The Effect of Proteolytic Enzymes on Bovine Factor V. I. Kinetics of Activation and Inactivation by Bovine Thrombin*

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ABSTRACT: This study focuses on the interaction between bovine factor V, purified 5000-fold over the starting plasma and free of contaminating coagulation factors, and bovine thrombin free of other proteolytic enzymes. Thrombin increases the activity of factor V more than twofold, a process blocked by the thrombin inhibitor hirudin. The reaction follows pseudo-first-order kinetics. Both the rate and extent of activation were proportional to thrombin concentration. The loss of factor V activity is 2.5 times more rapid following thrombin proteolysis than observed during incubation of native factor V. Acceleration of inactivation appears to be due to the formation of an unstable species rather than to further digestion since the decay is not blocked by hirudin. Thermal inactivation of factor V was unlikely since the activation energy of the decay process was 12,000 cal/mole. Both the rate and extent of the increase in activity induced by thrombin were inversely proportional to factor V concentration. This is attributed to substrate or product inhibition. Since factor V is necessary for the conversion of prothrombin into thrombin, these studies indicate both a positive and negative feedback mechanism which may help to regulate the rate and extent of blood coagulation.

Factor V[1] is necessary for the conversion of prothrombin into thrombin in both the intrinsic and extrinsic systems of blood coagulation in the presence of activated factor X, phospholipid, and calcium. Following incubation of factor V with thrombin, a marked increase in the ability of factor V to accelerate prothrombin conversion into thrombin was observed (Ware *et al.*, 1947; Hjort, 1957; Therriault *et al.*, 1957; Rappaport *et al.*, 1963). However, other investigators have disputed that thrombin catalyzes an increase in factor V activity (Hardisty, 1955; Surgenor *et al.*, 1960; Breckenridge and Ratnoff, 1965). Even with highly purified preparations of factor V (Esnouf and Jobin (1967) and Barton and Hanahan (1967)), disagreement exists as to the effect of thrombin. The experiments cited used crude thrombin preparations contaminated with activated factor X (Kerwin and Milstone, 1967), a protein known to complex with factor V. The ability to free thrombin of contaminating proteolytic enzymes and the availability of highly purified factor V make a reinvestigation of the action of thrombin on factor V feasible.

The present study indicates that thrombin catalyzes the formation of an altered form of factor V, which possesses more than twice the prothrombin-converting activity of the original protein. The altered form decays at 2.5 times the rate of native factor V owing to intrinsic instability rather than to further thrombin-induced proteolysis.

Methods

Purification of Bovine Factor V. The purification of factor V was accomplished by a modification in part suggested by Yin (E. T. Yin, 1967, personal communication) of the method of Esnouf and Jobin (1967). Bovine blood was collected in plastic buckets containing one-tenth volume of 0.1 M sodium oxalate. The plasma was obtained by centrifugation at 2300*g* for 15 min at 4°, then adsorbed with $BaSO_4$ (100 mg/ml) for 30 min at 27° with mechanical stirring. $BaSO_4$ was removed by centrifugation at 2000*g* for 15 min at 4°. After the addition of an equal volume of distilled water to the treated plasma, the pH was adjusted to 7.0 and TEAE-cellulose powder was added (3 g/100 ml of the undiluted plasma) and stirred for 20 min. at 27°. The TEAE-cellulose was collected by centrifugation at 3000*g* for 30 min at 4° and washed four times in a total of four volumes of 0.04 M potassium phosphate buffer (pH 7.0). After recentrifugation, the TEAE-cellulose was eluted by stirring in 0.25 volume of 0.4 M potassium phosphate buffer (pH 7.0) for 30 min at 27°. After centrifugation at 6000*g* at 4° for 20 min, the supernatant fraction was adjusted to 20° and solid $(NH_4)_2SO_4$ was slowly added to 25% saturation and equilibrated for 20 min prior to centrifugation at 20° for 20 min. The supernatant fraction was filtered and $(NH_4)_2SO_4$ was added to 70% saturation at 20° and equilibrated for 15 min

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[1] The designations of clotting activities conform to the recommendations of the International Committee for the Standardization of Blood Clotting Factors (*J. Amer. Med. Assoc. 170*, 325 (1959)).

prior to centrifugation at 20° for 20 min at 7000*g*. The precipitate was redissolved in 0.02 volume of 0.04 M potassium phosphate (pH 7.0). The $(NH_4)_2SO_4$ was removed by gel filtration on a Sephadex G-25 column or more conveniently by dialysis against 0.04 M potassium phosphate (pH 7.0) at 4° for 18 hr. The solution was applied to a 9 × 5 cm cellulose phosphate column previously equilibrated with 0.04 M potassium phosphate buffer (pH 7.0). A large protein peak containing no factor V activity was eluted by 0.15 M potassium phosphate buffer (pH 7.0) until the absorbancy of the eluate at 280 mμ was less than 0.020. Factor V activity was eluted with 0.4 M potassium phosphate buffer, pH 7.0. The best fractions were pooled, concentrated using an Amicon ultrafiltration apparatus with a UM-1 membrane (Blatt *et al.*, 1965), and frozen at −50° in the presence of 50% glycerol.

