Kinetic basis of prothrombin estimation particularly with reference to the rectilinearity of the log-log reference curve

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82 Figures, 23 Tables

19 65

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23. Kinetic Basis of Prothrombin Estimation Particularly with Reference to the Rectilinearity of the Log-Log Reference Curve

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The general procedure of the estimation of clotting factors depends upon the reasonable assumption that the clotting time obtained with an unknown amount of clotting factor will be equal to the clotting time of a dilution of normal plasma containing the same amount of the factor to be tested. The latter should be the only rate-limiting factor in the assay system.

In practice equal clotting times are rarely obtained from equal amounts of the reference sample and the sample to be tested. Hence, for quantitative estimation of coagulation factors a reference *curve* is constructed by estimating the clotting times of a series of known concentration as the rate-limiting factor. These clotting times are plotted against the concentrations and a smooth curve is constructed through the points thus obtained.

In practice the reference curve is usually thought to be a straight line when the logarithm of clotting time and the logarithm of concentration are plotted against each other.

$$\log t = a \log C + b$$

$$\text{or:} \quad t = b \cdot C^{a}$$

(t: clotting time; C: concentration of clotting factors; a and b: constants).

For two reasons this relationship has to be mistrusted. In the first place this kind of double logarithmic plotting is well-known in both chemistry and physics to render a straight line of a lot of paired experimental data because of its poor specificity. In the second place there is no reason whatsoever on a theoretical basis why this relationship should apply.

The log-log rectilinearity, when it occurs, has to be regarded as an important practical tool; however, as a result of its entirely empirical background it has some serious draw-backs.

1. It can never be used in a range where its applicability has not been tested experimentally.

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2. No importance can be attributed to the apparent constants which describe the clotting time as a function of clotting factor concentration, as no theoretical background exists to give a meaning to these constants.

In short, all this means that the log-log rectilinearity does not give any insight into the reaction mechanism of the clotting process.

For this reason we tried another approach.

Assuming a coagulation enzyme cascade, as proposed by Professor Macfarlane, we calculated that the clotting time might well indicate the inverse value of the reaction velocity in the rate-limiting step. In the most simple case possible the standard assumptions of enzyme kinetics would apply to this step.

This means:

1. The reaction proceeds by reversible combination of enzyme and substrate to a complex. This complex dissociates irreversibly into enzyme and product.
2. Only initial velocities are measured.
3. There is an excess of substrate.

Apparently this last condition is not fulfilled as both enzyme and substrate are present in a concentration of the same order of magnitude. A simple calculation shows that by dropping the assumption of excess of substrate the ordinary formula for the reaction velocity of an enzyme catalyzed reaction, viz.:

$$v = \frac{k_2 \cdot E \cdot S}{K + S}$$

(v: reaction velocity; E: enzyme concentration; S: substrate concentration; K and k_2: reaction constants), is modified into another formula:

$$v = \frac{k_2 \cdot E \cdot S}{K + E + S}$$

which, however, maintains velocity as a hyperbolic function of substrate concentration.

In order to avoid a long mathematical treatment, which will be published elsewhere, I will summarize by saying that in the simplest possible case a hyperbolic relationship between clotting velocity and concentration of the rate-limiting factor will exist. So clotting time will be in linear relationship to the inverse of the concentration of the rate-limiting factor.

In order to test these assumptions we estimated the clotting time of 50 normal plasmas, in 6 different dilutions in manifold. The data given are the mean of between 60 and 300 estimations for each point. Only the data obtained

with an artificially prepared factor II reagent will be shown. The results are essentially the same using congenitally deficient factor II or factor VII reagents.

The reaction mixture was: 0.2 ml of a plasma containing in excess all blood clotting factors involved (factor I, V, VII and X), except prothrombin (factor II), 0.1 ml human brain thromboplastin; the prothrombin was added in the form of a dilution of pooled plasma, which was supposed to contain 100% of prothrombin; the reaction was started by adding 0.1 ml $CaCl_2$ 50 mM.

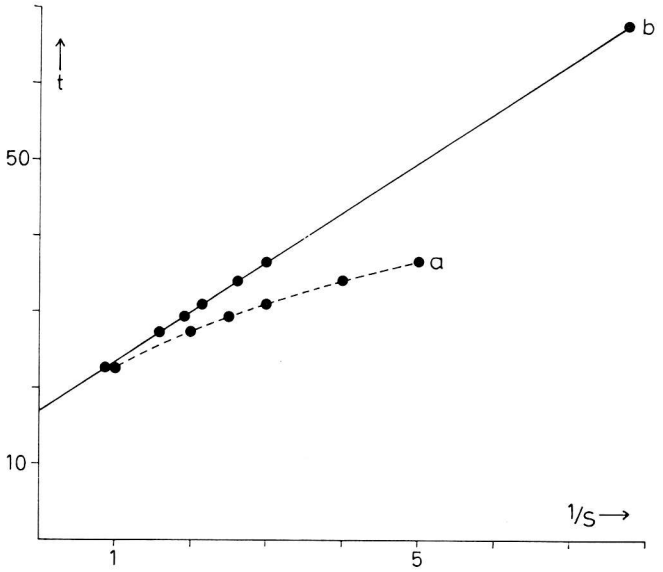

Fig. 1. Lineweaver Burk plot of prothrombin conversion. a) ● — — — ● clotting time plotted directly against the inverse of concentration of prothrombin added in the form of normal plasma; b) ●———● clotting time plotted against the inverse of the total concentration of prothrombin present in the assay, computed as indicated in the text.

As shown in Fig. 1, the results would not fit into a straight line.

