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Dilution Curve Studies in Prothrombin Complex Factors Deficiencies and Abnormalities

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Summary

Normal, coumarin, factor II deficient, factor VII deficient, factor X deficient, factor IX deficient, abnormal factor IX and abnormal factor X (factor X Friuli) plasmas were diluted 1 : 1, 1 : 2, 1 : 3, 1 : 5 and 1 : 10. Variable aliquots of such dilutions were then added to several tissue thromboplastins and the clotting time measured. Four thromboplastin reagents were used: Thrombotest, Normotest, Manchester Thromboplastin and Simplastin A. Using Manchester Thromboplastin absorbed normal plasma was added to the system too.

The "inhibitory effect" present in the system was evaluated by means of conventional units "i".

Using Thrombotest, Manchester Thromboplastin or Simplastin A a variable but clear "inhibition" ("i" > 0.5 units) was found in all plasmas. Using Normotest a slight "inhibition" was present only in coumarin plasmas and in factor VII or factor X deficient plasmas.

In factor IX deficient plasma no inhibition could be assessed since in all cases it gave a dilution curve identical to the normal plasma one.

The studies indicate that the dilution curve analysis has no value in the case of single congenital prothrombin factors deficiencies since these plasmas "behave" in the system in a way similar to coumarin plasma.

Zusammenfassung

Normalplasma und Cumarinplasma, ferner die Mangelplasmen von Faktor II, VII, X, IX sowie abnormales Faktor IX- und abnormales Faktor X- (Faktor X Friuli)-Plasma wurden 1 :1, 1 : 2, 1 : 3, 1 : 5 und 1 : 10 verdünnt. Wechselnde Aliquote dieser Verdünnungen wurden verschiedenen Gewebsthromboplastinen zugegeben und die Gerinnungszeiten bestimmt.

Vier Thromboplastin-Reagenzien kamen zur Verwendung: Thrombotest, Normotest, Manchester-Thromboplastin und Simplastin A.

Der Hemmeffekt wurde in Einheiten „i"ausgedrückt, die Gerinnungszeiten in Verdünnungskurven dargestellt und deren Bedeutung diskutiert.

Congenital prothrombin complex factors deficiencies represent a heterogeneous group of rare coagulation disorders of extreme scientific interest. In the plasma of the patients with these disorders only one factor is absent or reduced. On the contrary, patients given coumarin derivative drugs become deficient in all these factors.

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The laboratory control of anticoagulant therapy has been drawing considerable attention over the past years [*1,2,14,16,17,18,21*]. Recently the dilution curve has been proposed in the study of the sensitivity of tissue thromboplastins towards the coumarin plasma [*1,12,20*]. This approach would seem to be justified on the basis of those studies which indicate that an inhibitor (PIVKA = prothrombin inhibitor vitamin K absence) appears in coumarin treated patients [*10,11*].

It was maintained that the degree of sensitivity of a given thromboplastin to such inhibitor is proportional to the distance from the Y-axis at which the prolongation of the test plasma line and a rect parallel to the abscissa drawn from the point of the Y-axis intersected by the control plasma line, encounter [*12*]. If the two lines encounter at or near the Y-axis no inhibition is present. On the contrary, if the lines meet on the left of the Y-axis an inhibition is thought to be present in the system. Factor X was postulated to be the rate-limiting factor in the reaction [*11,12*].

The original studies were carried out using the Thrombotest reagent namely an ox-brain thromboplastin [*11,12*].

Subsequently other tissue thromboplastins have been used [*1*]. However the studies so far reported have dealt exclusively with coumarin plasmas.

No data are available on the type of dilution curve obtainable in prothrombin complex factors deficiencies.

Recently we have shown that an apparently "inhibitory" type of dilution curve is present in the abnormal factor X (factor X Friuli) coagulation disorder [*8*]. Since cross-correction studies and specific inhibitor studies failed to show the presence of any inhibitor in the Friuli plasma we suspected that the phenomenon was an artefact and that the same could be true for other congenital prothrombin complex factors deficiencies.

Should this be proven to be the case it would mean that in congenital single factor deficiencies no conclusion as to the presence or not of an inhibitor can be drawn on the basis of the dilution curve studies.