Assay of Factor V. Factor V activity was measured by using oxalate human plasma rendered factor V deficient by aging (Lewis and Ware, 1953). This reagent was prepared from 9 volumes of human blood mixed with 1 volume of 0.1 M sodium oxalate which was centrifuged at 2000*g* to remove red cells. The plasma was dialyzed against 0.1 M sodium oxalate–0.15 M NaCl for 18 hr and kept at 37° until the one-stage prothrombin time of this plasma was greater than 80 sec (usually 6–8 hr). Solutions to be tested were diluted with 0.02 M Veronal buffer in 0.15 M NaCl and 0.1 M sodium oxalate (pH 7.4). The assays were performed by adding 0.1 ml of test solution (diluted 1:10 as a standard), and 0.1 ml of aged plasma and adding a mixture of 0.1 ml of a suspension of acetone extract of human brain (thromboplastin) and 0.1 ml of 0.02 M $CaCl_2$. The time required for clot formation was measured with an automatic clot timer (Fibrometer, Baltimore Biological Laboratories). A calibration curve was constructed by assaying several dilutions of 20 normal human plasmas (assigned an average value of 1.00 unit/ml) and plotting the logarithm concentration factor V against logarithm of the average clotting times at each dilution.

Purification and Standardization of Thrombin. Thrombin was purified by the method of Yin and Wessler (1968) from crude bovine thrombin (Parke Davis). The purified as well as the commercial thrombin was standardized against a 2 mg/ml bovine fibrinogen (95% clottable) solution and compared with a standard NIH thrombin preparation (lot B_2) containing 23.7 NIH units/mg of dry protein. The standard calibration was made by plotting the reciprocal of the clotting times *vs.* thrombin concentration and a linear relationship was obtained. The esterolytic action of thrombin was also measured by quantifying the micromoles of methanol liberated from tosyl-L-arginine methyl ester by a colorimetric method (Siegelman *et al.*, 1962).

Activation of Factor V by Thrombin. Stock purified thrombin (580 units/ml), kept frozen in aliquots, was diluted to appropriate concentration with 0.02 M sodium Veronal–HCl buffer (pH 7.4) containing 0.15 M sodium chloride. The incubation mixture consisted of 0.9 ml of factor V and 0.1 ml of thrombin. At appropriate intervals, aliquots of 0.05 ml were withdrawn, immediately diluted at least tenfold with ice-cold 0.02 M Veronal–HCl buffer as above and, assayed for factor V activity in duplicates. For determination of the original activity of factor V prior to the addition of thrombin, at least two separate determinations were performed and assayed in duplicate. Thrombin at these concentrations did not clot fibrinogen (2 mg/ml) in 240 min. Temperature was maintained in a Gilson Omnibath to ±0.2°. All assays were performed within 5 min of the time of dilutions during which time no demonstrable change in activity occurs. Activity is expressed in units or in percentage of original activity prior to thrombin addition.

Other Methods. Protein concentration was determined by the absorbancy at 280 mμ (assuming an $E_{280}^{1\%}$ of 10) or by the method of Waddell (1956) for concentrations less than 0.1 mg/ml. Fibrinogen was measured by the method of Ratnoff and Menzies (1951). Factor II[2] was measured by the method of Owren and Aas (1951). Factors VIII and XI were determined by modification of the method of Proctor and Rappaport (1961) using congenitally deficient plasmas. Factor X was determined by the method of Bachman *et al.* (1958). Plasminogen was measured by the method of Alkjaersig *et al.* (1959). Proteolytic activity was measured against casein as in the determination of plasminogen but without the addition of streptokinase. Fibrinolytic activity was measured on untreated fibrin plates (Astrup and Mullertz, 1952).

Materials. Thrombin, purchased from Parke Davis, was purified as described in Methods. Hirudin, supplied by Sigma Chemical Co., was diluted in the same Veronal buffer as thrombin; 1 unit neutralized 1 NIH unit of thrombin. Brain thromboplastin was prepared by the method of Hjort (1957). Inosithin from Associated Concentrates was homogenized in appropriate buffer to make a stock solution of 2.8 g/100 ml and stored at −20°. Cellophane dialysis tubing (Visking) was boiled in 0.001 M EDTA and washed thoroughly prior to use. Bovine fibrinogen 95% clottable was procured from Gallard Schlesinger Chemical Manufacturing Corp. $BaSO_4$ was purchased from Merck and Co. Tosyl-L-arginine methyl ester was obtained from Cyclo Chemical. TEAE-cellulose (Cellex T) with an exchange capacity of 0.55–0.60 mequiv/g was supplied by Bio-Rad Laboratory and used as the dry powder. Cellulose phosphate (Whatman Chromedia P11) was obtained from Gallard Schlesinger Chemical Corp.; 1000 g was washed with 10–20 l. of 0.4 M potassium phosphate buffer (pH 7.0) until the filtrate was no longer yellow. It was then washed with distilled water until no phosphate was detectable as evidenced by a lack of turbidity upon mixing equal parts of the filtrate with 0.1 M $CaCl_2$. Finally, the cellulose phosphate was washed with 5–10 l. of 0.04 M potassium phosphate buffer (pH 7.0).

