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The determination of prothrombin using synthetic chromogenic substrates

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THE DETERMINATION OF PROTHROMBIN USING SYNTHETIC CHROMOGENIC SUBSTRATES; CHOICE OF A SUITABLE ACTIVATOR

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ABSTRACT

Normal prothrombin (factor II) is determined in whole plasma by activation either with staphylocoagulase, or with Taipan snake venom (TSV) or with human factor X_a. In a second reaction, the amount of activated prothrombin (factor II_a) is assayed by the amidolysis of synthetic chromogenic substrates. A convenient preparation of sufficiently pure human factor X_a is described. A linear relation was found between the prothrombin concentration and the amount of p-nitroanilides generated per unit of time. In normal plasma staphylocoagulase and the factor X_a preparation give similar results. As staphylocoagulase coestimates decarboxyprothrombin, it cannot be used to assess prothrombin during oral anticoagulation or vitamin K deficiency.

In this respect, Taipan snake venom and Echis Carinatus venom behave in a similar way as staphylocoagulase does. Moreover, TSV is inhibited by phospholipids. Ca^{++} has no effect on the activation of prothrombin neither by staphylocoagulase nor by Taipan snake venom.

INTRODUCTION

The chromogenic substrates used in coagulation factor assays are synthetic peptides, which liberate the yellow p-nitroanilide when they are cleaved by proteases. The different peptides are more or less specific for different activated clotting factors (1,2). For example Bz-Gly-Pro-Arg-PNa, and H-D-Phe-pip-Arg--PNa are more sensitive to thrombin than Bz-Ile-Glu-(γ-OR)-Glu-Arg-PNa, which is more sensitive to factor X_a. The methods developed with these substrates can be used for clinical and kinetic studies in which purified thrombin (3) or factor X_a are determined in the presence of antithrombin III (4) or heparin (5).

For a routine assay of prothrombin in plasma or anticoagulated patients, two conditions hold first: a. All prothrombin should be activated directly before testing in a time that is short compared to the time scale of inactivation by antithrombin III, and b. Only normal prothrombin should be converted into thrombin and decarboxyprothrombin should not (2,6,7,8). When using chromogenic substrates, a third requirement is that the prothrombin activating agent should not act directly on the chromogenic substrate. As staphylocoagulase generates thrombin activity from normal prothrombin as well as from decarboxyprothrombin, it is not suitable for the detection of prothrombin in dicoumarol plasma (9,10). Neither is Echis Carinatus venom. On the other hand, it was suggested by several authors (11,12,13) that Taipan snake venom (venom from Oxyuranus scutellatus) activates prothrombin like the physiological activator factor X_a does, and that this process is dependent on Ca^{++} and phospholipids. This venom therefore was a suitable candidate for the required activation of prothrombin. Another possible activator, of course, is factor X_a. We, therefore, developed an easy preparation procedure for human factor X_a and tested this factor for its usefulness in our assay system.

MATERIALS AND METHODS

Buffers

Buffer A: 0.075 M NaCl, 0.075 M imidazole, 0.075 M tris-HCl, pH 8.4.

Buffer B: 0.087 M NaCl, 0.0029 M sodium acetate, 0.0029 M sodium barbital, pH 7.4.

Normal plasma

Platelet-poor plasma was pooled from 30 healthy donors, 15 males and 15 females with a mean age of 30 years. The blood was collected in 0.13 M trisodium citrate (10%, v/v), centrifuged for 15 min at 1,500 x g at room temperature, after which the plasma was centrifuged for 30 min at 20,000 x g at 4°C. Plasma samples were stored at -30°C for several months.

Phospholipids

Phospholipids were prepared according to Bell and Alton (14) and dissolved in buffer B to a concentration of 250 µg/ml.

Venoms

Taipan snake venom (TSV), Echis Carinatus venom, and Russell's Viper venom were obtained from Sigma (USA) and dissolved in water in a concentration of 1 mg/ml. The two components of the latter (RVV-V and RVV-X) which activate factors V and X, respectively, were separated as described by Schiffmann et al. (15). Staphylocoagulase was prepared according to the method of Soulier (16), modified as described by Bas et al. (17). In the test the protein con-

centration was 3.75 mg/ml.

