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VII. The Relation between Clotting Time and Prothrombin Concentration

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Summary

The relation between clotting time and the inverse of prothrombin concentration is shown to be rectilinear.

Introduction

In previous articles (Hemker et al. 1965, Hemker et al. 1968) we developed a theory on the relation between the clotting time and the concentration of the coagulation factors of the extrinsic system.

We proposed that this relation is of the type

$$t_c = a. \ 1/c + b \tag{1}$$

where t_c, the coagulation time obtained with a concentration c,

    c, the concentration of the rate limiting coagulation factor,

    a,b, constants.

The relation holds true for situations where one factor is rate limiting (i. e. present in concentrations of 0–10%) and the other factors are present in relative excess (> 35%).

Until now we could not give direct experimental proof of the validity of formula (1) because of experimental limitations. For testing formula (1) one has to add known amounts of the rate limiting factor to a medium in which all other factors are present in excess. For example, small amounts of prothrombin to a prothrombin reagent. A prothrombin reagent until now always contained a small residual activity of prothrombin, equivalent to a small unknown concentration (L), as shown by the observation that a finite clotting time (buffer time, t_1) is obtained when no prothrombin is added to the sample. When one adds a concentration (C_{added}) the actual concentration acting in the reaction (C_{total}) = (C_{added} + L). C_{total} is the independent variable of formula (1) and it is unknown because L is unknown.

We were not able to falsify the hypothesis that the following formula is valid:

$$t_c = a.1/(C_{added} + L) + b \tag{2}$$

We worked out methods to determine a, L, and b from series of measurements of t_c at various C_{added} including C_{added} = 0, i. e. $t_c = t_e$ (buffer time) (Hemker et al. 1968, Hemker et al. 1972).

This amounts to making a three parameter fit to the experimental relationship t_c = f (C_{added}). It suffers from the drawback that it does not prove relationship (Hemker and

Muller 1968) to have a physical meaning. It only shows formula (2) to be a simple valid mathematical description of $t_c = f(C_{added})$ so that the assumptions on basis of which formula (2) was constructed cannot be refuted by experimental means.

These assumptions were

a) $t_c = a.1/C_{total} + b$ (3)

b) $C_{total} = C_{added} + L$ (4)

Because we recently found a method to prepare a factor II reagent devoid of any residual factor II (i. e. L=O) we are no longer dependent upon assumption b) and we can test assumption a) directly, which is done in this paper.

Materials and Methods

Bovine factor II reagent was prepared by adding a preparation containing amongst others the factors VII and X to $BaSO_4$ adsorbed bovine plasma containing 1 mM Na Oxalate, as described by Vermeer et al. 1976. The factor VII and X preparation was prepared from the serum obtained from normal bovine plasma upon coagulation with Echis Carinatus venom (1 mg/litre), 50 ml DEAE Sephadex A 50 slurry (in 0.06 M Tris-citrate buffer pH 7.1 was stirred in 3 litres of serum during 1 hour. The Sephadex was then packed into a column, washed with 1 litre of 0.15 M NaCl in 5 mM Tris HCl pH 7.0. Then the column was eluted with 1.0 M NaCl in 5 mM Tris- HCl pH 7.0. This yields factor X in a 55 % recovery and factor VII in a ~100 % recovery in the peak fraction.

It contains the following concentrations of coagulation factors: factor I: 278 mg%, factor II: 0%, factor V: 47%, factor VII 47.1%, factor X: 41.0%.