The opportunity we had to study extensively in recent years several patients with these rare disorders prompted this report.

Material and Methods

Plastic syringes and siliconized No. 19 needles.
Plastic tubes with 10 mm inner diameter.
Michaelis buffer at pH 7.3.
Silicone 200/350 (Società Generale Siliconi, Milan, Italy).

The following plasmas were used:

1) Pooled citrated plasma as obtained from at least five normal individuals of both sexes.

2) Coumarin plasma No. 1 was obtained from a patient on a long-term treatment with acenocoumarin and a prothrombin-time at the time of study of 33.9 sec. (10%) and a P/N ratio of 2.5.

3) Coumarin plasma No. 2 was obtained also from a patient on long-term therapy with acenocoumarin. The prothrombin time of this plasma at the time of study was 22.5 sec. (27%) and the P/N ratio was 1.6.

4) Fresh plasma of a patient known to have congenital hypoprothrombinemia. The prothrombin content of this plasma was about 10% of normal whereas all other clotting factors were within normal limits [*5*].

5) Fresh plasma of a patient with the abnormal factor X (factor X Friuli) coagulation disorder. The factor X content in this plasma was 5–15% of normal using tissue thromboplastins but was normal using a Stypven-cephalin mixture. All other clotting factors were within normal limits [*4,6*].

6) Deep frozen plasma of a patient with known congenital factor VII defect. The factor VII level in this plasma was 3% of normal whereas all other clotting factors were within normal limits [*9*].

7) Deep frozen plasma of a patient with classical hemophilia B. The factor IX content of this plasma was <1% of normal.

8) Deep frozen plasma of a patient with hemophilia B_+ or B_M. The factor IX content of this plasma was about 1% of normal. Immunologically the factor IX level was about 30% of normal. The ox brain thromboplastin prothrombin time in this patient was 86 sec. (Normal 40 sec.) [*7*].

9) Lyophilized factor X deficient plasma as supplied by Dade Laboratories, Miami, U.S.A.

All plasmas were non-contacted plasmas but for the factor X deficient plasma. In all cases however the dilutions were carried out in plastic tubes.

The dilutions used in each case were: 1 : 1, 1 : 2, 1 : 3, 1 : 5, 1 : 10.

In a few instances the 1 : 3 or the 1 : 10 dilution was skipped.

The dilution studies were carried out using the following thromboplastins:

1) Thrombotest lots 351 and 408, as supplied by Nyegaard Laboratories, Oslo, Norway.
2) Normotest lot 122 as supplied by Nyegaard Laboratories.
3) Manchester thromboplastin as received from Dr. Poller L., Withington Hospital, Manchester (England) on the following dates: 6/17/72; 9/13/72; 11/14/72. Each batch, upon receipt in Padua, gave a prothrombin time on our normal control plasma of 13.5–14 sec.

The tests were carried out as follows:

a) Thrombotest: the reagent was reconstituted with 2.2 ml. of 3.2mM $CaCl_2$ sol. and incubated at 37° C for 5 min. To 0.25 ml. of the reagent 0.05 ml. of the test plasma or dilution thereof was then added and the clotting time measured.
b) Normotest: the reagent was reconstituted with 2.2 ml. of dist. water and incubated at 37° C for 5 min. To 0.25 ml. of such reagent 0.01 ml. of the test plasma or dilution thereof was added and the clotting time measured.
c) Manchester thromboplastin: the reagent as received from Dr. Poller was mixed with equal parts of 0.024mM $CaCl_2$ and the mixture incubated at 37° C for 5 min. 0.1 ml. of such thromboplastin solution was added to a tube containing 0.1 ml. of the test plasma or dilution thereof and 0.1 ml. of adsorbed normal plasma and the clotting time measured. Adsorbed normal plasma was obtained by mixing 1 ml. of citrated plasma with 0.1 ml. of aluminium hydroxide suspension for 5 min. The supernatant was then separated by centrifuging at 3.000 r.p.m. for 5 min. and the procedure repeated since again. After this double adsorption the plasma did not clot upon addition of calcium and tissue thromboplastin (prothrombin time greater than 200 sec.).
d) Simplastin A: the reagent was reconstituted with dist. water and incubated at 37° C for 5 min. To 0.2 ml. of such reagent then were added 0.1 ml. of the test plasma or dilution thereof and the clotting time measured.

