# Kinetic aspects of the interaction of blood-clotting enzymes

Sonderdruck Reprint Tirage à part

THROMBOSIS ET DIATHESIS HAEMORRHAGICA

EDITORES: K. M. BRINKHOUS, CHAPEL HILL; F. BÜCHNER, FREIBURG/BREISGAU; E. DEUTSCH, WIEN; J. E. JORPES, STOCKHOLM; F. KOLLER, BASEL; J. ROSKAM, LIÈGE; J.-P. SOULIER, PARIS; I. S. WRIGHT, NEW YORK

VOL. XVII 31. V. 1967 No. 3/4

# Kinetic Aspects of the Interaction of Blood-Clotting Enzymes

## II. The Relation between Clotting Time and Plasma Concentration in Prothrombin-Time Estimations

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As a rule, clotting times obtained in prothrombin-time estimations become longer the more a plasma is diluted. This holds for the original Quick method as well as for any of its modifications. The estimation of the way in which clotting time ($t_c$) varies with the concentration (C) of the clotting factors in the plasma being tested, may be of considerable importance. Such estimation is done by analysis of the curve that gives $t_c$ as a function of C, which will reveal the nature of the function $t_c = f(C)$.

It has been claimed (1,2) that this function is hyperbolic, i. e. that the plot of t against 1/C is a straight line. This assumption yields useful results in the study of various kinds of thromboplastins (3, 4).

The purpose of this study was to assess the nature of $t_c = f(C)$ for the case of dilutions of normal plasma tested with the Thrombotest reagent; to see what influences affect the parameters of this function and to investigate briefly the results with other thromboplastins.

## Materials and Methods

*Normal Plasma* (N. Pl.): pooled platelet-free citrated plasma from at least 30 healthy normals (mean age around 30 years), freed of platelets by centrifugation for 30' at 12,500 g and stored at $-25°$ C in 1 ml aliquots. Three different batches of pooled normal plasma were used.

*Factor VII-deficient plasma:* plasma from a patient with congenital factor VII-deficiency, prepared in the same way as the normal plasma. This plasma contained 80% factor X.

*Factor X-deficient plasma:* plasma from a patient suffering from amyloidosis with consequent factor X-deficiency (5), kindly supplied by Dr. G. den Ottolander (Dijkzigt Hospital, Rotterdam).

*Thromboplastin:* human brain thromboplastin according to Owren and Aas (6).

*Roche Thromboplastin:* batch no. B604066; S. A. Hoffmann-La Roche & Co. A. G., Basle, Switzerland.

*Simplastin:* batch no. 0024016; Warner-Chilcott Laboratories, N. J., U. S. A.

*Thrombotest:* according to Owren: batch nos. 672 and 710; Nyegaard & Co. A/S, Oslo, Norway. Unless otherwise stated, the dry material from one large ampoule was dissolved in 11 ml of 3.2 mM $CaCl_2$. This material was a gift from Pharmachemie N. V., Haarlem, The Netherlands.

*The buffer* used for dilution was Michaelis buffer (pH 7.4).

The moment of clotting was assessed with a Kolle hook, and the time ($t_c$) between the beginning of the reaction and the moment of clotting was measured with a chronometer. The reactions were carried out at $37 \pm 0.1°$ C.

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The thrombotest reaction was carried out with 0.25 M Thrombotest reagent, to which 0.05 ml of plasma or diluted plasma was added.

*Dilution* (D) is defined as the ratio of the final volume of a plasma sample to its original volume. Thus, undiluted plasma has a dilution factor of unity (D = 1). D = n means that n–1 volumes of diluent were added to 1 volume of undiluted plasma.

A *t–D plot* is a plot in which the clotting time ($t_c$) obtained with plasma at a known dilution (D) is plotted along the Y-axis and the dilution along the X-axis. When the term "clotting-factor concentration" is used in this context, we mean the concentration of the clotting factor that is rate-limiting in these clotting-time estimations. This concentration is expressed as usual as a percentage of the concentration in the pooled normal plasma.

