Anniversary Issue Contribution

The initiation phase – A review of old (clotting-) times

H. Coenraad Hemker

Synapse bv – Cardiovascular Research Institute, Maastricht, The Netherlands

To browse through old work is a pleasure in the indian summer of a career, not devoid of nostalgia. As the large majority of our early work appeared in *Thrombosis and Diathesis Haemorrhagica*, the present issue is an appropriate occasion to do so and to review old results, holding them against the light of our present day knowledge – and apologising beforehand for the shameless self-citation that it automatically involves.

Even in the mild light of hindsight this soon turns out to be primarily an exercise in modesty and an occasion to recognise old errors. Our early attempts to extract a maximum of information from clotting times now may look a bit naïve. Yet some of the conclusions and insights still seem to hold and still may contribute to the understanding of what we now call the "initiation phase" of blood coagulation.

My first contribution to *Thrombosis and Diathesis Haemorrhagica* was in the supplement that reported the proceedings of the Gleneagles conference of the International Committee for the Nomenclature of Blood Clotting Factors in 1963. It was on PIVKA, protein induced by vitamin K absence(or antagonists) and reports my first appearance before the big personalities of coagulation of those days, such as Biggs, Brinkhous, Deutsch, Koller, Macfarlane, Seegers and Soulier. From sheer anxiety my right patella kept trembling during the full seven minutes that my talk lasted. And nobody paid more than a polite interest – with the notable exception of Peter Esnouf and François Josso.

My own initiation phase had begun some eight months before, when Fredi Loeliger had confronted me with a riddle in the control of oral anticoagulation, viz. the discrepancy between "prothrombin times" and single factor determinations. For those who are so modern as to know clotting times only from figures that come out of laboratory automatons, I will give some background information:

As everybody knows, the "prothrombin" defect in oral anticoagulation was, and still is, routinely assessed by the "prothrombin time", introduced by Armand Quick, often in a variant introduced by Owren, i.e. with the addition of $BaSO_4$ adsorbed cow-plasma, so as to make it dependent upon factors II, VII and X only. (For a review see e.g. [1]). We used a variant in which we added 0.1 ml of the plasma to be tested, 0.1 ml of $BaSO_4$ adsorbed cow plasma that contained human brain thromboplastin and recalcified at zero time with 0.1 ml of a 100 mM $CaCl_2$ solution in buffer. The "prothrombin" of Quick had turned out, in the meantime, to be a set of proteins but the "prothrombin time" remained, although "thromboplastin time" is more exact. This clotting time was recognised to be dependent upon the factors II, VII and X (factor IX standing offside at the high thromboplastin concentrations used).

Each of these factors can also be determined separately. In that case one takes 0.1 ml of plasma deficient in the factor that you want to determine; 0.1 ml of 1:10 diluted sample of the plasma to be tested and 0.1 ml thromboplastin solution in 100 mM $CaCl_2$. The 1:10 dilution was necessary to prevent that the other clotting factors present in the sample would influence the clotting time and in order to cover a useful range of clotting times. To relate clotting times to clotting factor concentrations, reference lines were made: the clotting time (t_c) at dilutions of normal plasma, by definition containing 100% of each factor, were plotted, on logarithmic paper, against the clotting factor concentration and the clotting time obtained with an unknown sample was related to a concentration using this graph.

Loeliger had found that in stable oral anticoagulation the four vitamin-K-dependent clotting factors were decreased to about the same level when tested individually (2). But in a "prothrombin time" type of experiment consistently about half of that concentration was found. Why? Was there an elusive, rate limiting fifth vitamin-K-dependent clotting factor?

In 1962 none of the clotting factors had been isolated to purity. It was not even certain that all of them were proteins. Walter Seegers and his school maintained that factors II, VII, IX and X were different aspects of one and the same prothrombin (1). Antibodies were not available, monoclonals did not exist. Clotting factors were defined as an activity required to make blood clot normally and that was congenitally absent in certain patients. Factor X e.g. was the entity lacking in the plasma of Mr. Stuart and obviously in every plasma that would not correct the defect, like that of Mrs. Prower (1). Therefore the possibilities to approach the problem were in practice limited to juggling with clotting times.