Chromogenic substrates

Chromozym TH^r was obtained from Boehringer Mannheim (Germany) and S 2238 from Kabi (Sweden).

Human factor X_a

Four ml normal plasma were mixed with 0.1 ml Echis Carinatus venom and $CaCl_2$ was added to a final concentration of 6.6 mM. After incubation for 2 hours at 37°C the clot was removed by centrifugation (10 min, 2,000 x g). In this way all fibrinogen and prothrombin were removed and no thrombin remains (18,19). The supernatant was adsorbed with $BaSO_4$ (100 mg/ml) and the sediment was washed three times with buffer B and subsequently the factors VII, IX, and X were eluted with 2 ml 0.2 M trisodium citrate pH 5.8. After each step the $BaSO_4$ was removed from the solution by centrifugation for 10 min at 2,000 x g. The eluate was dialyzed against 0.15 M NaCl and stored in 0.25 ml aliquots at -30°C. Within 6 months no loss of activity occurred, Factor X_a was generated from these samples by adding 1 µl of RVV-X and 30 µl 0.1 M $CaCl_2$ to 0.25 ml sample. The mixture was stored at room temperature overnight and was used the subsequent day for all tests. The procedure results in a complete activation of factor X. (20, 21, 22)

Measurement of amidolysis

The absorbance was recorded in an Aminco DW 2 spectrophotometer in the split beam mode with microcuvettes (400 µl) and d=1 cm. All reagents were kept at room temperature and pipetted directly in the cuvette at a constant temperature of 37°C. The measuring wavelength was 391 nm against 344 nm as a reference.The results were calculated as absorbance change per minute. The reaction mixtures (400 µl) contained: buffer, sample (1-4 µl), phospholipids (1.25 µg/ml,if added), $CaCl_2$ (10 mM, if added), and activator and were pipetted in this order. The activators which were used, were either staphylocoagulase (15 µl, corresponding to 148 µg/ml in the reaction mixture) or Echis Carinatus venom or TSV (75 µg/ml) or factor X_a. The mixtures containing TSV were incubated for 90 s and those containing factor X_a for 120 s before the substrate was added. The substrate concentrations were 148 µM (S 2238) and 187.5 µM (chromozym TH^r).

Coagulation assays

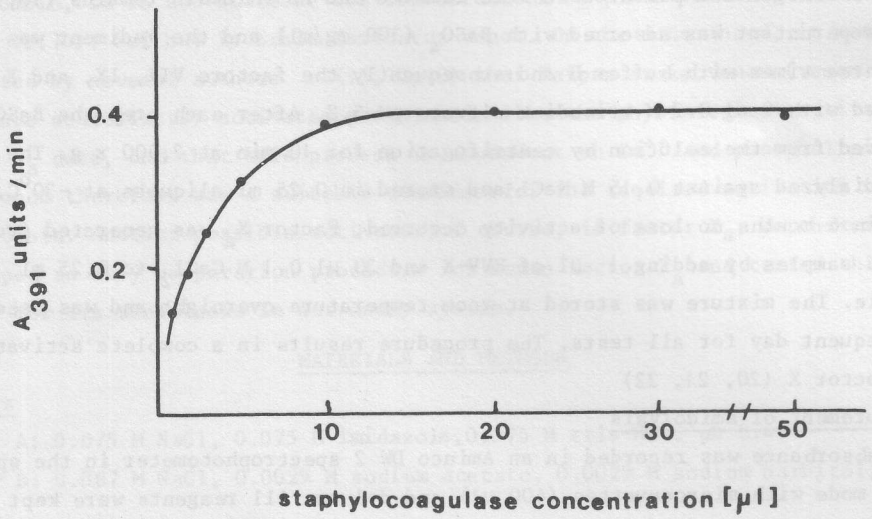
The one-stage prothrombin assay and the two-stage Echis Carinatus assay were performed as described by Vermeer et al. (19).