## Experimental Results

A t-D plot of normal plasma is essentially a reference curve in which $t_c$ is plotted against the inverse of clotting-factor concentration.

In this special t-D curve, the clotting-factor concentration is by definition 100% at D = 1, 50% at D = 2, 33% at D = 3, and generally $100 \div n\%$ at D = n. The t-D plot of normal plasma will be called the *normal t-D plot*. The shape of the normal t-D plot with the Thrombotest reagent is shown in Figs. 1, 2, and 3. In these Figures each point represents the mean of 50 estimations; four times the standard error of the mean is rendered as the diameter of the circle representing the clotting time.

The estimations were done in 5 groups, each group consisting of tenfold estimations of the thrombotest time in 7 different dilutions. The dilutions of each of the 5 groups were made independently on different days. The dilutions of normal plasma shown in Fig. 1 were made with buffer in plastic tubes; for those in Fig. 2 glass tubes were used. The dilutions in Fig. 3 were made in plastic tubes with $BaSO_4$ absorbed oxalated plasma instead of buffer. Fig. 4 shows the dilution plot of normal plasma (black dots) as well as the dilution plots of mixtures of $BaSO_4$ absorbed plasma and normal plasma; the ratio of normal plasma to absorbed plasma was 2:1 and 1:2, respectively. Fig. 5 indicates that there is a shortening of the thrombotest time when diluted plasma is stored in glass tubes. This Figure also shows that this effect is enhanced by

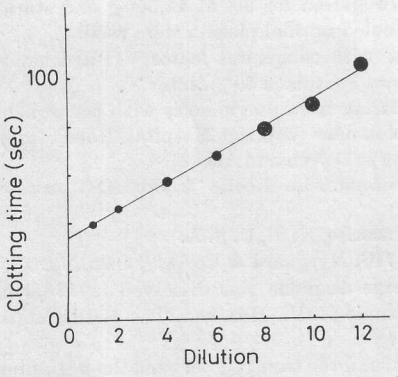


Fig. 1

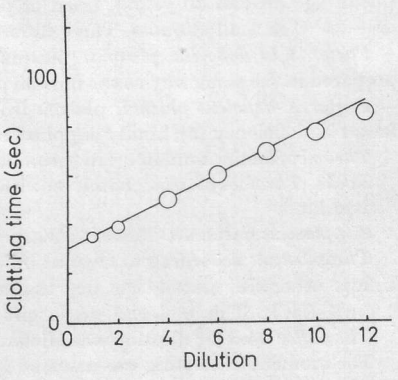


Fig. 2

Fig. 1. *The normal t-D plot.* Pooled normal plasma, diluted with buffer in plastic tubes, tested with Thrombotest reagent.

Fig. 2. *The normal t-D plot.* Pooled normal plasma, diluted with buffer in glass tubes, tested with Thrombotest reagent.

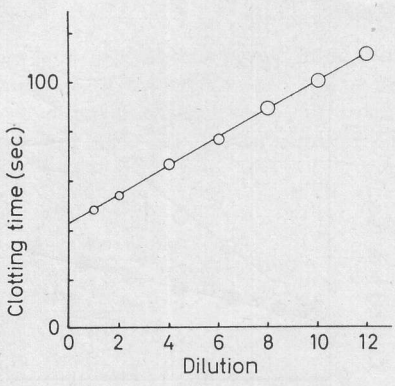


Fig. 3

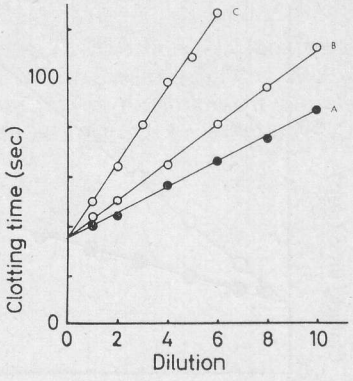


Fig.4

Fig. 3. *The normal t-D plot.* Pooled normal plasma, diluted with $BaSO_4$ absorbed bovine plasma in plastic tubes, tested with Thrombotest reagent.