Factor II was prepared according to Owen et al. (1974). The preparation used was >98 % pure and devoid of demonstrable other coagulation activities. The coagulation tests and the preparation of normal pool plasma were carried out as described (Hemker et al. 1972). The three parameter fit of formula (2) to a series of t_c, C_{added} data pairs was carried out with the computer program described in (Hemker et al. 1972), as well as by the analytical method by hand as described (Hemker et al. 1968). Both methods directly yield a, L, and b. The constant b is given as t_{min}, i. e. the coagulation time of the system at infinite concentration of the rate limiting coagulation factor ($C_{added} \rightarrow \infty$). The other constant given is not a, but $K_m = b/a$, which has the dimension of a concentration and the physical meaning of being that concentration at which half maximal reaction velocity is obtained.
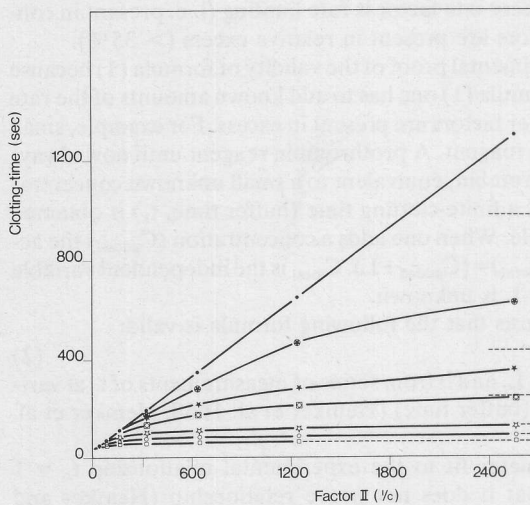

Fig. 1 Reference curves from prothrombin reagents with known added amounts of prothrombin.

☐ reagent + 2 % factor II
○ reagent + 1 % factor II
✩ reagent + 0.5 % factor II
⊠ reagent + 0.2 % factor II
✳ reagent + 0.1 % factor II
✪ reagent + 0.05 % factor II
● reagent + 0 % factor II

Experiments

With the aid of bovine normal plasma we investigated the t_c-C_{added} relationship with the reagent devoid of factor II (Fig. 1, upper curve). It appears that the relation between t_c and $1/c$ is rectilinear. Table 1 shows a statistical test on the rectilinearity of this relationship. It appears

Table 1   Test of the linearity of the function t=f(1/c).

C	1/c	t (sec)	s (sec)	s (%)	t_{calc}
10.00	10	37.3	1.1	3.0	38.5
4.00	25	54.4	2.7	5.0	49.8
1.00	100	103.3	2.9	2.8	106.1
0.40	250	218.1	6.2	2.8	218.8
0.10	1000	782.0	15.3	2.0	782.2

C is the prothrombin concentration in 0.01 U/ml. t is the mean clotting time from 20 determinations. s is the standard deviation. t_{calc} is the calculated clotting time from the regression line through the experimental points (t=0.7512 × 1/c+31.0). The hypothesis that the line is not straight can be rejected below the $\alpha = 0.01$ level.

Table 2   Values of L, t_{min} and K_m calculated for prothrombin reagents with known added amounts of prothrombin.

C (%)	L_1 (%)	L_2 (%)	t_{min} (sec)	K_m (%)	t_1 (sec)	s. d. (%)
2	1.84	2.00	28.2	2.32	62	2.00
1	1.98	1.40	28.8	2.23	88	1.40
0.2	0.29	0.18	31.3	2.23	237	0.18
0.1	0.15	0.14	33.3	1.89	426	0.14
0.05	0.045	0.05	34.5	1.93	979	0.05
0	0	0	28.8	2.24	~	0

C: amount of prothrombin added to the reagent. L_1: C as from the t_c-C_{added} data pairs by the method of ref. 3. L_2: as L_1, but calculated by hand with the method of ref. 1. t_1: clotting time at $C_{added}=0$ (buffer time). t_{min}, K_m: see text. s. d.: standard error of the mean of four determinations at each concentration from the relationship computed (Hemker et al. 1972).
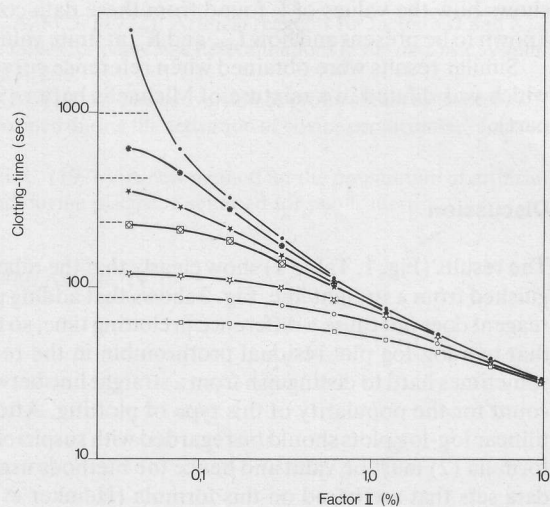