While doing this, I recalled the Lineweaver-Burke plot of enzyme kinetics: inverse reaction velocity against inverse sub-

Correspondence to:
Prof. H. C. Hemker
Synapse BV, University Maaastricht/CARIM
PO Box 616, Maastricht, Limburg
6200 MD, The Netherlands
Tel.: +31 43388 1675, Fax: +31 43388 4159
E-mail: hc.hemker@thrombin.com

Received April 17, 2007
Accepted May 9, 2007

Prepublished online June 12, 2007
doi:10.1160/TH07–04–0284

Thromb Haemost 2007; 98: 20–23

strate concentration is a straight line. I reasoned that dilution is an inverse concentration of whatever might be rate-limiting in a clotting time. Also I thought that clotting times could be inversely related to clotting velocity – whatever "clotting velocity" might in fact mean. So I made a plot of clotting time against plasma dilution – the t-D plot. To my pleasant surprise it appeared to be a straight line (Fig.1)! The intercept of this line and the abscissa – always by analogy to the Lineweaver-Burke plot – then should indicate a minimal clotting time (t_{min}), i.e. the clotting time that could not be further shortened by increasing clotting factor concentrations. Indeed we found that, in patients in which the clotting factors were low due to parenchymatous liver disease without vitamin K deficiency, such dilution curves extrapolated to roughly the same point as normal plasma did and that, logically, the slope of the line indicated the clotting factor content; not, however, in vitamin K deficiency or in oral anticoagulation: there the extrapolated minimum time was systematically much longer (Fig.1).

We reasoned – after exclusion of a number of alternatives – that an inhibitor of prothrombin conversion must be present. The minimal clotting time then would represent infinite clotting factor concentration but infinite inhibitor concentration as well. Further calculations and experiments showed that it should be a competitive inhibitor. Logically, competitive inhibition suggests a substrate analogue and therefore an analogue of prothrombin. So we thought of a prothrombin-like protein induced by the lack of functional vitamin K and we postulated a precursor of prothrombin that escaped into the circulation because its final synthesis step was blocked (3). It was a long shot, but a hit.

Alas we did not bring home more than half of the game. I was so eager to demonstrate that the inhibitor was a prothrombin-like protein that I made a fundamental mistake. It was considered a very specific property of prothrombin to adsorb easily to bentonite, and so did our inhibitor. Prothrombin also was known to adsorb to $BaSO_4$ but our inhibitor hardly would. In my zeal to stress the similarities with prothrombin I added so much adsorbent that it finally did. I did not mention the quantity used in the letter to *Nature* and neither did I explore this difference further. What if I had? With hindsight, it is infuriating that, at that moment, we did not do the experiment that we published only in 1970, i.e. the activation of the euglobulin fraction of plasma (to exclude antithrombins) from orally anticoagulated patients (4). There we saw that much more thrombin formed than could be accounted for by the prothrombin content measured in a one-stage test. First, a quick thrombin generation occurred, in proportion with the amount of normal prothrombin present, thereafter a slow generation followed, well in excess of that amount, and obviously due to the activation of an abnormal prothrombin. All the techniques to do this simple experiment were already available in 1963, and it would have taken a week to finish the experiments. If we had done that, we would have shown that in plasma from orally anticoagulated patients two types of prothrombin existed, one determined in a one-stage clotting assay and readily adsorbed to $BaSO_4$, the other slowly converted and escaping notice in the presence of antithrombins. This might have been more direct evidence than the rather abstract kinetic reasoning, that indeed could not convince everybody. Hendrik Dam himself e.g. remained reluctant to accept our reasoning, because he could not