RESULTS

A. The determination of prothrombin after activation with staphylocoagulase

Staphylocoagulase activated normal prothrombin and decarboxyprothrombin almost instantaneously in the absence of Ca^{++} and phospholipids. Sufficiently purified staphylocoagulase had no demonstrable amidolytic activity of its own. In fig. 1, the concentration of staphylocoagulase is plotted against the initial velocity of amidolysis. The results obtained with S 2238 were similar to those obtained with chromozym TH^r, shown in fig. 1, and indicate that 10 µl staphylocoagulase give a maximal activation of prothrombin in 1.25% final concentration of normal plasma.

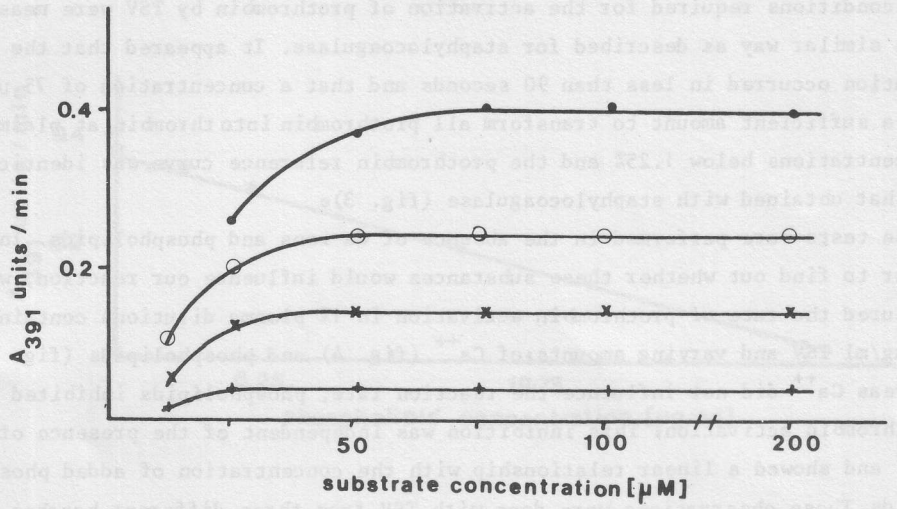
FIG.1.



The relation between the rate of thrombin-induced amidolysis and the amount of staphylocoagulase. The reaction was performed with a 1.25% normal plasma dilution (final concentration) and with chromozym TH^r as a substrate. The other components of the reaction mixture were as described in Materials and Methods.

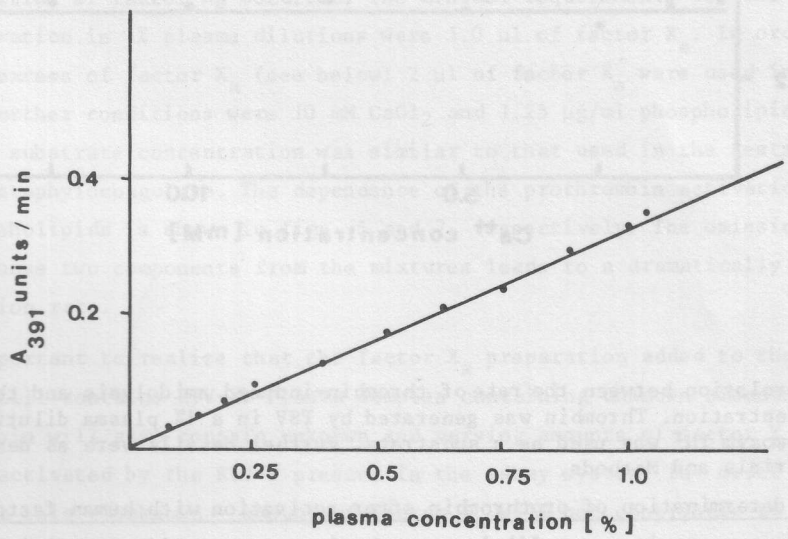
With an excess of staphylocoagulase (15 µl) together with different prothrombin concentrations, the dependence of the observed peptidase activity on the substrate concentrate was tested (fig. 2). It appeared that at plasma concentrations below 1.25% 50 M substrate was a sufficient amount to give a maximal reaction velocity. With an excess of both the activator (15 µl) and the substrate (187.5 µM) we measured the initial reaction velocities in a great number of plasma dilutions. As is shown in fig. 3, there is a linear relationship between the initial reaction velocity and the plasma concentration between 0 and 1 %. This curve may be used as a reference curve for the determination of unknown prothrombin concentrations. Because the concentration of prothrombin should be the only rate-determining factor, all other components in the reaction mixture should be present in excess.