Fig. 4. *The normal t-D plot.* The normal t-D plot (A) compared with the t-D plot of 66% normal plasma in $BaSO_4$ absorbed plasma (B) and 33% normal plasma in $BaSO_4$ absorbed plasma (C).

kaolin but is absent in factor XII-deficient plasma. Fig. 6 depicts the results demonstrating that the use of $BaSO_4$ absorbed plasma as a diluent shifts the optimal $Ca^{++}$ concentration to higher values. Figs. 7, 8 and 9 show the normal t-D curves when prothrombin times are estimated with human brain thromboplastin, Simplastin and

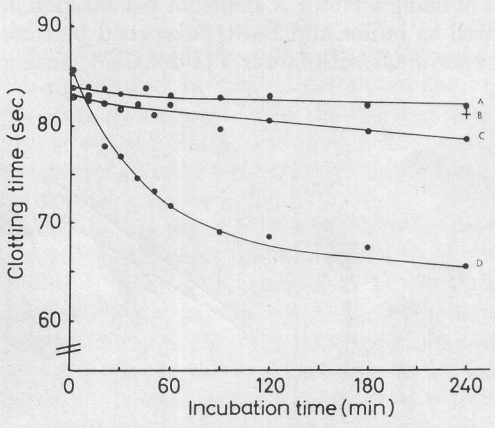


Fig. 5

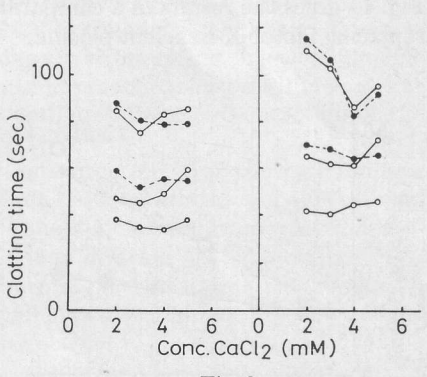


Fig. 6

Fig. 5. *Thrombotest times after incubation of 1:10 diluted plasma.* A. factor XII-deficient plasma in glass tube. B. Normal plasma in a plastic tube. C. Normal plasma in a glass tube. D. Normal plasma in a plastic tube with kaolin (1.25 mg/ml).

Fig. 6. *Thrombotest times of normal plasma at various $Ca^{++}$ concentrations.* Plotted along the X-axis is the $Ca^{++}$ concentration in the fluid with which the thrombotest solution was made up. Lower curve: undiluted plasma. Middle curves: normal plasma diluted 1:5. Upper curves: normal plasma diluted 1:10. Solid line in left hand graph: dilutions made with Michaelis buffer; idem in right hand graph: dilutions made with 10 mM oxalate in Michaelis buffer. Broken line in both graphs: dilutions made with $BaSO_4$ absorbed plasma.

