra-

tion of enzyme, substrate, etc.). We consistently found this difference, even in experiments in which we approached Esmon's conditions as closely as possible, and this renders explanation c less likely.

In order to find out whether also factor X_a has some affinity to factor V_a, the experiments of Table III should be repeated with reaction mixtures containing factor II, Ca^{2+} and PIVKA-X_a or factor X_a, whether or not supplemented with factor V_a, phospholipid or a combination thereof. Up to now, however, experiments of this kind have been hampered by the fact that it is very hard to prove that a PIVKA-X preparation does not contain trace amounts of factor X.

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