Studies on Blood Coagulation Factor V

VI. The Inactivation of Factor V and Prothrombinase

From the Laboratory for Coagulation Biochemistry and Cardiovascular Biochemical Research, Clinic for Internal Medicine, University Hospital, Leiden, The Netherlands

H. C. Hemker, M. J. P. Kahn
Factor V is listed as an instable coagulation factor. The term "instable" is used confusingly for two distinctly different properties of this factor. In the first place it points to the fact that in human plasma factor V is consumed during the coagulation process; in the second place it means that factor V upon storage disappears relatively rapidly from normal blood and plasma, which is presumably a consequence of its liability to denaturation. The two processes will be called briefly consumption and denaturation henceforth.

It is our aim here to compare denaturation and consumption and to show that these indeed are different processes. This is done by the analysis of the kinetics of the disappearance of factor V and prothrombinase activity from different kinds of preparations. This subject has hardly been touched in pre-existent literature. Weiss (1965) has studied the dependency of the velocity of denaturation upon the concentration and the nature of bivalent cations present. He found that Ca⁺⁺-ions are responsible for the maintenance of factor V activity. He also found that inactivation brought about by lack of Ca⁺⁺-ions is partly reversible, whereas the denaturation under alkaline conditions is not. Although assumptions are made by Weiss on the kinetics of inactivation, these assumptions are not unequivocally supported by the published data. A re-investigation of this point therefore was thought to be worth-while.

Blombäck and Blombäck (1963) also reported a stabilizing effect of bivalent cations on factor V. This was also mentioned in earlier reports of Quick (1947) and Stefanini (1950). Weiss, however, was the first to determine the bivalent cation to be Ca⁺⁺.

Materials and Methods

Normal human plasma was prepared as described by Hemker et al. (1968). The final concentration of citrate was 20 mM.

Bovine plasma was prepared in the same way as human plasma from bovine blood obtained at the slaughter house by collecting in 1/50 vol. of tri Na-citrate 0.55 M.

Factor V and factor X determinations were carried out in one-stage assays by comparison of the coagulation-time obtained by adding the (diluted) sample to a reaction mixture specifically deficient in the factor to be tested only, with the coagulation times obtained in that reaction mixture with a series of dilutions of normal plasma. For a detailed description see Hemker et al. (1968).

Al(OH)₃-adsorption was used to free plasma from the factors II, VII, IX and X; it was carried out according to Biggs and Macfarlane (1962).

Ba-Stearate adsorption and elution of factor V from Bsteareate was carried out as described by Kahn and Hemker (1969).

A factor V preparation (TEAE eluate) was prepared by adsorption of factor V onto TEAE from Al(OH)₃ treatend normal human plasma and subsequent elution as described in detail by Kahn and Hemker (1970).
Prothrombinase was generated in mixtures of serum, Al(OH)₃ adsorbed plasma and a phospholipid suspension (Bell and Alton, 1964), containing CaCl₂ (6.25 mM) at pH 7.9. The prothrombinase activity as it develops and declines was estimated by subsampling 0.1 ml of the incubation mixture in 0.1 ml of normal plasma together with 0.1 ml of CaCl₂ 25 mM. The method was essentially equal to the thromboplastin generation test as described by Biggs and Macfarlane (1962).

When maximal activity developed one part of the mixture was rapidly cooled to 0° C to prevent inactivation. Serial dilutions were made from this part, and the clotting times obtained in a mixture of 0.1 ml of each of these dilutions with 0.1 ml normal plasma and 0.1 ml of 25 mM CaCl₂, were used to construct a reference curve. The remaining amount of the mixture was kept at 37° C and tested at regular intervals. By comparison of the clotting times from this part of the incubation mixture with the reference curve, obtained with the other part, the development of the activity of the prothrombinase mixture could be expressed as a percentage of the maximal activity attained.

**Experimental Results**

*The Inactivation of Factor V in Plasma*

Factor V activity disappears rapidly from human citrated plasma left at room temperature or higher. From Fig. 1 it can be seen that in first approximation this inactivation follows first order reaction kinetics, the plot of the logarithm of the residual activity against time being approximately straight.

![Figure 1](image)

Fig. 1. Disappearance of factor V from normal human plasma. The data presented are plotted as the logarithm of the residual activity against time. The pH was checked and did not change during the experiment.