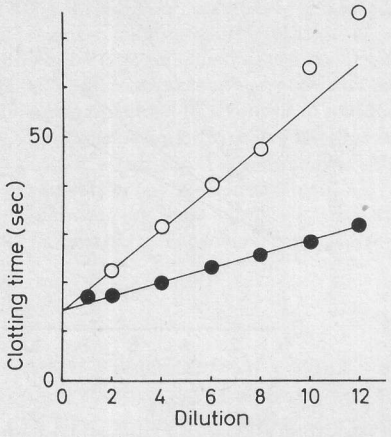


Fig.7

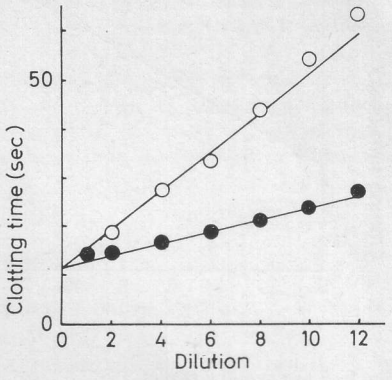


Fig.8

Fig. 7. *Normal t-D graph of prothrombin times obtained with human thromboplastin.* Diluent: buffer (circles) or $BaSO_4$ absorbed bovine plasma (dots). Reaction mixture: 0.1 ml (dilution of) normal plasma; 0.1 ml thromboplastin; 0.1 ml $CaCl_2$ 25 mM.

Fig. 8. *Normal t-D graph obtained with Thromboplastin Roche.* Diluent: buffer (circles) or $BaSO_4$ absorbed bovine plasma (dots). Reaction mixture: 0.1 ml (dilution of) normal plasma; 0.1 ml thromboplastin; 0.1 ml $CaCl_2$ 25 mM.

Thromboplastin Roche. The dilutions were made with buffer as well as with $BaSO_4$ absorbed plasma. Fig. 10 shows the results of using a factor X-deficient plasma rich in factors II and VII as a diluent, as compared to buffer and $BaSO_4$ absorbed plasma. Fig. 11 gives the results of a comparable experiment with factor VII-deficient plasma replacing factor X-deficient plasma.

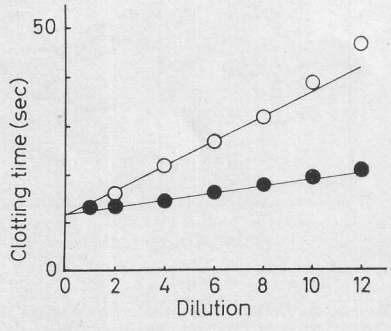


Fig. 9

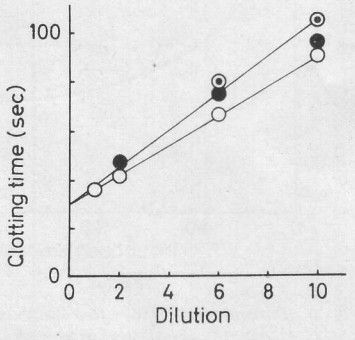


Fig. 10

Fig. 9. *Normal t-D graph obtained with Simplastin.* Diluent: buffer (circles) or $BaSO_4$ absorbed bovine plasma (dots). Reaction mixture: 0.1 ml (dilution of) normal plasma; 0.1 ml thromboplastin; 0.1 ml $CaCl_2$ 25 mM.

Fig. 10. *The normal t-D plot obtained with Thrombotest with various diluents.* ○———○ buffer as a diluent; ⊙———⊙ $BaSO_4$ absorbed plasma as a diluent; ●———● factor X-deficient plasma as a diluent; At D = 2, point ⊙ and point ● coincide.

*Theoretical Considerations*

The t-D plot has some interesting features (see also 7). The line obtained with dilutions of normal plasma can be used as a reference curve to read the clotting- factor concentrations leading to an estimated clotting time ($t_u$) in an unknown sample (see Fig. 12). The clotting-factor concentration in the sample can be calculated as $d/u \times 100\%$.

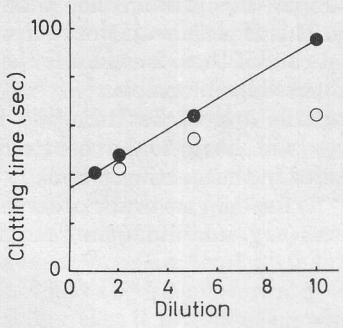


Fig. 11

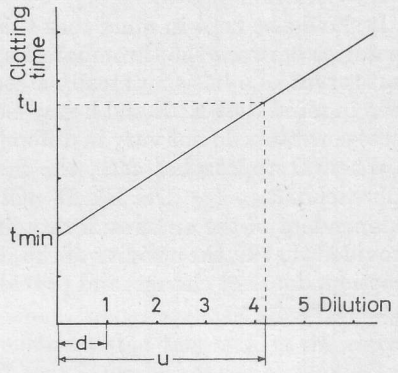


Fig. 12

Fig. 11. *The normal t-D plot obtained with thrombotest with various diluents.* ●———● buffer as a diluent; ○———○ factor VII-deficient plasma as a diluent.