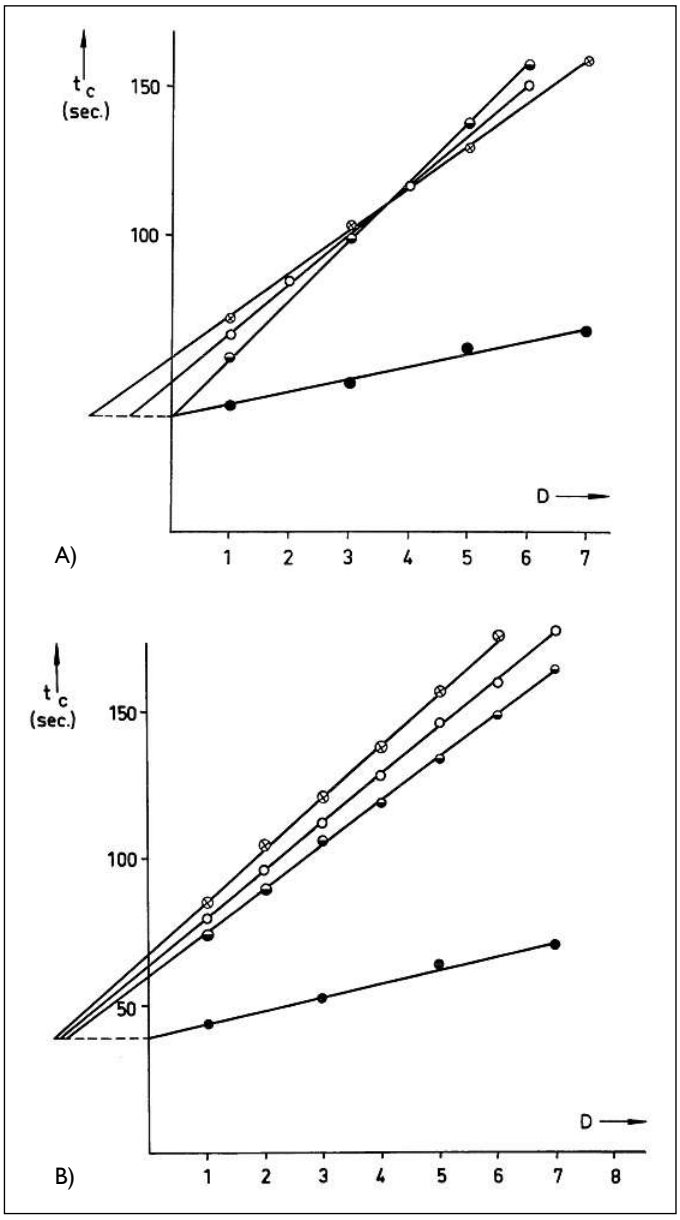

Figure 1: Thrombotest dilution curves. A) Mixture of cirrhosis plasma and vitamin-k-deficient plasma. ●–● t-D plot of normal plasma; ◒–◒ t-D plot of cirrhosis plasma; ⊗–⊗ t-D plot of vitamin-K-deficient plasma; ❍–❍ t-D plot of a mixture of equal parts of cirrhosis plasma and vitamin-k-deficient plasma. B) Mixture of dicumarol plasma and vitamin-k-deficient plasma. ●–● T-D plot of normal plasma; ⊗–⊗ t-D plot of dicumarol plasma; ◒–◒ t-D plot of vitamin-K-deficient plasma; ❍–❍ t-D plot of a mixture of equal parts of dicumarol plasma and vitamin-k-deficient plasma. (Printed with permission from ref. [10]).

reproduce the PIVKA phenomenon in his chickens – for the good reason that in chickens the precursor does not appear in the circulation. In cows it does, but their PIVKAs have no inhibitory properties. On the whole our hypothesis on the mode of action of vitamin K was neither contradicted nor recognised as something particularly worthwhile.

The real progress in the matter came in 1968, more than five years after our letter, when Ganrot and Nilehn demonstrated two types of prothrombin by immuno-electrophoresis (5) and got Johan Stenflo interested in the problem, which, several years

later, led to the discovery of gamma carboxy glutamic acid (see further [6]). Here I must mention that already in 1965 François Josso showed me immuno-electrophoretic experiments that represented two types of prothrombin that differed in Ca^{++}-binding but, regrettably, timely publishing was not among his numerous qualities (7).