This might have — and almost had — caused us to drop this line of approach, until we recognized that we had to apply a correction factor for the residual amount of prothrombin activity present in the reagent. This causes a constant small amount of prothrombin to be present in all tests besides the amount added in the form of diluted normal plasma. This would explain the general form of curve a in Fig. 1, as the addition of a constant small amount of factor II will become more important as dilutions get higher causing a large deviation from the expected straight line especially in this region.

With the additional assumption of the reagent containing prothrombin, which obscures the relationship between clotting time and the amount of prothrombin

added, it could be shown that the difference between the clotting time obtained with buffer (t_0) and the clotting time obtained with a concentration (S) of normal plasma (t_C) devided by the concentration added (S) had to be in linear relationship to the clotting time found (t_C).

$$\frac{t_0 - t_C}{S} = \frac{1}{B} \cdot t_C - \frac{1}{E.\,h.\,B.}$$

E: enzyme concentration
h: constant
B: amount of prothrombin present.

I shall again avoid the mathematics which led to this conclusion. Much to our surprise it appeared that the experimental data fitted nicely into the straight waistcoat which our lengthy theoretical approach had tailored ready-made for them.

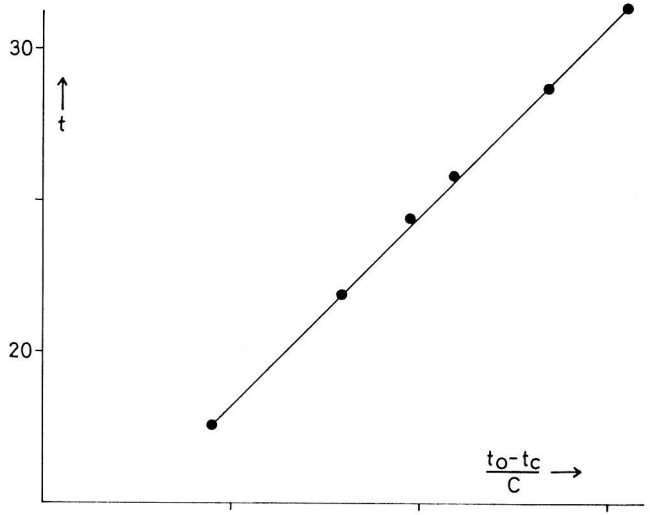

Fig. 2. Correction curve used for computing the amount of prothrombin present in the reagent.

It could be proven that the direction coefficient of the line in Fig. 2 expressed the inverse of the concentration of prothrombin in the reagent, so that this concentration could be calculated. It appeared that the artificially prepared factor II reagent contained 1,33% prothrombin. In congenitally factor II deficient plasma 1,51% prothrombin was found, whereas in congenitally factor VII deficient plasma, 1,63% factor VII was present.

As a recheck of the validity of this method, it proved to be possible to get a straight line when clotting time was plotted against the inverse of the total concentration of prothrombin present in an assay, this total concentration being calculated as the sum of the concentration present in the reagent and the concentration added to the normal plasma (Fig. 1, curve b). The practical consequences of these findings are:

1. It should be possible to find the Michaelis constant (K_m) of the reaction under study. As K_m is a constant representing the concentration of substrate at which half-maximal reaction velocity is obtained, we might be able to get an absolute standard of prothrombin concentration (or factor VII concentration) in this way.
2. When between 1% and 10% of coagulation factor is present in the plasma tested, the hyperbolic curve can be replaced by a logarithmic curve without including gross errors. Hence the log-log plot in daily clotting practice is only to be used in the range between 10% and 100% prothrombin concentration (Fig. 3).
3. Plasma concentrations below 10% (less than 1% prothrombin concentration in the diluted plasma) computed on a log-log plot appear to be overestimated (Fig. 3).

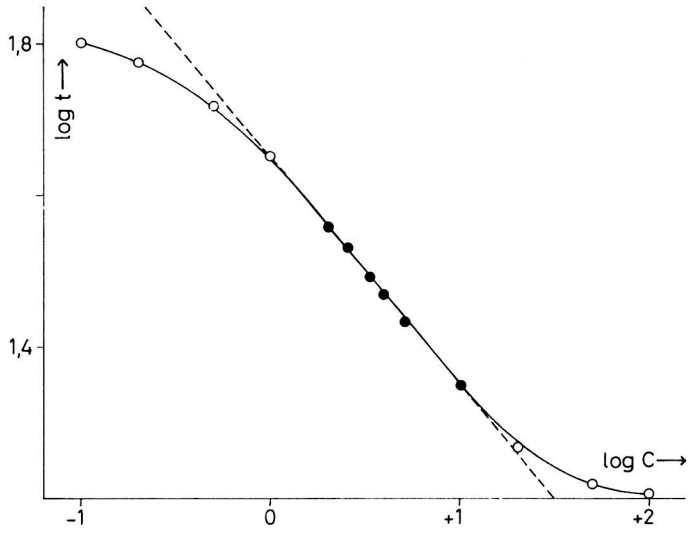

Fig. 3. A double logarithmic reference curve for prothrombin estimation. This figure shows that with 1 to 10% of prothrombin present (black dots) the double logarithmic plot simulates a perfect linear relationship.

4. Because of the hyperbolic relationship between factor concentration and reaction time it is clear that methods in which high concentration of the factor to be tested are present (e.g. from 20% final concentration upwards) are extremely inaccurate, as a big difference in clotting factor concentration causes only small differences in clotting time. This shows the obvious need to dilute a blood sample at least ten times before it is possible to differentiate between carriers and normals.