<table>
<thead>
<tr>
<th>temp.</th>
<th>t½ (min)</th>
<th>k</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>2.5</td>
<td>277</td>
</tr>
<tr>
<td>46</td>
<td>4.6</td>
<td>147</td>
</tr>
<tr>
<td>43</td>
<td>10.5</td>
<td>66</td>
</tr>
<tr>
<td>40</td>
<td>21.7</td>
<td>22</td>
</tr>
<tr>
<td>37</td>
<td>47.0</td>
<td>15</td>
</tr>
<tr>
<td>30</td>
<td>170.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

t½ is the half life time of factor V activity in minutes, k is calculated as 693 / t½.
From the slopes of the lines in Fig. 1 one can estimate first order reaction constants of the inactivation reaction at different temperatures. The estimated values are shown in Table 1. From them a rectilinear Arrhenius plot can be constructed (Fig. 2). From the slope of this plot the activation energy $E$ of the denaturation reaction is estimated to be 44 kcal/mol. $Q_{10}$, i.e. the factor by which the reaction velocity rises when the temperature is raised 10°C, is about 10. In all cases for which we calculated velocities of inactivation, activation energies of the inactivation reaction and $Q_{10}$, we used plots analogous to those in Figs. 1 and 2.

![Arrhenius plot](image)

Fig. 2. Arrhenius plot of the inactivation reaction of factor V in normal human plasma. Log $k$ estimated as indicated in Table 1 from the slopes of the lines in Fig. 1, is plotted against inverse of the absolute temperature.

When the velocity of the inactivation is measured in the presence of glycerol (50%) or MgSO$_4$ (0.1 M) the course of the inactivation reaction again is not distinguishable from that of a first order reaction (Kahn and Hemker, 1970b). An Arrhenius plot constructed from the first order rate constants obtained under these conditions, shows that at all temperatures glycerol slows down the rate of inactivation by a factor of about 3.5 and MgSO$_4$ does the same by a factor of between 1.5 and 2. No changes in the activation energy of the inactivation reaction were observed, however (Fig. 3).

Purified fractions showed inactivation velocities that were 8 to 5 times higher than those in normal plasma. As can be seen from Table 2, $E$ of the inactivation reaction
showed a tendency to increase under these circumstances, but the significance of this observation remains questionable.

![Arrhenius plot](image)

**Fig. 3.** Arrhenius plot of the inactivation reaction of human factor V in the presence of glycerol or MgSO₄. ○○○ in the presence of 50% glycerol; ●●●● in the presence of 0.5 M MgSO₄.

Drawn line: the line from Fig. 2 given for comparison.

**Table 2. Constants of inactivation of factor V under different circumstances.**

<table>
<thead>
<tr>
<th></th>
<th>k</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal plasma</td>
<td>100</td>
<td>44</td>
</tr>
<tr>
<td>Normal plasma diluted 1 in 2 with buffer</td>
<td>130</td>
<td>52</td>
</tr>
<tr>
<td>Normal plasma diluted 1 in 2 with glycerol</td>
<td>28</td>
<td>42</td>
</tr>
<tr>
<td>Normal plasma with 10 mM MgSO₄</td>
<td>60</td>
<td>44</td>
</tr>
<tr>
<td>Al(OH)₃ adsorbed normal plasma</td>
<td>108</td>
<td>38</td>
</tr>
<tr>
<td>Ba-stearate adsorbed normal plasma</td>
<td>120</td>
<td>36</td>
</tr>
<tr>
<td>Ba-stearate eluate</td>
<td>370</td>
<td>53</td>
</tr>
<tr>
<td>TEAE eluate in 50% glycerol and 10 mM MgSO₄</td>
<td>430</td>
<td>54</td>
</tr>
<tr>
<td>Bovine plasma</td>
<td>0.3</td>
<td>66</td>
</tr>
</tbody>
</table>

k = monomolecular reaction constant of inactivation at 37°C in arbitrary units.

E = activation energy of inactivation in kcal/mol.
The data from the inactivation of bovine factor V did not give a straight line in a semi-logarithmic plot; consequently this reaction cannot be first order. These data gave an approximately straight line when the inverse of the remaining activity was plotted versus time. This suggests a bimolecular reaction (Fig. 4) (cf. ref. 14). The bimolecular reaction constants were determined from these plots. The activation energy of this reaction was estimated to be about 66 kcal/mol. The velocity of this inactivation was appreciably lower than that of human factor V (Fig. 5). All of these experiments were carried out at pH 7.5. No noticeable change in inactivation velocity was found between pH 6.4 and 8. Factor V is reported to be very unstable at pH’s below 5 and above 9.5 (Weiss, 1965).