In the meantime we continued to try and apply enzyme kinetics to clotting times and published the results in a series of articles: Kinetic aspects of the interaction of clotting factors (8–15). The first of the series was an attempt to give an underlying theory to the enzyme kinetics of clotting times. In itself it was an amusing exercise to sort out the differences between classical kinetics and that of blood clotting: no initial velocity measurements but end-point measurements of product (fibrin), not necessarily an excess of enzyme over substrate, no real blanks because of the presence of residual amounts of clotting factor in deficient plasmas etc. My error was not so much in pursuing this line of research but very much in the forbidding way I presented it. If I would have had the intention to scare away even the most interested clotting physiologist or clinician I could not have done better. That first article brought me a solid reputation of incomprehensibility that has haunted me for years. Upon rereading I now primarily spot the junior scientist that wanted to show off.

Is there nevertheless anything that remains useful in this series of articles? Perhaps yes. In the second article of the series (9) we verified the validity of the straight line relationship between thromboplastin time and plasma dilution. To make our point (rather our line), the experimental data were the mean of a minimum of 50 clotting times each. Two times the standard deviation of the mean fitted within the diameter of the dots in the graphs. Just counting the experimental points in that article shows that it must be based on the determination of well over 5,000 (hand made) clotting times. It left no doubt that the graph of clotting time against the inverse of plasma concentration is a straight line. There is no doubt either that nobody found this especially interesting. When some ten years later the comparison of thromboplastins became an issue, the calculation (international normalized ratio, INR) was based on an assumed rectilinear relationship between the logarithms of clotting time and plasma concentration, which, in the mean time we had shown to be distinctly S-shaped (12, 16).

As I said then, and still think today, "This finding of a simple straight line relationship between clotting time and plasma dilution is by no means simple to explain" (9). It must tell something about the kinetics of thrombin generation during the initiation phase but what it told remained enigmatic. In the last article of the series (15), which better should have come first, we showed that clotting occurs when the area under the thrombin-time curve attains a certain fixed value (at a given fibrinogen concentration). A fixed amount of enzymatic "work" apparently is required to obtain clotting. This foreshadows the endogenous thrombin potential (ETP): The initiation phase is apparently a micro-thrombin generation experiment in its own right, with thrombin concentrations that are so low that they escape detection in a normal thrombin generation experiment.

The clotting time marks the moment when enough thrombin has acted long enough to make fibrinogen clot. The straight t-D curve therefore shows that the amount of thrombin formed before clotting occurs is proportional to the plasma dilution. This may sound logical, but in fact it is not. In a normal thrombin generation experiment, i.e. in the production phase, the amount of thrombin formed increases as plasma is diluted! At a 1:24 dilution as much free thrombin is formed as at 2:3 dilution, primarily because both prothrombin and antithrombin are diluted (E. De Smedt, to be published). This suggests that, under the conditions of a "prothrombin time" measurement, antithrombin does not play a role, which is in line with the observation that the thromboplastin time is hardly influenced by heparin and implies that free thrombin hardly occurs during the initiation phase. Probably (meizo-)thrombin will activate factor V on the phospholipid surface, and probably most of the thrombin that gets into solution immediately binds to fibrinogen and is not released when fibrin is formed. The strong binding of thrombin to fibrin is long since known (17), and the dependence of clotting time on the fibrinogen concentration is compatible with a reaction mechanism in which the thrombin-fibrin intermediate does not dissociate but converts another fibrinogen (8). As an aside, one may question why then the activated partial thromboplastin time is influenced by heparin? Elsewhere we argued that this is because thrombin in free solution is an essential element in the generation of prothrombinase as soon as factor VIII is involved in the mechanism (see [18]).

In a further article of the "Kinetic aspects" series we completed and extended the experiments that led to the discovery of PIVKA (12) and in a fourth one, as forbidding as the first one, we treated the kinetics of competitive inhibition in clotting tests (13).