Fig. 2   Reference curves from prothrombin reagents with known added amounts of prothrombin. Log-log plot. For further details see the legend to Fig. 1.
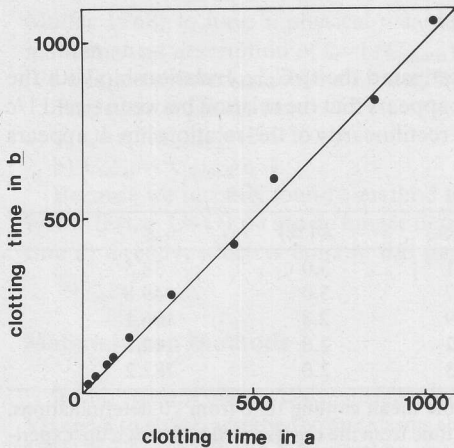
Fig. 3   Influence of the order of addition of prothrombin to the reaction mixture. Experiment a: 9 volumes of *prothrombin reagent* were supplied with one volume of purified prothrombin in Michaelis buffer in varying dilutions. The mixture was left for 1 hour. Then the clotting time was recorded of 0.1 ml of this mixture with 0.1 ml thromboplastin, 0.1 ml $BaSO_4$ adsorbed plasma and 0.1 ml of $CaCl_2$ 33 mM. Experiment b: 9 volumes of *BaSO₄ adsorbed plasma* were supplied with one volume of purified prothrombin to obtain the same range of concentrations as in experiment a. Mean of three determinations.

impossible to prove that the relationship is not rectilinear. On the other hand the log-log plot obviously is curved (Fig. 2, upper curve).

Next we added small amounts of purified prothrombin or plasma to the reagent and assessed that this change in order of addition of prothrombin to the final reaction mixture did not alter the eventual clotting time (Fig. 3).

It was, therefore, concluded that prothrombin in the reagent behaves similar to prothrombin in the sample so that formula (2) indeed can be used. Then we added small amounts of prothrombin to the reagent and made reference curves with dilutions of normal plasma (Figs. 1 and 2). The parameters t_{min}, K_m and L in Table 1 were those fitted to these data sets. This table also shows how the values of L found from these data compared to the amounts of prothrombin known to be present and how t_{min} and K_m at finite values of L compared to those found at L=0.

Similar results were obtained when reference curves were prepared from purified factor II which was diluted in a mixture of Michaelis buffer (9 parts) and $BaSO_4$ adsorbed plasma (1 part).

Discussion

The results (Fig. 1, Table 1) show clearly that the relation between t_c and $1/c$ cannot be distinguished from a straight line. Fig. 3 shows that adding prothrombin with the sample or with the reagent does not cause a difference in clotting time, so that formula (4) seems valid. Fig. 2 shows that in a log-log plot residual prothrombin in the reagent causes sigmoid curves, which are sometimes hard to distinguish from a straight line between 1.0 and 10 % factor II. This may account for the popularity of this type of plotting. Attempts to find a theoretical basis for rectilinear log-log plots should be regarded with suspicion. When both formulas 3 and 4 are valid, formula (2) must be valid and hence the methods used to assess L, t_{min} and K_m from t_c-C_{added} data sets that are based on this formula (Hemker et al. 1965, 1968) must be valid. Table 1

shows that the three parameter fit of formula (2) of the experimental t_c-C_{added} data sets gave values of L that were indeed in fair agreement to the amounts of prothrombin added. This justifies the use of this method in determining the residual prothrombin activity in deficient plasma's: one simply uses the deficient plasma as a reagent, determines the reference curve and from this calculates the value of L. From Table 2 it is also seen that those parameters that represent chemical constants of the system (t_{min} and K_m) are indeed virtually independent of the amount of prothrombin added.

We conclude that the two basic assumptions which we used to explain the observed fit of formula (2) to the experimental data are independently proven to be valid, i. e.
1. the relation between clotting time and prothrombin concentration is as described by formula (1);
2. this relation is usually obscured by residual prothrombin activity in the reagent.

Résumé

On montre que la relation entre le temps de coagulation et l'inverse de la concentration de la prothrombine est rectiligne.

Zusammenfassung

Es wird gezeigt, daß sich Gerinnungszeit und die reziproke Prothrombinkonzentration rektilinear verhalten.

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