[2] Clotting activities are defined as containing 1.0 unit/ml on the basis of calibration curves separately determined on 20 normal plasmas.

TABLE I: Purification of Bovine Plasma Factor V.

Step	Vol (ml)	Total Factor V Act. (units)	Purifcn Factor[b]	Sp Act. (units/mg)	Yield (%)
Bovine plasma[a]	1800	2600	1	0.0175	100
TEAE-cellulose eluate	630	2040	111	1.94	78.5
$(NH_4)_2SO_4$ precipitate (70% saturation)	37	2220	183	3.20	85.5
Cellulose phosphate eluate (0.4 M phosphate, pH 7.0)	565	900	2760	48.50	34.5

[a] The original bovine plasma assayed at 1.44 units/ml. [b]Based on original bovine plasma.

TABLE II: Analysis of Purified Bovine Factor V and Thrombin for Possible Contaminants.

Coagulation Act.	Purified Bovine Factor V[a] (unit/ml)	Purified Bovine Thrombin[b] (units/ml)
I (fibrinogen)	*c*	*c*
II (prothrombin)	0.01	0.01
V (accelerator globulin)	1.30	0.00
VIII (antihemophilic globulin)	0.00	0.0080
X (Stuart factor)	0.00	0.0018
XI (plasma thromboplastic antecedent)	0.00	0.00
Proteolytic activity		0.00
Fibrinolytic activity		0.00
Plasminogen		0.00
Thrombin	<0.00001	.10

[a] Purified bovine factor V, 26.2 units/mg, used for assays. [b] Purified bovine thrombin, 418 NIH units/mg, used for assays. [c] No fibrin observed in preparations after 4 hr after adding 20 NIH units of thrombin at 37°.

Results

Purification and Characterization of Factor V. The results of a typical purification procedure are summarized in Table I. The over-all yield was 34.5% of the original factor V activity in the bovine plasma, specific activity 48.5 units/mg, and the relative purification was 2760-fold, similar to that reported by Esnouf and Jobin (1967). Both the original bovine plasma and the $(NH_4)_2SO_4$ precipitate are stable when frozen and the latter can be lyophilized after desalting or extensively dialyzed at 4° without loss of activity. The TEAE-cellulose eluate is unstable and loses up to half of its activity on freezing. The cellulose phosphate eluate loses activity on freezing but may be kept indefinitely at −50° in 50% glycerol without loss of activity.

The cellulose phosphate eluate adjusted to a concentration equivalent to that of the starting bovine plasma, 1.30 units/ml, was tested for contamination with other plasma coagulation proteins, as shown in Table II. No significant amount of clotting factors I, II, VIII, X, XI, or VII were noted. This is extremely important since thrombin is known to act on fibrinogen and factor VIII. No thrombin was demonstrable in the purified factor V preparation.

Characterization of Purified Thrombin. The purified thrombin was also tested for functional purity (Table II). No contamination with factors I, II, V, VIII, X, or XI was noted. In addition, the preparation contained no proteolytic activity as measured with casein, no fibrinolytic activity, and contained no plasminogen. The absence of fibrinolytic activity is crucial since plasmin is known to inactivate factor V. Considerable care was taken to exclude any contamination with active factor X, known to interact with factor V (Esnouf and Jobin, 1967). The commercial thrombin used as a source from which to purify the thrombin contained 0.47 unit of total factor X for each NIH unit of thrombin. Of the total factor X, 80% was in the activated form. In contrast, the purified thrombin contained only 0.0018 unit of total factor X/NIH unit and less than 0.0010 unit of activated factor X. In addition, the ratio of proteolytic activity (clotting of fibrinogen) to esterolytic (tosyl-L-arginine methyl ester) was eight times as high in the purified thrombin as in the commercial starting material indicating that substantial esterase activity other than thrombin had been removed during purification. The specific activity of the purified thrombin was 418 NIH units/mg of protein (absorbancy 280 mμ) and while not physically homogeneous, appeared functionally satisfactory for kinetic studies.