FIG.2.



The relation between the rate of thrombin induced amidolysis and the substrate concentration.The reaction was performed with normal plasma dilutions (final concentration) of: 1.25 % (• —— •), 0.5% (o —— o), 0.25% (x —— x), and 0.125% (+ —— +). Staphylocoagulase was used as prothrombin activator. Further details were as described in the legend to fig. 1.

FIG.3.



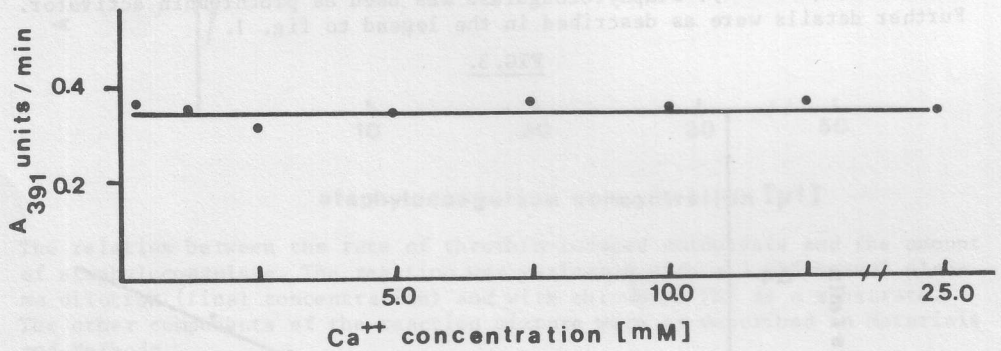
Prothrombin reference curve prepared from normal plasma dilutions. The plasma concentrations are expressed as the final concentrations in the reaction mixture. Further details were as described in the legend to fig. 1.

B. The determination of prothrombin after activation with Taipan snake venom

The conditions required for the activation of prothrombin by TSV were measured in a similar way as described for staphylocoagulase. It appeared that the activation occurred in less than 90 seconds and that a concentration of 75 µg/ml was a sufficient amount to transform all prothrombin into thrombin at plasma concentrations below 1.25% and the prothrombin reference curve was identical to that obtained with staphylocoagulase (fig. 3).

These tests were performed in the absence of Ca-ions and phospholipids. In order to find out whether these substances would influence our reaction, we measured the rate of prothrombin activation in 1% plasma dilutions containing 75 µg/ml TSV and varying amounts of Ca^{++} (fig. 4) and phospholipids (fig. 5). Whereas Ca^{++} did not influence the reaction rate, phospholipids inhibited the prothrombin activation. This inhibition was independent of the presence of Ca^{++} and showed a linear relationship with the concentration of added phospholipids. These observations were done with TSV from three different batches.

FIG.4.

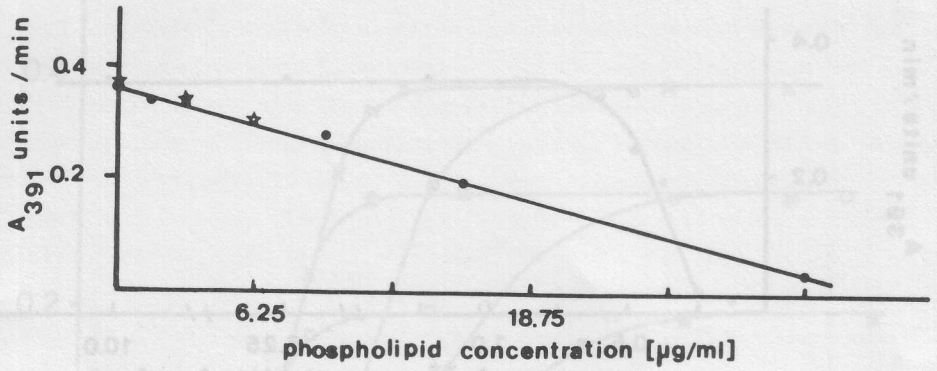


The relation between the rate of thrombin-induced amidolysis and the $CaCl_2$ concentration. Thrombin was generated by TSV in a 1% plasma dilution. Chromozym TH^r was used as a substrate. Further details were as described in Materials and Methods.