All tests were carried out in triplicate and the average clotting time maintained as the final time.

The clotting times then were plotted on regular graph paper having on the abscissa the reciprocal of plasma dilutions and on the ordinate the clotting times in seconds.

The inhibition "i" present in the system then was evaluated in conventional units (1 U = 4 cm) by measuring on the abscissa the distance on the left of the Y-axis at which the test plasma dilution line met the rect parallel to the abscissa drawn from the point of the intersection of the normal plasma line with the Y-axis.

Results

Results are summarized in Table 1 and in Figs. 1–8.

Using Thrombotest a clear inhibition ("i" $\geqslant$ 0.5) was present in coumarin plasmas and in all congenitally deficient plasmas (Figs. 1, 2). The degree of inhibition present in the moderately anticoagulated plasma was greater than that present in the adequately anticoagulated plasma, 2.2 U. versus 0.8 U. (Fig. 1).

Using Normotest inhibition was mild but still present in every case but for the factor II deficient and the abnormal factor X (factor X Friuli) plasmas (Figs. 3, 4).

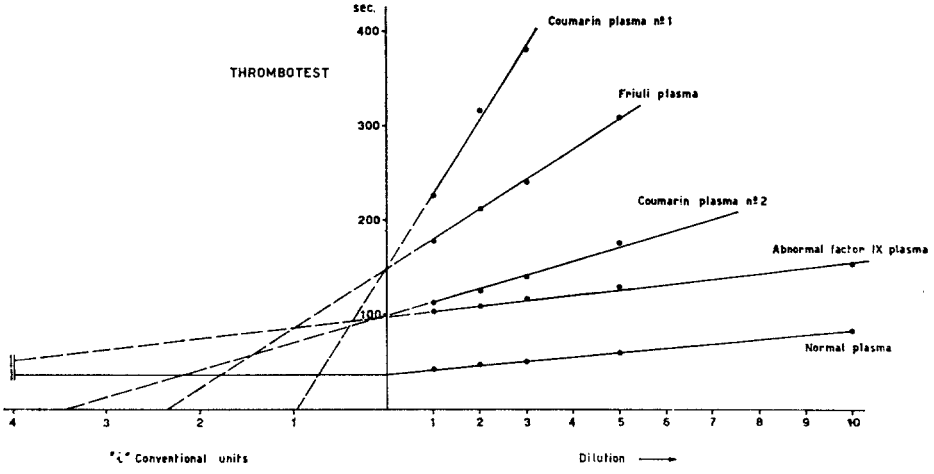

Fig. 1: Behavior of Coumarin plasmas, abnormal factor IX and abnormal factor X (factor X Friuli) plasmas in the *Thrombotest dilution curve system.* In each instance a clear "inhibition" seems present.

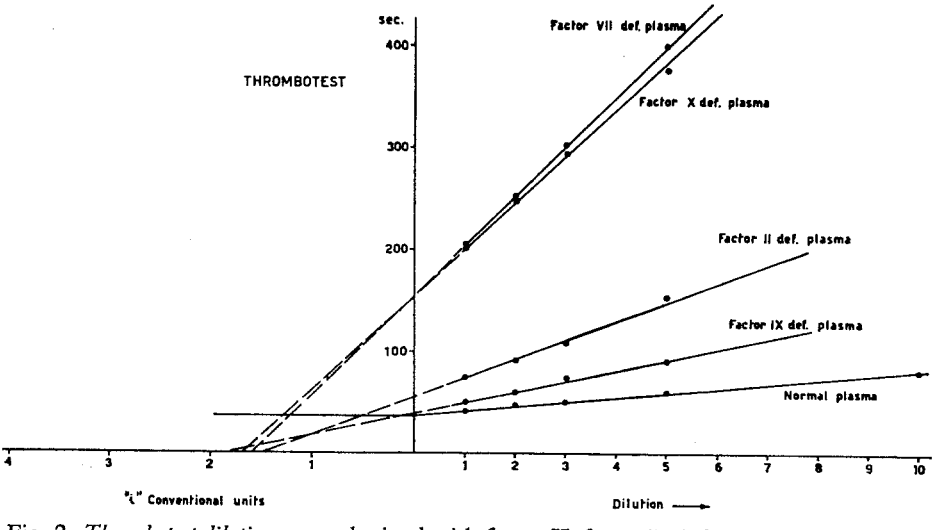

Fig. 2: *Thrombotest dilution curves* obtained with factor II, factor VII, factor IX and factor X deficient plasmas.