Characteristics of the Decay of Factor V Activity

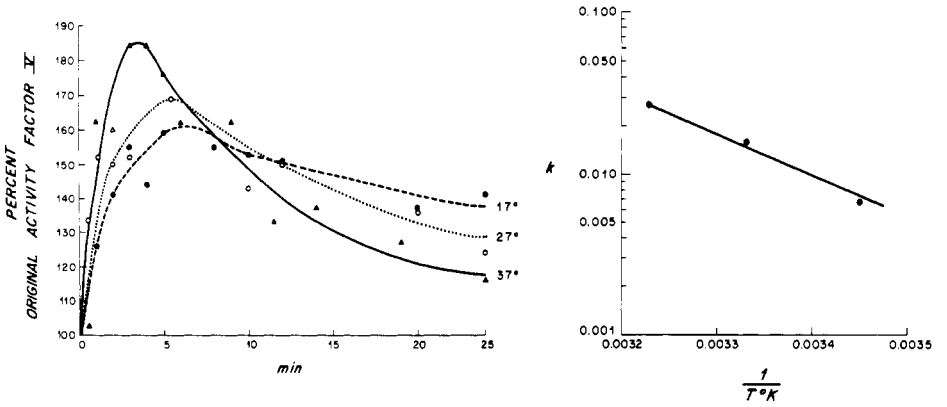

FIGURE 1: Activation of factor V by thrombin as a function of temperature. Purified bovine factor V (7.80–8.40 units/ml) was incubated with purified bovine thrombin at a final concentration of 1.0 NIH unit. On the left, activity is expressed in terms of original activity prior to adding thrombin. The temperatures of the reaction mixture are indicated. The Arrhenius plot for rate constant of inactivation, k, in min^{-1} is shown on the right. The half-life in minutes ($t_{1/2}$) at each temperature was calculated by plotting the log of factor V activity *vs.* time for the descending limb of the activation curve. The pseudo-first-order rate constant was calculated from the equation $k\ (min^{-1}) = 0.693/t_{1/2}$. The activation energy was calculated from the slope of the curve and equals 12,000 cal/mole.

following Thrombin Activation. The interaction of thrombin with factor V was studied by systematically varying the conditions of the reaction. When 1 NIH unit of thrombin was added to 8.0 unit/ml of factor V (Figure 1) there appeared to be a rapid increase in activity followed by a significant decay of activity. When the reaction was conducted at different temperatures, it was evident that at higher temperatures, both the rate and extent of the activity increase were greater and the rate of decay more rapid (Figure 1, left). The decrease in activity following thrombin activation was first order with half-lives of 26, 44, and 103 min at 37, 27, and 17°, respectively, with respect to factor V. An Arrhenius plot of the first-order rate constant *vs.* the reciprocal of the absolute temperature allowed determination of the activation energy of the reaction as 12,000 cal/mole (Figure 1, right). The question of whether the presence of thrombin was responsible for the activity loss was next explored utilizing the thrombin inhibitor hirudin. In preliminary experiments, it was shown that 1 unit of hirudin neutralizes 1 NIH unit of thrombin not only in its action against fibrinogen but in its ability to increase factor V activity. When hirudin was added at the peak of factor V activity (Figure 2) in a concentration known to totally inhibit thrombin, no change in the rate of decay occurred as indicated by the colinear plot. Therefore, it appears that thrombin *per se* was not responsible for the loss of activity of factor V following activation. In addition, no change in the rate of decay with varying thrombin concentrations was noted. To further elucidate the explanation for the loss of activity following thrombin action, the rate of that decay was compared with the inactivation rate constant in the absence of thrombin (Figure 3). The loss of activity following thrombin activation was 2.5 times as rapid as in the absence of thrombin, suggesting that the thrombin-altered factor V was less stable than the native factor V.

Kinetics of the Thrombin-Catalyzed Activation of Factor V. Studies of the kinetics of activation were performed at 17°. Since the decay reaction was much slower (half-life = 103 min) than the activity increase (maximum in 5 min), the loss of activity over the time span of the kinetic measurements could be neglected. When a concentration of factor V similar to that in the original plasma (0.90 unit/ml) was employed in activation experiments with thrombin, an increase of activity

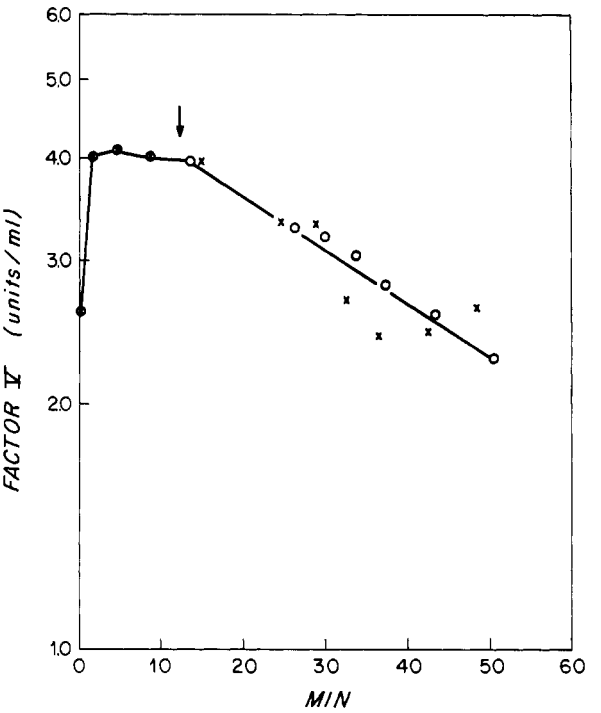

FIGURE 2: Loss of factor V activity after thrombin activation in the presence of hirudin. Purified bovine factor V (2.60 units/ml) was incubated with purified bovine thrombin at a final concentration of 0.83 NIH unit at 27°. At 12 min as indicated by the arrow, one-half of the incubation mixture was removed and hirudin was added in a final concentration of 1 unit/ml while an equal volume of buffer was added to the remainder. Samples were assayed for factor V activity alternately. (X) No hirudin added and (○) hirudin added. The half-life was 46 min in the presence and absence of hirudin.

of about 2.5-fold was demonstrated (Figure 4) which occurred progressively with a maximum recorded at 5 min. When the logarithm of the maximal activity (A_{max}) divided by unactivated factor V ($A_{max} - A_t$) was plotted, the results were linear with respect to time. Thus, the activation of factor V follows pseudo-first-order kinetics. To ascertain whether thrombin concentration was rate determining, a study of the effects of concentration of thrombin on the activation of factor V was performed. Using a constant concentration of factor V, the rate of activation decreases with decreasing thrombin concentration (Figure 5). In addition, the extent of activation of factor V decreases with lower thrombin concentrations.