C. The determination of prothrombin after activation with human factor X_a

For our purposes it was sufficient to obtain a preparation containing one single specific component that activates prothrombin. Bovine factor X_a caused a direct cleavage of the chromogenic substrates and could not be used therefore. In the human factor VII,IX,X concentrate (see Materials and Methods), factor X could be activated (20, 21, 22). The solution containing

FIG.5.

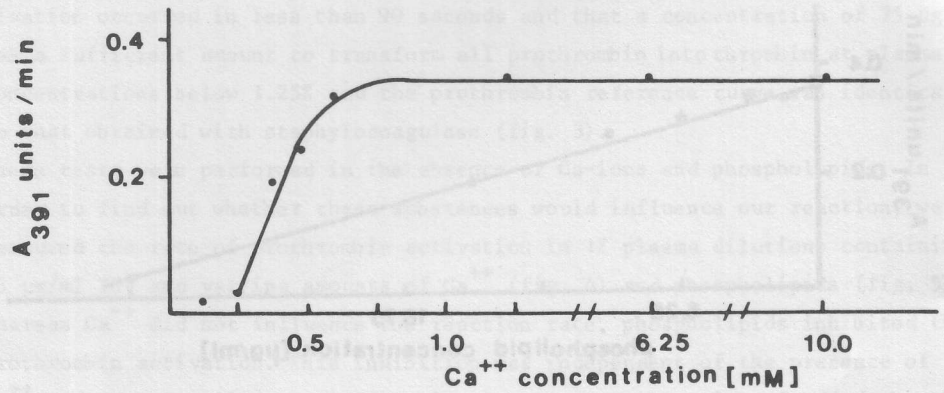


The relation between the rate of thrombin-induced amidolysis and the phospholipid concentration in the presence (x —— x) and absence (• —— •) of $CaCl_2$. Details were as described in the legend to fig. 4.

factor X_a was stable for several hours at room temperature which indicates that no inhibition of factor X_a occurred. The minimal requirements for the prothrombin activation in 1% plasma dilutions were 1.0 µl of factor X_a. In order to have an excess of factor X_a (see below) 2 µl of factor X_a were used in all tests. Further conditions were 10 mM $CaCl_2$ and 1.25 µg/ml phospholipids. The required substrate concentration was similar to that used in the tests with TSV and staphylocoagulase. The dependence of the prothrombin activation on Ca^{++} and phospholipids is shown in figs. 6 and 7, respectively. The omission of one of those two components from the mixtures leads to a dramatically decreased reaction rate.

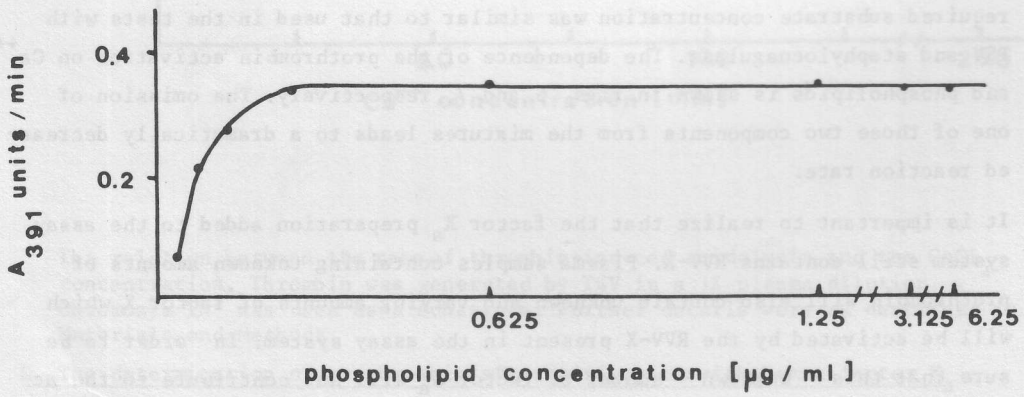
It is important to realize that the factor X_a preparation added to the assay system still contains RVV-X. Plasma samples containing unknown amounts of prothrombin will also contain unknown and varying amounts of factor X which will be activated by the RVV-X present in the assay system. In order to be sure that this - unknown - amount of factor X_a will not contribute to the activation of prothrombin, the reaction rate of this activation should be the maximal, i.e.: an excess of externally added factor X_a should be present in the assay mixtures. This requirement was controlled by measuring the time-course of the thrombin generation in plasma dilutions in the presence of factor X_a (thus containing RVV-X) and in the presence of a similar concentra-