Fig. 12. *The t-D plot* (see text).

It is clear that the shortest time theoretically available in this system is $t_{min}$, the time indicated by the intercept of the reference curve and the Y-axis. When $t_c$ equals $t_{min}$, u equals O, so the clotting-factor concentration giving this clotting time should equal infinity (d/u approaching infinity as u approaches zero). This means that $t_{min}$ is the clotting time that would be obtained with an infinite concentration of the rate-limiting clotting factor.

Now let us assume that we have a plasma containing 50% of the rate-limiting clotting factor in its undiluted form, and that we make serial dilutions of this plasma. The undiluted test plasma (D = 1) will then give the same clotting time as the twice-diluted normal plasma (D = 2). In general, a given dilution (D = n) of the 50% plasma will give the same clotting time as the normal plasma diluted twice as much (D =2n). In the t-D plot the abnormal plasma will give a straight line, but a straight line twice as steep as the line obtained with normal plasma.

In general, when a dilution series is made of a test plasma with an unknown amount of clotting factors present, the steepness of the line obtained with the test plasma, relative to the steepness of the line obtained with normal plasma, will express the amount of rate-limiting clotting factor in the plasma tested. This is demonstrated in Fig. 4. Plasmas known to contain 66% and 33% of the $BaSO_4$ adsorbable clotting factors, show dilution graphs with cotangents that amount to 66% and 33% of the cotangent of the normal t-D line. In equation (cf. equation 30 from ref. 7).

$$(1)\ t_c \,.\, h = (1 + \frac{K}{E}) \,.\, \frac{100}{x} \,.\, D + \frac{1}{E}$$

where:

$t_c$ = clotting time at dilution D
h = a constant
K = a reaction constant
E = an enzyme concentration
x = the amount of "substrate" clotting factor in the plasma tested, expressed as a percentage
D = dilution

It should be kept in mind that this formula is derived on the basis of the observed analogies between the thrombotest system and the "simple" model of enzyme action that serves as a basis for steady-state enzyme kinetics. This is permissable only if we keep in mind that K, E and h may be complex constants and if there is evidence that these constants do not vary in different estimations compared in one graph.

When D approaches zero, the first term of the formula approaches zero and $t_c$ approaches $t_{min}$ (= 1/h. E). It will be clear that $t_{min}$ will have to be constant, independent of the amount of rate-limiting clotting factor in the starting plasma (x), provided that E, the activity of the substance behaving as the enzyme in the observed reaction, does not change, and provided that no modifiers (e. g. inhibitors) are present in the system.

## Discussion

Figs. 1 and 3 show that in a thrombotest experiment the relation between clotting time and dilution is linear when either buffer or absorbed plasma is used as a diluent. Fig. 2 shows that the thrombotest time of high dilutions of normal plasma tends to shorten when dilutions are made in glass tubes; this causes a slight deviation of the straight-line relationship in this case.

Comparison of Figs. 1 and 3 indicates that dilution with absorbed plasma causes a lengthening of the thrombotest time relative to an equal dilution with buffer. This may be due to (a) the presence of antithrombin III in the absorbed plasma, (b) the presence of a factor V inactivator in the absorbed plasma (8) and (c) the presence of a $Ca^{++}$ binding entity in the absorbed plasma.