The experiments thus far were on thromboplastin times, i.e. on experimental systems, where the three factors II, VII and X were diluted simultaneously. Next we wanted to investigate the behaviour of the clotting time as a function of each of the factors of the extrinsic system separately (14). This was complicated by the fact that the deficient plasmas that were used as reagents never were completely devoid of the deficient factor. The reagent-plasma always would clot, even when not a trace or normal plasma was added. However, we found a trick to measure the amount of residual factor in the deficient plasma and then could show that the clotting time is inversely proportional to each of the concentrations of the factors of the extrinsic system. Only years later, when we could make completely deficient plasmas, we were able to check that we had not been mistaken (16).

In the light of the inverse relation between clotting time and the amount of thrombin formed (17), today I would simply interpret these results to show that, in a thromboplastin-time type of experiment, the amount of thrombin formed during the initiation phase is proportional to the rate limiting clotting factor, whether this is factor II, V, VII or X. In the original article we went into great length to demonstrate that the relations found were not in accordance with the original cascade hypothesis but required a mechanism in which prothrombinase was a reversible stoichiometric product of Xa and Va and was inactivated during the clotting process.

By that time, together with Macfarlane and Esnouf in Oxford, we had found that prothrombinase formed as a reversible complex of factors Va and Xa on phospholipid (21). It was natural that we wanted to show that our results in plasma confirmed that

reaction scheme. In the light of what we now know about tissue factor pathway inhibitor, proteins S and C and other complications of the tissue factor-initiated clotting mechanism, our attempts to reconcile our clotting times with our experiments on the mechanism of prothrombinase seem rather bold. I suspect that there may have been some mistake or error that made things come out right. Nevertheless, I still would agree that our results did not confirm to the original cascade hypothesis. In a further article we used the kinetics with single factors to localise the site responsible for the prolongation of the thromboplastin time by PIVKA at the level of factor Xa (13).

In retrospect I must consider that our attempts to squeeze information out of the relation between clotting times and clotting factor concentrations have been of little practical value. They could have been used for the standardisation of prothrombin times and for the construction of exact reference curves for one-stage clotting factor determinations (20), but they were not. Neither did they contribute to our recognition of tenase as a complex of the antihaemophilic factors on phospholipids (21, 23).

Enzyme kinetics with purified factors became a main line of research when we had moved to Maastricht in1975. Jan Rosing was the first to demonstrate that factor Va serves to enhance the turnover of prothrombin by factor Xa and that phospholipids decrease the Km (24). Van Dieijen showed similar functions of factor VIIIa and phospholipids in tenase (25). In Maastricht also, Vermeer and Soute continued the work on vitamin K and developed it into a flowering research line (see e.g. [26]).

In retrospect we probably should have paid more attention to thrombin generation experiments then we did (4, 22), but that somehow was not in the tradition of the Leiden group. Only in the late 1980s, after some years in a higher administrative function that were not nearly as exciting as research is, a sabbatical brought me to Paris, where I began my collaboration with Suzette Béguin and stage two – two stage experiments – could be started.

References

1. Stormorken H, Paul A. Owren & the golden era of haemostasis. Sandvika, Gazettebok, 2005.

2. Loeliger EA, van der Eb, Mattern MJ, et al. Behaviour of factors II, VII, IX and X during long-term treatment with coumarin. Thromb Diath Haemorrh 1963; 9: 74–89.

3. Hemker HC, Veltkamp JJ, Hensen A, et al. Nature Of Prothrombin Biosynthesis: Preprothrombinaemia In Vitamin K-Deficiency. Nature 1963; 200: 589–590.

4. Hemker HC, Muller AD, Loeliger EA. Two types of prothrombin in vitamin K deficiency. Thromb Diath Haemorrh 1970; 23: 633–637.

5. Ganrot PO, Nilehn JE. Plasma prothrombin during treatment with Dicumarol. II. Demonstration of an abnormal prothrombin fraction. Scand J Clin Lab Invest 1968; 22: 23–28.

6. Stenflo J. From gamma-carboxy-glutamate to protein C. J Thromb Haemost 2006; 4: 2521–2526.

7. Josso F, Lavergne JM, Gouault M, et al. Various molecular states of factor II (prothrombin). Their study by means of staphylocoagulase and anti-factor II antibodies. I. Factor II in patients treated with vitamin K antagonists. Thromb Diath Haemorrh 1968; 20: 88–98.