FIG.6.



The relation between the rate of thrombin-induced amidolysis and the $CaCl_2$-concentration. Human factor X_a was used as the prothrombin activator in a 1% dilution (final concentration). Chromozym TH^r was used as a substrate. Further details were as described in Materials and Methods.

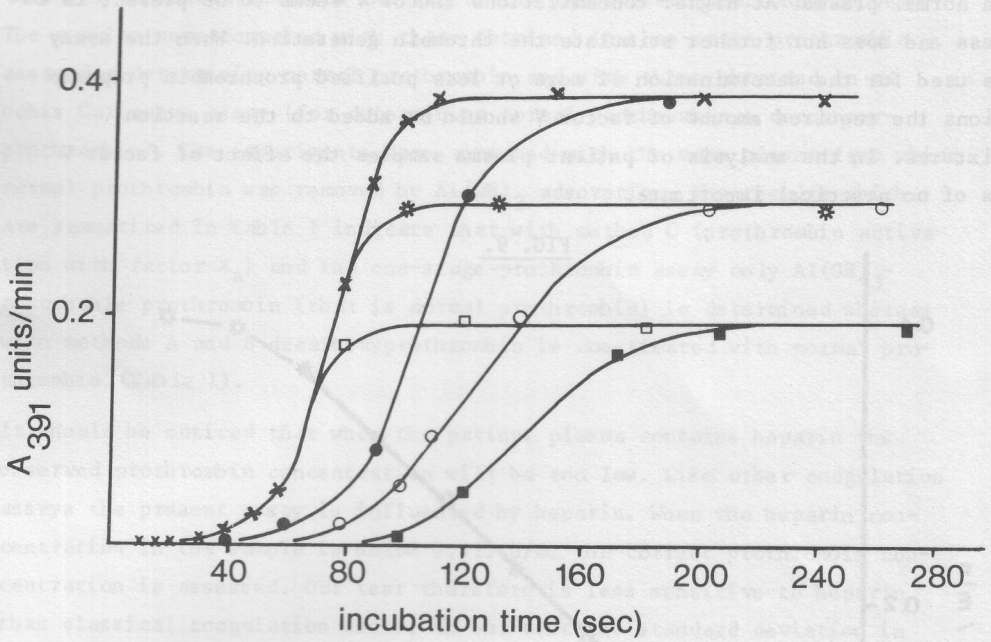
FIG.7.



The relation between the rate of thrombin-induced amidolysis, and the phospholipid concentration. Details were as described in the legend to fig. 6.

tion of RVV-X alone (fig. 8).

FIG.8.