In attempting to calculate K_m and V_{max}, the effect of varying factor V (substrate) concentration was next studied with a constant concentration of thrombin (1 NIH unit) (Figure 6). Contrary to expectation, the higher the factor V concentration, the lower the rate of activation by thrombin consistent with substrate or product inhibition. At low concentrations (0.37 unit/ml) significantly greater activation is noted, and at high concentrations (2.7–5.8 units/ml) substantially decreased activation is noted compared with the concentration existing in plasma (0.55–1.53 units/ml). This phenomenon is currently under investigation.

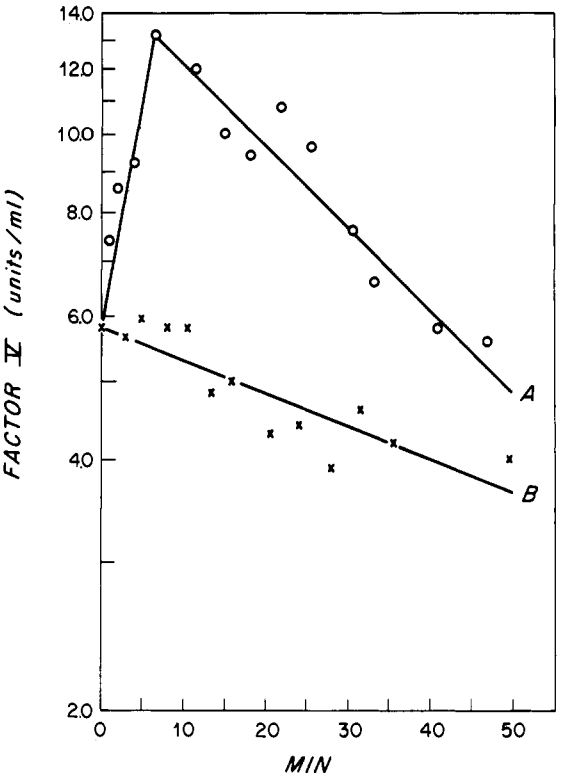

FIGURE 3: Loss of factor V activity in the absence of thrombin and following thrombin activation. Purified bovine factor V was used with an initial concentration of 5.80 units/ml and was incubated with purified bovine thrombin in a final concentration of thrombin of 0.75 NIH unit at 37° in expt A. An equal amount of buffer instead of thrombin was added to the other aliquot of the incubation mixture (B). Without thrombin, the inactivation constant, $k = 0.0092$ min^{-1}; with thrombin addition $k = 0.0230$ min^{-1}.

Discussion

The purification of factor V described in this paper leads to a preparation 3000–6000-fold purified over the original bovine plasma. Although Esnouf and Jobin (1967) found homogeneity by ultracentrifugation and immunoelectrophoresis and Barton and Hanahan (1967) found a single band of cellulose acetate electrophoresis, bovine factor V is not yet homogeneous by disc electrophoresis (R. W. Colman, 1967, unpublished results). However, it is devoid of proteins such as fibrinogen or factor VIII which are known substrates of thrombin and might interfere with the reaction between thrombin and factor V. Thrombin used in these experiments, while not physically pure, is devoid of activated factor X and plasmin, enzymes known to interact with factor V. The use of commercial thrombin in many previous studies has led to ambiguous results due to the presence of a large amount of activated factor X.

At all temperatures studied, the reaction of thrombin with factor V involves two phases, a rapid activity increase followed by a slower decay. The increase in activity observed is very likely due to the proteolytic action of thrombin. The fact that the thrombin inhibitor hirudin (Markwardt, 1963) which blocks the action of thrombin on fibrinogen also inhibits totally its action on factor V is consistent with this hypothesis. Supporting evidence comes from the work of Papahadjopoulous *et al.* (1964) who demonstrated a change in molecular size on Sephadex G-200 of factor V after interaction with thrombin. The reasons for loss of activity following thrombin are still unknown, however, but the rate constant for inactivation following thrombin activation is 3–10% of the rate constant of all the other proteolytic enzymes which destroy factor V (Colman, 1969), implying a different process. The loss of activity of factor V following thrombin action which is 2.5-fold increased over the rate of decay of native factor V probably is not due to further thrombin action. This conclusion is suggested by the observations that the rate of destruction is not proportional to thrombin concentration nor is it altered by blocking the action of thrombin with the specific inhibitor hirudin at the height of the activation. The observation that the activation energies of thermal denaturation range from 40,000 to 100,000 cal per mole (Sizer, 1943) suggests that the activity decrease is not due to extensive thermal inactivation. It appears that thrombin catalyzes the formation of an altered form of factor V with higher activity but a decreased inherent stability compared with native factor V. The low value for the energy of activation suggests that the loss of activity is associated with rather limited changes in conformation. Previous investigators (Bersagel and Nockolds, 1965; Barton and Hanahan, 1967) have commented on the instability of factor V following thrombin activation, but there have been no quantitative determinations or studies of possible mechanisms of inactivation.