Repeatedly, a partially purified preparation of human factor V that was relatively stable in 50% glycerol, lost its activity when glycerol was dialyzed out against 10 mM MgSO₄, but regained activity when dialyzed again against 10 mM MgSO₄ in 0.5 mM tris-H₂SO₄ buffer containing 50% glycerol. The yields found in 10 different experiments were: starting material 100% (by definition); after dialysis (90 h, 5° C) against 0.01 M MgSO₄ in buffer: 1.8% (range 0.3–10%); after second dialysis (12 h, 5° C) against 50% glycerol and 0.01 M MgSO₄: 14% (range 2–27%).

The Inactivation of Prothrombinase

Because factor V is consumed during the process of coagulation, i.e. after having formed part of the prothrombinase complex, the study of the inactivation of prothrombinase forms an essential part of the study of the degradation of factor V.

The inactivation of human as well as of bovine prothrombinase apparently follows second order kinetics (Fig. 6). From Fig. 7 it can be calculated that the activation energy of this inactivation reaction is about 10 kcal per mol, which is substantially less than the value found for the inactivation of factor V in non-clotting plasma. The reac-
Fig. 6. Arrhenius plot of the inactivation reaction of bovine factor. The values of \( k \) were obtained from Fig. 6 and comparable graphs. b: data obtained with bovine material; h: line obtained with human material (Fig. 2) shown for comparison.

The inactivation velocity of the inactivation of prothrombinase is much higher than the velocity of inactivation of factor V in plasma.

To investigate whether a serum or a plasma protein takes part in the inactivation of prothrombinase, we made prothrombinase in mixtures in which the ratios of serum to \( \text{Al(OH)}_2 \)-adsorbed plasma were varied. From Table 3 it can be seen that the increase of this ratio causes a decrease in the reaction constant of the inactivation of human prothrombinase. This suggests that \( \text{Al(OH)}_2 \)-adsorbed plasma contains a factor that enhances the inactivation rate. When \( \text{Al(OH)}_2 \)-adsorbed bovine plasma is used as a source of factor V, the reaction constant of inactivation of prothrombinase drops appreciably. To determine whether it is factor V or some other factor that influences the rate of inactivation of prothrombinase, we added \( \text{Al(OH)}_2 \)-adsorbed plasma obtained from a patient with a congenital factor V deficiency to the reaction mixture. This did not influence the rate of inactivation. When serum prepared from this plasma was used instead of normal serum, the generation and inactivation of prothrombinase could not be distinguished from that in a control experiment in which normal serum was used. The rate of inactivation thus seems to be determined by the concentration of factor V.
Table 3. Inactivation velocity of prothrombinase at different ratios of adsorbed plasma and serum.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Apparent reaction constant of inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum : plasma</td>
<td></td>
</tr>
<tr>
<td>1 : 9</td>
<td>196</td>
</tr>
<tr>
<td>1 : 4</td>
<td>206</td>
</tr>
<tr>
<td>1 : 2</td>
<td>222</td>
</tr>
<tr>
<td>1 : 1</td>
<td>100</td>
</tr>
<tr>
<td>2 : 1</td>
<td>63</td>
</tr>
<tr>
<td>Serum : bovine plasma</td>
<td></td>
</tr>
<tr>
<td>1 : 1</td>
<td>52</td>
</tr>
<tr>
<td>Serum : human plasma : V-def. plasma</td>
<td>89</td>
</tr>
<tr>
<td>1 : 1 : 1</td>
<td></td>
</tr>
<tr>
<td>Serum : human plasma : buffer</td>
<td>98</td>
</tr>
<tr>
<td>1 : 1 : 1</td>
<td></td>
</tr>
</tbody>
</table>

The concentration of factor X in the serum used was 120%.

Discussion

The experimental evidence presented in the foregoing shows that the inactivation of human factor V in non-clotting plasma in first approximation can be described as a first-order reaction. The activation energy of the reaction is high: 44 kcal/mol, which is in accordance with the values mentioned for protein denaturation (Joly, 1965).

Weiss (1965) and Blombäck and Blombäck (1963) reported the finding of reversible denaturation of human factor V in plasma, dependent upon the presence of Ca++ ions. Esnouf found that bovine factor V can be reversibly inactivated. We found reversible inactivation in purified human preparations dependent upon the presence of glycerol. These results indicate that the step in the denaturation process in which the molecule loses its biological activity must be reversible. The reversibility is variable and never complete, indicating that under these circumstances the reversible step is part of a more complicated chain of reactions in which irreversible reactions occur.