The experiment shown in Fig. 6 demonstrates that the optimal $Ca^{++}$ ion concentration in experiments in which the dilutions are made with $BaSO_4$ absorbed plasma, tends to be higher than when the dilutions are done with either buffer or oxalated buffer. Although this finding does not rule out the possible contribution of antithrombin III or other inactivators, it stresses the importance of the $Ca^{++}$ binding properties in the $BaSO_4$ absorbed plasma as one of the factors determining the thrombotest clotting time.

The acceleration of clotting by glass contact appears to be present in the system tested in the thrombotest experiments as well as in the intrinsic system (9, 10, 11, 12, 13). Fig. 5 shows that this activation is a time-dependent phenomenon enhanced by kaolin and absent in factor XII-deficient plasma. Further studies on this subject are in progress in our laboratory. The fact that more dilute samples of plasma are more readily activated, seems to be accounted for by the relative abundance of wettable surface in diluted samples. At any rate, it is clear that reliable estimation of the normal t-D curve is possible only with the exclusion of surface activation.

Figs. 7, 8 and 9 show that the kind of relationship found with the Thrombotest reagent, can be found with other thromboplastins as well. In this case, however, dilution of factor V appears to cause a lengthening of clotting times, which seems to be

relatively more pronounced in high dilutions and therefore the t-D curves prepared with buffer as a diluent tend to bend upwards at the higher values of D. The experimental results therefore suggest that the normal t-D curve is a straight line under conditions including an abundance of factor V and no contact activation.

This finding of a simple straight-line relationship between clotting time and plasma dilution is by no means simple to explain.

The over-all reaction of clotting in the extrinsic system is known to comprise a minimum of 8 reactants (tissue factor, factor VII, factor X, factor V, phospholipid, factor II, factor I and calcium), 3 of which are varied simultaneously when dilutions of normal plasma are added to the thrombotest reaction mixture (factors VII, X and II). If the over-all clotting rate of the mixture is determined by the concentrations of all 3 factors, it is hard to see how a basically simple reaction between clotting time and dilution would result (14).

When dilutions of normal plasma are added as a source of the factors II, VII, and X, the ratio of the concentrations of these factors remains equal to the ratio found in the original normal plasma (the "normal ratio"). This normal ratio might well represent a functional excess of 1 or 2 of these factors over 1 or more of the others. The existence of a functional excess is determined by the molar ratios of the factors and by the reaction constants of their interaction, values which are not known at the moment.

The experiment shown in Fig. 10 provides evidence that factor X is the clotting factor that is rate-limiting when factors II, VII, and X are present in a normal ratio. This is clearly demonstrated, for instance, by the 1 : 10 dilution of the normal plasma in this experiment. With buffer or absorbed plasma as a diluent, the concentration of factors II, VII, and X in the sample is 10%, with factor X-deficient plasma as a diluent the concentration of factor X is again 10%, whereas the concentration of factors II and VII is 100%. Yet this tenfold increase of factors II and VII does not appreciably shorten the clotting time. This indicates that the normal ratio of factors II, VII, and X is such as to ensure a functional excess of factors II and VII over factor X in this experimental situation.

This means that a thrombotest experiment is essentially a factor X estimation as long as the ratio of factor X to factor VII and/or II does not appreciably exceed the normal ratio. So the factor measured by estimating the slope of a t-D curve is factor X.

Of course, this concept is not contradictory to the finding that isolated factor II or factor VII deficiencies exhibit long thrombotest times, because in these cases the ratio F. X : F. II or F. X : F. VII greatly exceeds the normal ratio and factor II or factor VII become rate-limiting.

The results shown in Fig. 11 stress this point. In the 1 : 10 dilution of normal plasma with factor VII-deficient plasma, i. e. in a situation in which 109% of factor II, 82% of factor X* and 11% of factor VII are present, the clotting time is appreciably shorter than in the comparable dilution with either buffer, $BaSO_4$ absorbed plasma, or factor X-deficient plasma. Yet the clotting time is longer than in an experiment with 100% factor VII and 82% factor X because of the fact that the ratio of factor X to factor VII greatly exceeds the normal ratio in this particular experiment. The situation is summarized in Table 1.