Plasma	Thrombo-test	Normo-test	Manchester Thrombo-plastin	Simplastin A	Comment
Coumarin plasma N. 1	0.8 U.	0.5 U.	0.7 U.	0.8 U.	
Coumarin plasma N. 2	2.2 U.	0.5 U.	1.5 U.	0.6 U.	
Factor II deficient plasma	0.5 U.	0.4 U.	1.8 U.	1.3 U.	
Factor VII deficient plasma	1.3 U.	0.6 U.	3.6 U.	3.7 U.	
Factor IX deficient plasma	0.1 U.	/	/	/	"i" not determinable; dilution curve practically identical with normal curve.
Abnormal factor IX plasma	>4.0 U.	0.3 U.	0.5 U.	0.6 U.	
Factor X deficient plasma	1.4 U.	0.8 U.	2.3 U.	2.2 U.	
Abnormal factor X (factor X Friuli) plasma	1.8 U.	0.3 U.	1.0 U.	1.3 U.	
Average inhibition Coumarin plasmas	1.5 U.	0.5 U.	1.1 U.	0.7 U.	
Average inhibition congenital conditions	1.8 U.	0.5 U.	1.8 U.	1.8 U.	Factor IX deficiency excluded.

Table 1: "I" capacity in conventional units as observed in the plasmas studied with the different thromboplastins.

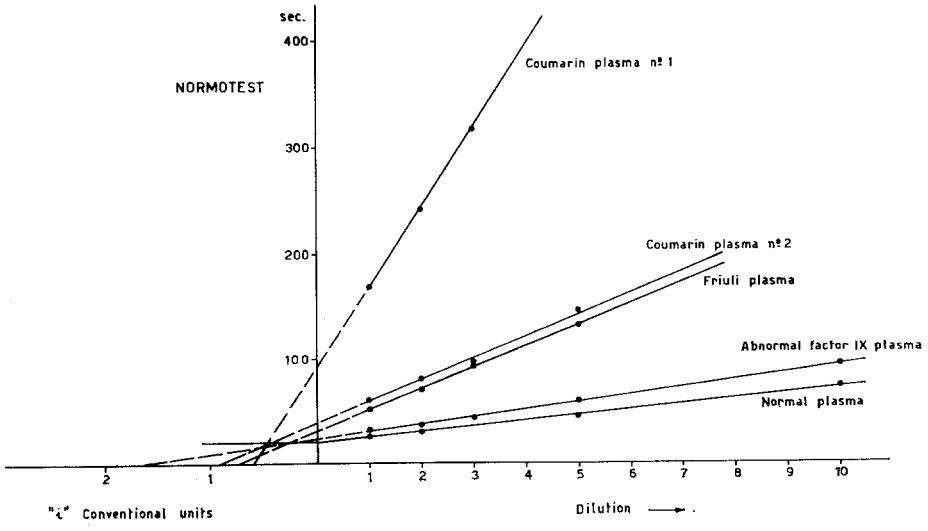

Fig. 3: Behavior of normal plasma, coumarin plasmas, abnormal factor IX and abnormal factor X (factor X Friuli) plasmas in the *Normotest dilution curve system.*