The kinetics of factor V activation by thrombin have not been systematically studied previously. The only

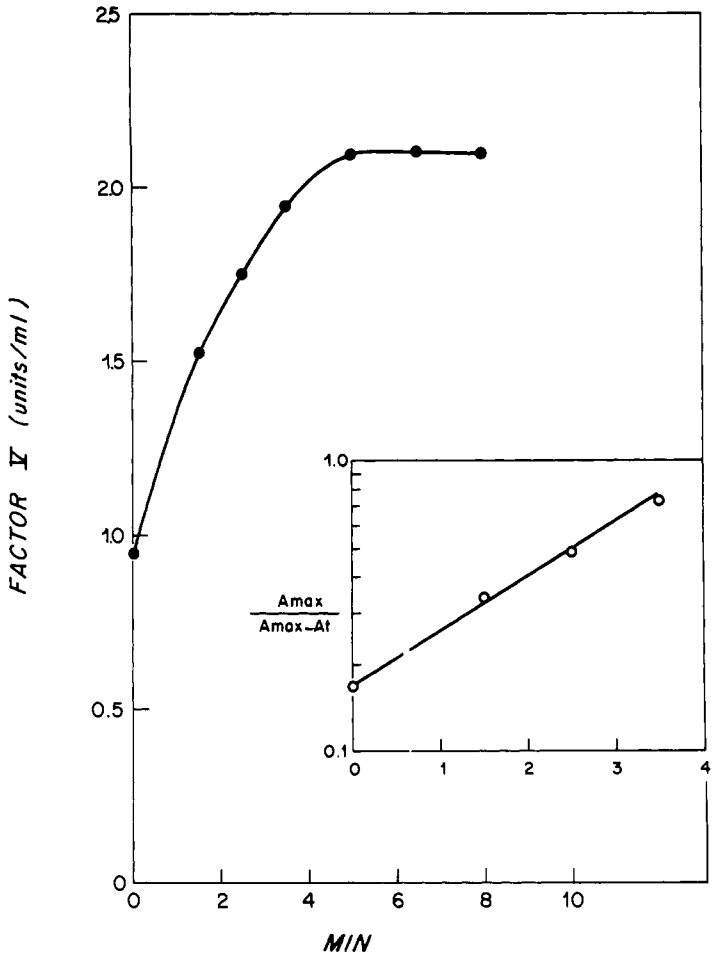

FIGURE 4: Kinetics of factor V activation by thrombin. Purified bovine V (0.90 unit/ml) was incubated with purified bovine thrombin at a final concentration of 0.83 NIH unit at 17°. Kinetic analysis of the data is shown in the insert where the abscissa is the time of activation. On the ordinate, A_{max} = maximum factor V at 5-min incubation and A_t = factor V activity at the various times of observation.

activation by thrombin achieved with a factor V preparation of comparable purity with that in the present study was by Barton and Hanahan (1967) who state: "The apparent increase in the present experiments was immediate and no activation time curve could be attained." By studying thrombin action at lower temperatures, factor V activity increase could be followed as a function of time and the reaction was observed to follow pseudo-first-order kinetics. Studies with various thrombin concentrations indicate that the apparent first-order rate is dependent upon thrombin concentration. Not only the rate, but the extent of the reaction, depends upon the thrombin concentration. The effect on the maximum degree of activation is probably due to the competing decay reaction which becomes more significant with slower rates of activation observed with the lower concentrations of thrombin. Hjort (1957), utilizing crude bovine factor V, found a substantial increase with 1 NIH unit of thrombin compared with 0.1 NIH unit in agreement with the present observations.

Since thrombin is produced by the conversion of prothrombin by a complex of factors V, X, phospholipid, and calcium, the finding of better than twofold increase by thrombin of the efficiency of factor V in prothrombin conversion constitutes a positive feedback or autocatalytic reaction which may accelerate the

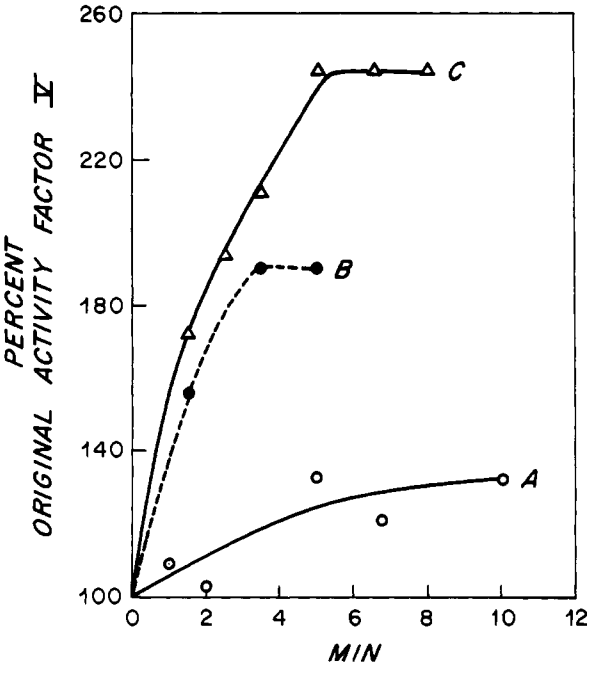

FIGURE 5: The effect of thrombin concentration on thrombin activation of factor V. Purified bovine factor V (0.70 unit/ml) was employed in all experiments at 17°. The final concentration of thrombin in NIH units per milliliter in the incubation mixtures was (A) 0.10, (B) 0.33, and (C) 0.83.