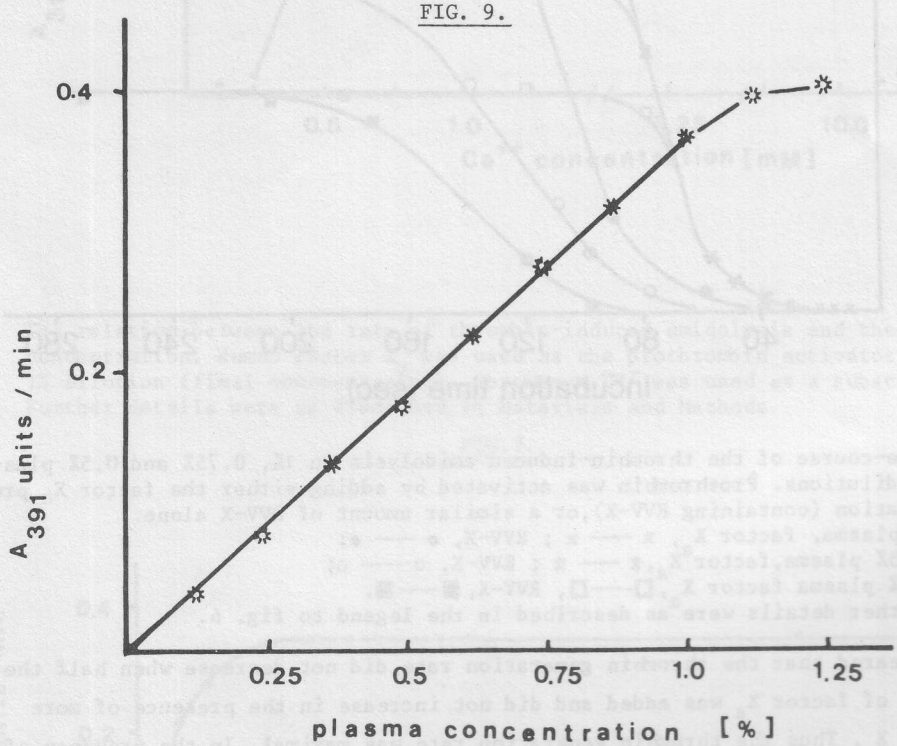


Time-course of the thrombin-induced amidolysis in 1%, 0.75% and 0.5% plasma dilutions. Prothrombin was activated by adding either the factor X_a preparation (containing RVV-X),or a similar amount of RVV-X alone.
1% plasma, factor X_a, x —— x ; RVV-X, • —— •;
0.75% plasma,factor X_a,* —— * ; RVV-X, o —— o;
0.5% plasma factor X_a,□——□, RVV-X,■——■.
Further details were as described in the legend to fig. 6.

It appeared that the thrombin generation rate did not decrease when half the amount of factor X_a was added and did not increase in the presence of more factor X_a. Thus the thrombin generation rate was maximal. In the presence of RVV-X alone, the thrombin generation was slower, dependent on the concentration RVV-X (not shown here), and the difference with the maximal thrombin generation rate increased at lower plasma dilutions. From these experiments we concluded that within 120 s all prothrombin can be converted into thrombin by the externally added factor X_a and that the reaction rate is not influenced by the presence of varying amounts of factor X in the plasma samples to be assayed. Therefore, a reference curve as shown in fig. 9. may serve to establish the prothrombin concentration in unknown plasma as well as in dicoumarol plasma and in factor X-deficient plasma samples. On the other hand, the presence of factor V in the plasma sample will influence the rate of thrombin

generation and then that of amidolysis. It was found that this effect is of no importance when the sample concentration of factor V is above 1 % of that in normal plasma. At higher concentrations factor V seems to be present in excess and does not further stimulate the thrombin generation. When the assay is used for the determination of more or less purified prothrombin preparations the required amount of factor V should be added to the reaction mixtures. In the analysis of patient plasma samples the effect of factor V is of no practical importance.

FIG. 9.



Prothrombin reference curve prepared from normal plasma dilutions. Details were as described in the legend to fig. 6.

D. The determination of prothrombin in plasma from anticoagulated patients

In the plasma of patients under anticoagulant therapy, generally two forms of prothrombin occur: normal prothrombin, which is active in coagulation and decarboxyprothrombin, which is an inactive prothrombin precursor. Both forms of prothrombin can be converted into thrombin with Echis Carinatus venom. A quick determination of normal prothrombin in this kind of plasma is of high clinical importance. Therefore, we prepared pooled

plasma from 20 long-term anticoagulated patients and determined the prothrombin concentration with the aid of the three tests mentioned above. The results were compared with those obtained in a one-stage prothrombin assay for determining normal prothrombin and with those obtained in an Echis Carinatus assay (for determining normal prothrombin + decarboxyprothrombin). The experiments were repeated with the same plasma from which normal prothrombin was removed by $Al(OH)_3$ adsorption. The results which are summarized in table I indicate that with method C (prothrombin activation with factor X_a) and the one-stage prothrombin assay only $Al(OH)_3$-adsorbable prothrombin (that is normal prothrombin) is determined whereas with methods A and B decarboxyprothrombin is coestimated with normal prothrombin. (Table I).