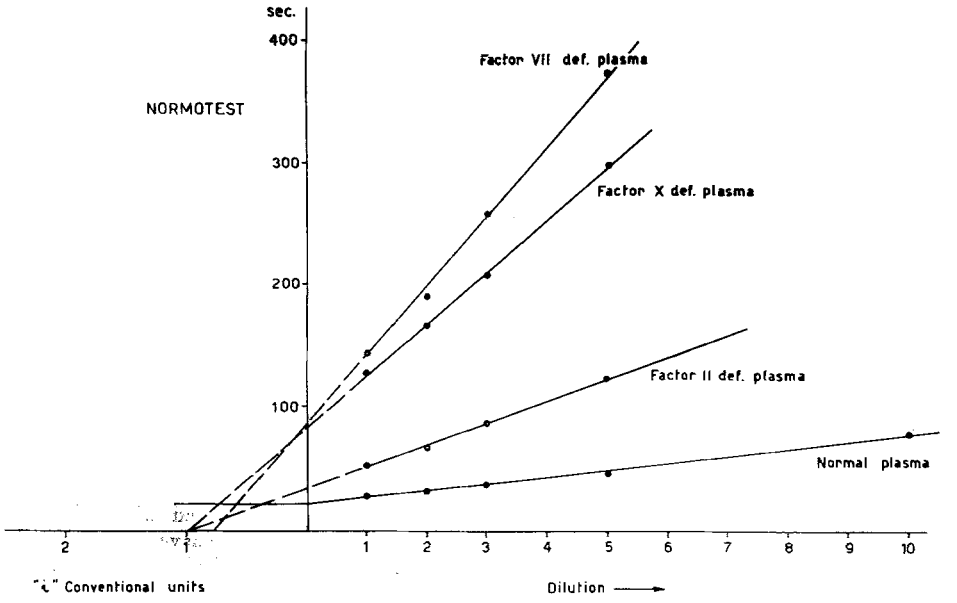

Fig. 4: *Normotest dilution curves* obtained with factor II, factor VII or factor X deficient plasmas and with normal plasma. The curve obtained with the factor IX deficient plasma was practically identical with the normal plasma one and has not been plotted.

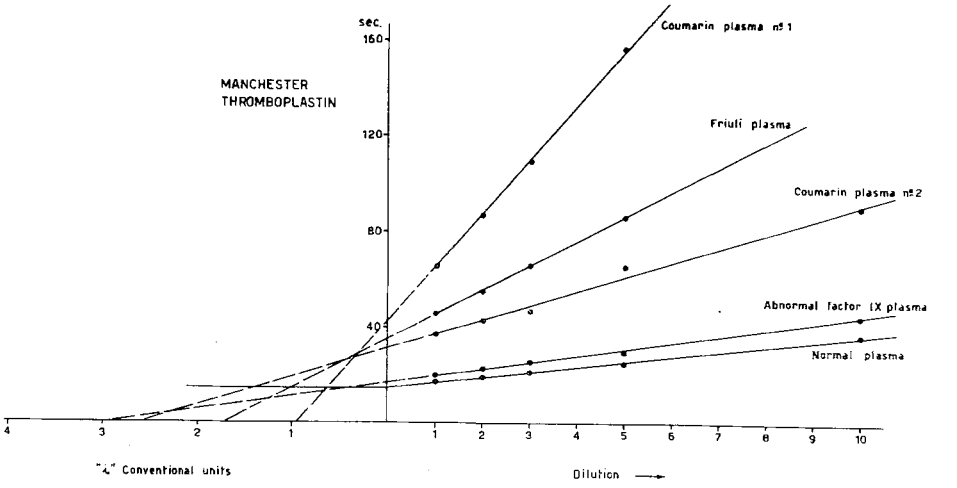

Fig. 5: Behavior of coumarin plasmas and of abnormal factor IX or abnormal factor X (factor X Friuli) plasmas in the *Manchester Thromboplastin* (human brain) *dilution curve system.*

Using Manchester thromboplastin a clear inhibition was seen for all plasmas (Figs. 5, 6).

In the case of Factor VII deficient plasma the clotting times of 1 : 2 diluted plasma was always shorted than that of undiluted plasma (Fig. 6). A similar type of curve was obtained using a second Factor VII deficient plasma (Dade factor VII deficient plasma).