8. Hemker HC, Hemker PW, Loeliger EA. Kinetic aspects of the interaction of blood-clotting enzymes. I. Derivation of basic formulas. Thromb Diath Haemorrh 1967; 13: 155–175.

9. Hemker HC, Siepel T, Altman R, et al. Kinetic aspects of the interaction of blood-clotting enzymes. II. The relation between clotting time and plasma concentration in prothrombin- time estimations. Thromb Diath Haemorrh 1967; 17: 349–357.

10. Hemker HC, Veltkamp JJ, Loeliger EA. Kinetic aspects of the interaction of blood clotting enzymes. III. Demonstration of an inhibitor of prothrombin conversion in vitamin K deficiency. Thromb Diath Haemorrh 1968; 19: 346–363.

11. Hemker HC, Hemker PW. Kinetic aspects of the interaction of blood clotting enzymes. IV. Kinetics of competitive inhibition in clotting tests. Thromb Diath Haemorrh 1968; 19: 364–367.

12. Hemker HC, Muller AD. Kinetic aspects of the interaction of blood clotting enzymes. V. The reaction mechanism of the extrinsic clotting system as revealed by the kinetics of one-stage estimations of coagulation enzymes. Thromb Diath Haemorrh 1968; 19: 368–382.

13. Hemker HC, Muller AD. Kinetic aspects of the interaction of blood-clotting enzymes. VI. Localization of the site of blood-coagulation inhibition by the protein induced by vitamin K absence (PIVKA). Thromb Diath Haemorrh 1968; 20: 78–87.

14. Hemker HC, Vermeer C, Govers-Riemslag J. Kinetic aspects of the interaction of blood clotting enzymes. VII. The relation between clotting time and prothrombin concentration. Thromb Haemost 1977; 37: 81–85.

15. Hemker HC, Kop J, Willems GM. Kinetic aspects of the interaction of blood clotting enzymes. VIII. The relation between clotting time and clotting velocity. Thromb Haemost 1979; 41: 309–313.

16. Hemker HC, Meer Jvd, Loeliger EA. Kinetic basis of prothrombin estimation particularly with reference to the rectilinearity of the log-log reference curve. Thromb Diath Haemorrh 1965; 17 (Suppl): 247–252.

17. Seegers WH, Nieft M, Loomis EC. Note on the adsorption of thrombin on fibrin. Science 1945; 101: 520–1.

18. Hemker HC, Al Dieri R, De Smedt E, et al. Thrombin generation, a function test of the haemostatic-thrombotic system. Thromb Haemost 2006; 96: 553–561.

19. Hemker HC, Esnouf MP, Hemker PW, et al. Formation of prothrombin converting activity. Nature 1967; 215: 248–251.

20. Hemker HC, Swart AC, Alink AJ. Artificial reagents for factor VII and factor X, a computer programme for obtaining reference tables for one-stage determinations in the extrinsic system. Thromb Diath Haemorrh 1972; 27: 205–211.

21. Hemker HC, Kahn MJ. Reaction sequence of blood coagulation. Nature 1967; 215: 1201–1202.

22. Hemker HC, Hemker PW, Torren Kvd, et al. The evaluation of the two-stage prothrombin assay. Thromb Diath Haemorrh 1971; 25: 545–554.

23. Varadi K, Hemker HC. Kinetics of the formation of the factor X activating enzyme of the blood coagulation system. Thromb Res 1976; 8: 303–317.

24. Rosing J, Tans G, Govers-Riemslag JW, et al. The role of phospholipids and factor Va in the prothrombinase complex. J Biol Chem 1980; 255: 274–283.

25. Van Dieijen G, Tans G, Rosing J, Hemker HC. The role of phospholipid and factor VIIIa in the activation of bovine factor X. J Biol Chem 1981; 256: 3433–3442.

26. de Metz M, Vermeer C, Soute BA, et al. Identification of phospholipid as an essential part of bovine vitamin K-dependent carboxylase. J Biol Chem 1981; 256: 10843–10846.