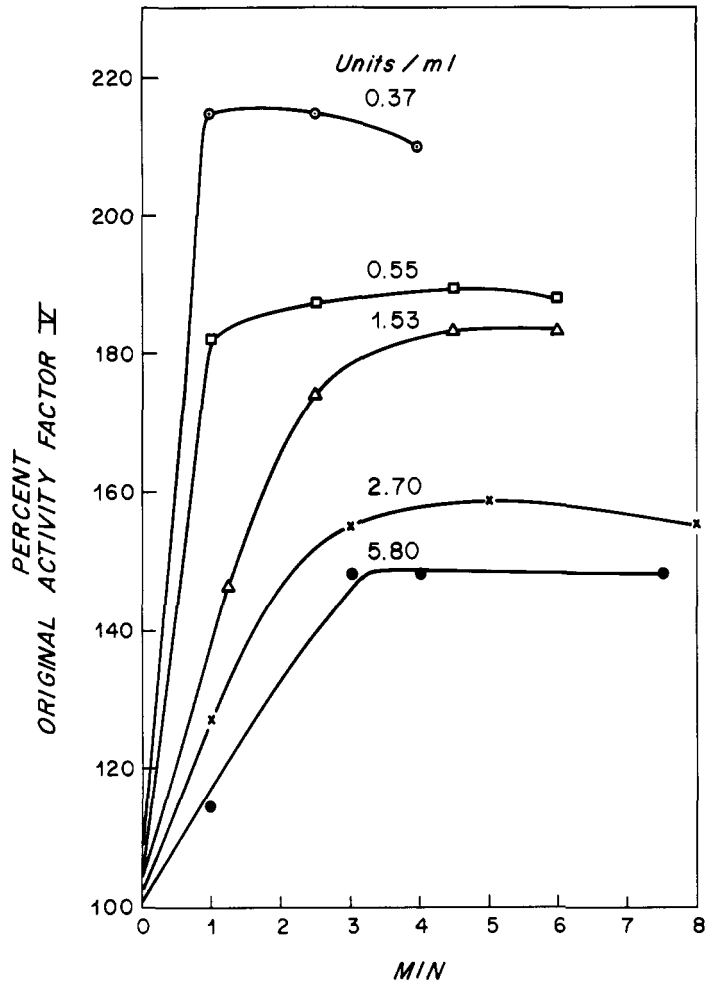

FIGURE 6: The effect of factor V concentration on thrombin activation. Purified bovine factor V was used in the concentrations indicated. The final concentration of thrombin in all experiments was 1 NIH unit/ml and the incubations were performed at 17°.

process considerably. However, this process may be limited by two considerations. Thrombin-altered factor V is much more unstable than native factor V, and this may lead to rapid depletion of the available factor V in the organism. In certain pathologic conditions such as disseminated intravascular coagulation, thrombin may be responsible for depletion of factor V by formation of an unstable form.

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References

Alkjaersig, N., Fletcher, P., and Sherry, S. (1959), *J. Biol. Chem. 234*, 832.

Astrup, T., and Mullertz, S. (1952), *Arch. Biochem. Biophys. 40*, 346.

Bachmann, F., Duckert, F., and Koller, F. (1958), *Thromb. Diath. Haemorrhag. 2*, 24.

Barton, P. G., and Hanahan, D. J. (1967), *Biochem. Biophys. Acta 133*, 506.

Bersagel, D. E., and Nockolds, E. R. (1965), *Brit. J. Haematol. 11*, 395.

Blatt, W. F., Feinberg, M. P., Hopfenberg, H. B., and Saravis, C. A. (1965), *Science 150*, 224.

Breckenridge, R. T., and Ratnoff, O. D. (1965), *J. Clin. Invest. 44*, 302.

Colman, R. W. (1969), *Biochemistry 8*, 1445 (this issue; following paper).

Esnouf, M. P., and Jobin, F. (1967), *Biochem. J. 102*, 660.

Hardisty, R. M. (1955), *Brit. J. Haematol. 1*, 323.

Hjort, P. (1957), *Scan. J. Clin. Lab. Invest. 27*, 7.

Kerwin, D. M., and Milstone, J. H. (1967), *Thromb. Diath. Haemorrhag. 17*, 247.

Lewis, M. L., and Ware, A. G. (1953), *Proc. Soc. Exptl. Biol. Med. 84*, 640.

Markwardt, F. (1963), Bluterinnungshemmende Wirkstoffee aus blutsaugenden Tieren, Hena, Fischer.