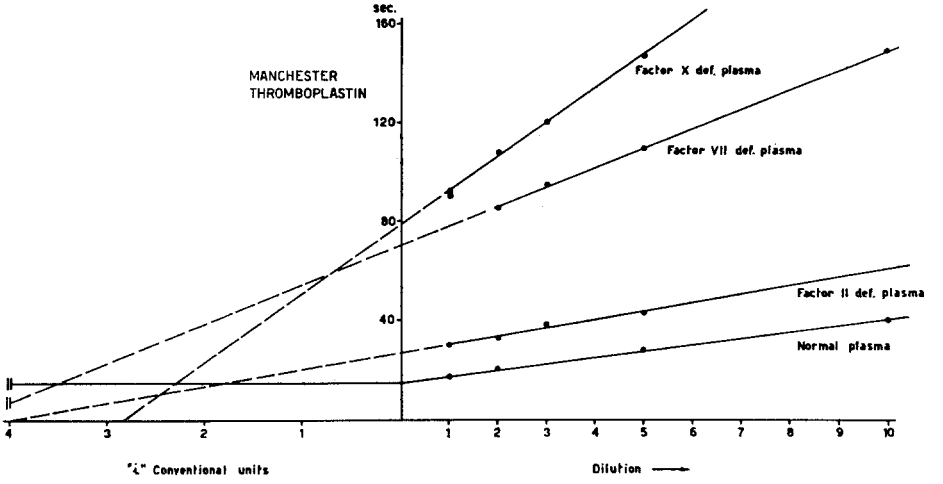

Fig. 6: *Manchester Thromboplastin* (human brain) dilution curves obtained with factor II, factor VII or factor X deficient plasmas and with normal plasma. The curve obtained with the factor IX deficient plasma was practically identical with the normal plasma and has not been plotted.

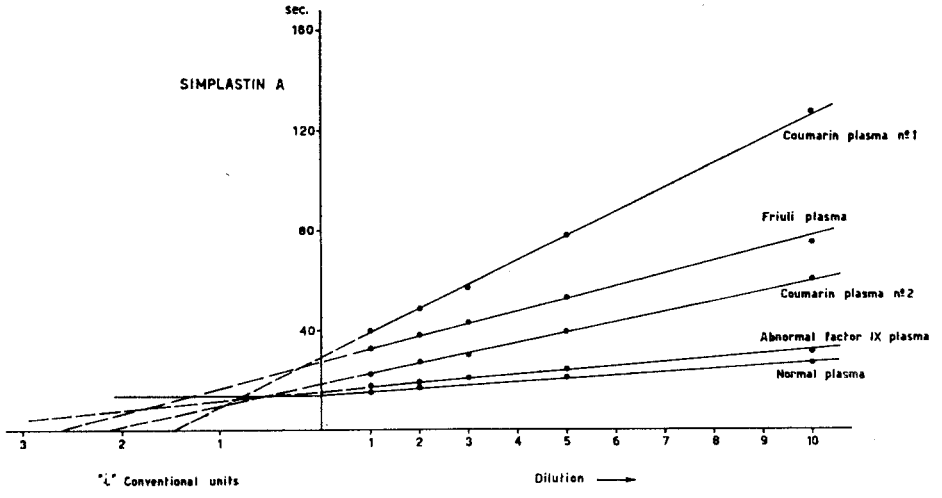

Fig. 7: Behavior of coumarin plasmas and of abnormal factor IX and abnormal factor X (factor X Friuli) plasmas in *the Simplastin A* (rabbit brain and lung) *dilution curve system.*

Using Simplastin A a sure inhibition was evident in all plasmas too. In factor IX deficient plasma no inhibition could be observed since it gave a dilution curve practically identical to the normal one in all instances (Figs. 7, 8).

The average "inhibition" found in the two coumarin plasmas was 1.5 U., 0.5 U., 1.1 U. and 0.7 U. respectively for Thrombotest, Normotest, Manchester thromboplastin and Simplastin A.

The average "inhibition" found in the congenital prothrombin complex factors deficiencies or abnormalities was 1.8, 0.5, 1.8 and 1.8, respectively.