The velocity of denaturation of human factor V is high. Half-life-time in normal human citrated plasma at 37°C is about 45 min (compared to 10 days for factor II). This velocity is markedly reduced by the addition of Mg++ ions and of glycerol.

The inactivation of factor V from bovine plasma shows the characteristics of a bimolecular reaction, suggesting a dimerization as the rate limiting step. The high value found for the activation energy again suggests a denaturation reaction.

The kinetics of the inactivation of prothrombinase in both human and bovine blood show the features of a bimolecular reaction.

The reaction velocity is about 10 times higher than that of the denaturation of factor V in intact human plasma. The activation energy of the process is about 10 kcal/mol, which indicates that here a process differing from a denaturation reaction is setting the pace. The results shown in Table 3 strongly suggest that the rate of inactivation in dependent upon a protein that is absent in serum and in congenitally factor V deficient plasma. The factor that enhances prothrombinase breakdown therefore in all probability is identical with factor V.
At the moment there is much evidence that prothrombinase is a complex consisting of a molecule of factor $X_a$ and a molecule of factor $V_a$ bound next to each other on a phospholipid surface. It cannot be readily imagined that two prothrombinase complexes combine in order to inactivate, given the surface bound nature of these units. This leaves us with the possibility that free factor $V_a$ combines with prothrombinase to give inactive prothrombinase.

\[
\text{Prothrombinase} + V_a + X_a \rightleftharpoons \text{Prothrombinase}
\]

\[
\text{Prothrombinase} + V \rightarrow \text{Inactive prothrombinase}
\]

In conclusion it may be said that the denaturation of factor $V$ in human plasma is a process that is essentially different from the inactivation of factor $V$ in the course of the coagulation process. The disappearance of factor $V$ activity from human plasma can be described as a protein denaturation. From bovine plasma factor $V$ disappears in a second-order reaction with a high activation energy, presumably a denaturation in which a dimerization is rate-limiting. Prothrombinase inactivates in both human and bovine material in a second order reaction, presumably because a second molecule of factor $V$ combines with the prothrombinase complex.

**Summary**

The disappearance of factor $V$-activity from human plasma on storage can be described as a first order reaction with an activation energy of about 44 kcal/mol.

The disappearance of factor $V$-activity from bovine plasma in vitro is a second order reaction with an activation energy of about 66 kcal/mol.

The inactivation of human prothrombinase during coagulation is a second order reaction; the activation energy is about 10 kcal/mol. It is concluded that this inactivation involves a reaction of the factor $V$ moiety of prothrombinase with free factor $V$.

**Résumé**

La disparition de l'activité du facteur $V$ pendant la conservation du plasma humain est une réaction de premier ordre avec une énergie d'activation d'environ 44 kcal/mol.

La disparition de l’activité du facteur $V$ dans le plasma bovin in vitro est une réaction de second ordre avec une énergie d’activation d’environ 66 kcal/mol.

L’inactivation de la prothrombinase humaine pendant la coagulation est une réaction de second ordre; l’énergie d’inactivation est d’environ 10 kcal/mol. On en conclut que l’inactivation est due à une réaction du facteur $V$ de la prothrombinase avec du facteur $V$ libre.

**Zusammenfassung**

Das Verschwinden der Faktor-V-Aktivität aus menschlichem Plasma während der Lagerung kann als eine Reaktion erster Ordnung mit einer Aktivierungsenergie von ungefähr 44 kcal/mol beschrieben werden.

References

(1) Bell, W. N., H. G. Alton: Brain extract as a substitute for platelet suspensions in the thrombo-
(1967).
(5) Hemker, H. C., J. J. Velthamp, E. A. Looiiger: Kinetic aspects of the interaction of blood
clotting enzymes. III. Demonstration of the existence of an inhibitor of prothrombin con-
(7) Kahn, M. J. P., H. C. Hemker: Studies on blood coagulation factor V. Preparation and prop-
erties of an artificial factor V reagent by adsorption with Ba-stearate. Coagulation 3: 55
(1970a).
(8) Kahn, M. J. P., H. C. Hemker: Studies on blood coagulation factor V. A partially purified
(9) Kahn, M. J. P., H. C. Hemker: Studies on blood coagulation factor V. The interaction of salts
(10) Kahn, M. J. P., H. C. Hemker: Studies on blood coagulation factor V. Changes of molecular
weight accompanying activation of factor V and the procoagulant protein of Russell's viper
(12) Stefanini, M.: New one-stage procedure for the quantitative determination of prothrombin

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