Owren, P. A., and Aas, K. (1951), *Scan. J. Clin. Lab. Invest. 3*, 201.

Papahadjopoulous, D., Hougie, E., and Hanahan, D. J. (1964), *Biochemistry 3*, 264.

Proctor, R. R., and Rappaport, S. I. (1961), *Am. J. Clin. Pathol. 36*, 212.

Rappaport, S. I., Schiffman, S., Patch, M. J., and Ames, S. B. (1963), *Blood 21*, 221.

Ratnoff, O. D., and Menzies, C. J. (1951), *J. Lab. Clin. Med. 37*, 316.

Siegelman, A. M., Carlson, A. S., and Robertson, T. (1962), *Arch. Biochem. Biophys. 97*, 159.
Sizer, I. W. (1943), *Advan. Enzymol. 3*, 35.
Surgenor, D. M., Wilson, N. A., and Emergy, A. S. (1960), *Thromb. Diath. Haemorrhag. 5*, 1.
Therriault, D. G., Gray, J. L., and Jensen, H. (1957), *Proc. Soc. Exptl. Biol. Med. 95*, 207.
Waddell, W. J. (1956), *J. Lab. Clin. Med. 48*, 311.
Ware, A. G., Murphy, R. C., and Seegers, W. H. (1947), *Science 106*, 618.
Yin, E. T., and Wessler, S. (1968), *J. Biol. Chem. 243*, 112.

The Effect of Proteolytic Enzymes on Bovine Factor V. II. Kinetics of Activation and Inactivation by Papain, Plasmin, and Other Proteolytic Enzymes*

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ABSTRACT: The effect of various proteolytic enzymes on the kinetics of activation and inactivation of purified bovine factor V was studied and compared to the effects of thrombin on factor V. Like thrombin, the addition of Russell viper venom to factor V results in a rapid increase in specific activity followed by a slow loss of factor V activity. Human plasma kallikrein, *Bothrops jaracara*, and *Agkistrodon rhodostoma* venom have no effect on factor V. Plasmin and trypsin destroy this plasma protein, a process inhibited by soybean trypsin inhibitor. Papain is unique in activating factor V at low concentrations, a reaction inhibited by iodoacetate, and destroying it at higher concentrations. Each of these proteolytic enzymes was examined to ascertain its effect on the course of thrombin activation and inactivation. Russell viper venom and trypsin do not interfere with thrombin action of factor V, but papain directly inhibits thrombin activation of factor V by successful competition for thrombin-susceptible bonds. Plasmin decreases the extent of activity increase catalyzed by thrombin by increasing the rate of inactivation of thrombin-altered factor V.

Purified bovine factor V[1] freed of contaminating coagulation factors is activated more than twofold by bovine thrombin lacking other proteolytic enzymes (Colman, 1969). Although factor V loses activity rapidly following its activation, this was shown not to be due to further proteolytic action by thrombin. To ascertain the specificity of the effects of thrombin on factor V, a variety of proteolytic enzymes with similar substrate specificity to that of thrombin were studied. Experiments were conducted to ascertain whether these enzymes were capable of activating or destroying factor V and whether they interfered with the thrombin-catalyzed activation.

Materials and Methods

Enzymes. The purification, assay,[2] and properties of bovine thrombin were described previously (Colman, 1969). Papain, obtained from Worthington Biochemical Corp. (specific activity of 2.5 units/mg of protein), was activated immediately prior to use in a solution containing 0.005 M cysteine and 0.001 M EDTA and assayed by measuring the hydrolysis of benzoyl-L-arginine ethyl ester in a Radiometer automatic titrator equipped with an automatic buret and titrigraph. Plasmin prepared from human plasma by the method of Kline and Fishman (1961) was activated by streptokinase according to the method of Troll and Sherry (1955). The assay, hydrolysis of acetyl-L-arginine methyl ester, and conversion of the activity into casein units were performed as described by Sherry *et al.* (1965). Plasmin was also assayed by the proteolytic digestion of casein by the method of Alkjaersig *et al.* (1959) without the addition of streptokinase. Human plasma kallikrein was prepared by the method of Colman *et al.* (1969) and assayed by quantifying the micromoles of methanol liberated by the hydrolysis of tosyl-L-arginine methyl ester by a colorimetric method (Siegelman *et al.*, 1962). Russell viper venom obtained from Burroughs Wellcome was assayed by its ability to activate factor X (Bachmann *et al.*, 1958). *Agkistrodon rhodostoma* venom purchased from Miami Serpentarium and *Bothrops jaracara* venom (Reptilase) obtained from Sigma Chemical Co. were both assayed by their ability to clot fibrinogen. Bovine trypsin once crystallized supplied by Worthington was assayed by hydrolysis of tosyl-L-arginine methyl ester in a Radiometer automatic titrator equipped with an automatic buret and titrigraph.

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[1] The designations of clotting activities conform to the recommendations of the International Committee for the Standardization of Blood Clotting Factors (*J. Amer. Med. Assoc. 170*, 325 (1959)).

[2] All clotting activities are defined as containing 1.0 unit/ml on the basis of calibration curves separately determined on 20 normal plasmas.