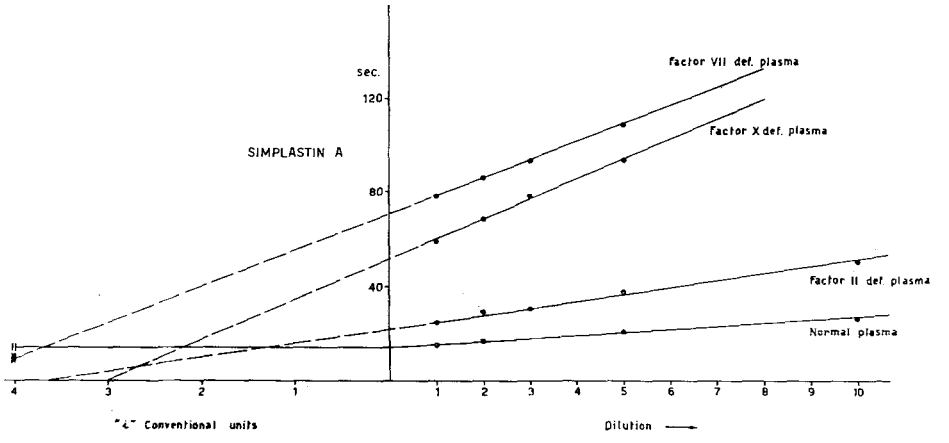

Fig. 8: *Simplastin A dilution curves* obtained with factor II, factor VII or factor X deficient plasmas and normal plasma. The curve obtained with the factor IX deficient plasma was practically identical to the normal plasma one and was not plotted.

Discussion

The administration of coumarin to a patient results in a multiple factor defect. Factors II, VII, IX and X are all decreased. The level of three of these factors can be assessed by a common prothrombin time.

The Thrombotest was originally thought to be sensitive to factor IX deficiency too [*18*]. Subsequently this was shown not to be the case [*2,21*]. The Thrombotest is maintained to be the test most sensitive to prothrombin conversion inhibitors [*20*]. This is claimed to be true both for the abnormal factor IX inhibitor and for the coumarin induced inhibitor PIVKA [*20*].

The theoretical value of the dilution curve in coagulation studies rests on kinetic studies. However these kinetic studies deal with single enzyme and single substrate systems [*3,15*].

The reaction involving the activation of factor X via the factor VII and tissue thromboplastin complex (extrinsic pathway) may be a multiple step process and therefore the formula may not apply. It is clear that the dilution curve approach in single congenital prothrombin complex factor deficiency is not valid. It does not allow any differentiation between coumarin plasmas and congenital deficient or abnormal plasmas. No inhibitor was present in these plasmas but for the case of the abnormal factor IX defect. Furthermore no vitamin K deficiency was present in the patients whom the plasma were obtained from. In spite of these facts, in all cases a clear inhibition is evident with all thromboplastins but for Normotest. Such "inhibition", as a whole, is much less evident with Normotest even though a mild "inhibition" may be present with this reagent too. This seems at variance with the claim that Normotest is not sensitive to the coumarin induced inhibitor or other inhibitors [*19*]. The composition of Normotest is unknown but available data suggest it contains rabbit brain thromboplastin together with a source of factor V and factor I. If this is so the reagent appears to be similar to Simplastin A [*20*].

With human brain and rabbit brain and lung thromboplastin the "inhibition" present as compared with Thrombotest was moderately decreased in coumarin plasma but was exactly identical in the congenital deficiencies.

It is also interesting to note that the highest "inhibition" ("i" > 3 U.) was not found in coumarin plasmas but in the abnormal factor IX plasma with Thrombotest and in factor VII deficient plasma with Manchester Thromboplastin and with Simplastin A. The results obtained using the factor VII deficient plasmas are of interest.

The type of curve obtained is usually considered typical of an excess of substrate or of a non-competitive type of inhibition [*3, 10*]. This could indicate that whenever factor VII is low the resulting low factor VII + tissue thromboplastin complex finds an excess of factor X to be activated.

Our studies seem to cast serious doubts as to the value of the dilution curve in coagulation studies.

We do not deny the possibility that the procedure might be of interest in some instances. It is clear however that no sure conclusion can be drawn. It seems to us that serious evaluation of the problem is warranted before assigning a particular sensitivity to one tissue thromboplastin or to another according to their behavior in a dilution curve system.

Our studies indicate that an "inhibitory" type may be observed in almost all congenital prothrombin complex factors deficiencies. The congenital coagulation disorders or abnormalities studied by us are all known prothrombin complex factor deficiencies or abnormalities but for prothrombin Barcelona [*13*]. No data are available so far on the behavior of this prothrombin abnormality in a dilution curve system. It would be very interesting to know whether the results obtainable in Prothrombin Barcelona plasma are similar to those obtained by us for Friuli plasma. A mixing experiment has already indicated, like for factor X Friuli, that no inhibition is present in such plasma [*13*].

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