It is interesting to see that an increase of factor VII concentration does not shorten the clotting time, whereas contact activation does. The most logical explanation of this seems to be in terms of factor VII activation. We know that factor VII can be

*) 82% factor X, because the original factor VII-deficient plasma contains only 80% factor X.

Table 1. Influence of Selective Variation of Clotting Factors VII and X on the Thrombotest Time.

| Sample | Composition (%) | | | Clotting Time (sec) |
|---|---|---|---|---|
| | F. II | F. VII | F. X | |
| A normal plasma | 100 | 100 | 100 | 38 |
| B factor VII-def. pl. | 110 | 1.2 | 80 | >500 |
| C factor X-def. pl. | 105 | 120 | 0.8 | >500 |
| A in buffer 1:10 | 10 | 10 | 10 | 93 |
| A in B 1:10 | 109 | 11 | 82 | 63 |
| A in C 1:10 | 105 | 118 | 11 | 96 |

The clotting factor concentrations are given as final concentrations in the sample tested, expressed as a percentage of a normal pool.

converted into activated factor VII (F. VIIa) by a constituent of the Thrombotest reagent ("tissue factor") or by a contact activation product (13, 15). In a contact-free thrombotest experiment a small *fixed* amount of tissue factor is present in every test. If the substrate of the tissue factor (i. e. F. VII) is present in excess, this situation will lead to a velocity of VIIa formation that is determined by the concentration of tissue factor rather than by the concentration of factor VII.

Because contact activation product acts on factor VII just as tissue factor does, contact activation will cause a higher velocity of factor VII conversion and thus give rise to a shorter clotting time. This is of course a grossly qualitative explanation. Work on the actual kinetics of this process is in progress in our laboratory.

## Summary

It is demonstrated that clotting time in a thrombotest experiment bears a linear relationship to the inverse of factor X concentration under conditions in which the ratio of factor X to the factors II and VII is equal to or smaller than the ratio in normal plasma.

A similar relationship occurs with other thromboplastins as long as factor V and fibrinogen are present in excess, i. e. when the dilutions are made with $BaSO_4$ absorbed plasma.

Contact activation causes deviation from the observed straight-line relationship.

## Résumé

Il a été montré qu'il existe une relation linéaire entre le temps de coagulation déterminé par le thrombotest et l'inverse de la concentration en facteur X sous condition que le rapport du facteur X aux facteurs II et VII soit égal ou inférieur au rapport normal plasmatique.

Une relation similaire apparait avec d'autres thromboplastines tant que le facteur V et le fibrinogène sont en excès c'est-à-dire tant que les dilutions sont faites avec du plasma adsorbé par le $BaSO_4$.

L'activation par contact cause une déviation de la relation linéaire observée.

## Zusammenfassung

Es wird gezeigt, daß zwischen der Gerinnungszeit im Thrombotest und dem reziproken Wert der Faktor-X-Konzentration eine lineare Beziehung besteht unter Be-

dingungen, in welchen der Quotient von Faktor X zu Faktor II und VII gleich oder geringer ist als im Normalplasma.

Eine ähnliche Beziehung besteht mit anderen Thromboplastinen so lange, als Faktor V und Fibrinogen im Überschuß vorhanden sind, d. h., wenn die Verdünnungen mit bariumsulfatadsorbiertem Plasma hergestellt werden.

Die Kontaktaktivierung bewirkt eine Abweichung von der beobachteten geradlinigen Beziehung.

*Acknowledgements*

The authors wish to thank Miss J. M. C. Wimmers and Messrs. P. W. Hemker and A. C. W. Swart for fruitful discussions and suggestions. They also wish to thank Miss A. D. Muller and Mr. P. P. Devilée for invaluable technical assistance.

Part of this work was supported by the Dutch Organization for Pure Scientific Research (Z. W. O.).

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Received for publication September 26, 1966