It should be noticed that when the patient plasma contains heparin the observed prothrombin concentration will be too low. Like other coagulation assays the present assay is influenced by heparin. When the heparin concentration in the sample is below 0.125 U/ml the correct prothrombin concentration is assessed. Our test therefore is less sensitive to heparin than classical coagulation tests. In our test the standard deviation in duplicate was about 1%.

DISCUSSION

In principle it has several advantages to determine clotting factor concentrations with the aid of chromogenic substrates:

1. The determination is independent of the many factors that influence the clot formation in normal plasma.
2. Many samples can be assayed automatically in a short period of time with the standard equipment of a laboratory of clinical chemistry.
3. Kinetic data can be determined directly.

Most coagulation tests are performed to estimate the effect of oral anticoagulants in patients. All routine methods in non-specialized hospitals are not only dependent on the measuring of clot formation, but also on the simultaneous determination of several clotting factors and the properties of a poorly defined tissue thromboplastin. So we tried to develop a more accurate method for the specific determination of normal prothrombin independent of the concentration of decarboxyprothrombin or other coagulation factors. As two chromogenic substrates were available which could only be cleft with thrombin, and not with any of the other activated factors of human origin, our only

problem was to find an enzyme that activated normal prothrombin and not decarboxyprothrombin. Four activators were compared: staphylocoagulase, Taipan snake venom, Echis Carinatus venom and a preparation containing human factor X_a. Staphylocoagulase is known to activate both normal prothrombin and decarboxyprothrombin (9) and factor X_a is supposed to activate only normal prothrombin.

In contrast to the data in the literature (11, 12, 13, 23) TSV turned out to activate decarboxyprothrombin and in general behaved much more like staphylocoagulase and like Echis Carinatus venom than like factor X_a. A second disadvantage of TSV was the fact that the thrombin generation rate proved to be dependent on the phospholipid concentration in the reaction mixture.

On the other hand, it turned out that human factor X_a was an activator with the required specificity for normal prothrombin. In dicoumarol plasma this test measures an amount of prothrombin that is comparable to the value obtained with the one-stage coagulation assay (table I). After removal of normal prothrombin by an small amount of $Al(OH)_3$, almost no prothrombin could be detected with one of these tests. When the same plasma dilutions were assayed with staphylocoagulase or TSV as prothrombin activators, the data were similar to those in the Echis Carinatus assay and represent the sum of prothrombin and decarboxyprothrombin. So we concluded that in the assay system in which human factor X_a was used as a prothrombin activator, only normal prothrombin is detected.

TABLE I

	Prothrombin determination (%) with chromogenic substrate and prothrombin activator:			Prothrombin determination (%) with	
	staphylocoagulase	TSV	factor X_a	one-stage coagulation assay	Echis Carinatus assay
I	54.0	53.0	20.0	19.5	56.5
II	30.0	30.5	2.0	2.0	32.0

Comparison of varying prothrombin determination methods in dicoumarol plasma before (I) and after (II) $Al(OH)_3$ adsorption. For this adsorption 0.1% (w/v) $Al(OH)_3$ was used. For further details, see text.

The test can be performed in a few minutes and only very small amounts of plasma are required. The high plasma dilution prevents awkward clot formation in the cuvettes. The reaction velocity of amidolysis shows a linear relation with the prothrombin concentration in plasma dilutions between 0 and 1%. Moreover, the activating solution (factor X_a) is easy to produce and stable; further purification is without advantages for measurements in plasma. The activation of factor X may be carried out either with purified RVV-X or with crude RVV, which is commercially available. Finally, this method of prothrombin determination is independent of the presence of other plasma components.

Studies, in which varying methods of prothrombin determination in a number of anticoagulated patient plasmas are compared, are in current progress.

ACKNOWLEDGEMENT

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