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*

Robert W. Colman

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.

* From the Department of Medicine, Harvard Medical School, and the Hematology Research Laboratory of the Medical Service of the Massachusetts General Hospital, Boston, Massachusetts 02114. *Received October 28, 1968.* This investigation was supported in part by the National Institutes of Health, Grant No. HE-11519, and by a grant from the Massachusetts Chapter of the American Cancer Society.

[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.

Chemicals. Soybean trypsin inhibitor three-times crystallized was purchased from Worthington Biochemical Corp. Iodoacetate was supplied by Merck and Co. Tosyl-L-arginine methyl ester, acetyl-L-arginine methyl ester, and benzoyl-L-arginine ethyl ester were obtained from Cyclo Corp.

Factor V: Preparation, Assay, and Activation. The method of purification, characterization, and assay of bovine factor V as well as its activation by thrombin are described by Colman (1969). The same protocol was used for all proteolytic enzymes as for thrombin and at the concentrations tested no effect on factor V assay was noted.

Results

Effect of Various Enzymes on Activation and Inactivation of Factor V. A summary of the effects of the various enzymes on the activation and destruction of factor V is presented in Table I. Besides thrombin, two other enzymes, papain (0.95 unit/ml) and Russell viper venom, were found to activate factor V as manifested by a ratio of maximum to initial activity of greater than 1.00. Following activation by thrombin, Russell Viper venom, and papain, a first-order decay process ensued with rate constants varying from 0.67 to 3.4 $\times$ 10^{-2} min^{-1} indicating a relatively slow inactivation. Following activation by Russell viper venom, the rate of decay did not increase with increasing enzyme concentration. Factor V activated by Russell viper venom is no longer susceptible to thrombin but the presence of the venom does not interfere with thrombin activation. Papain at high concentrations (4.9 units/ml) destroys factor V without previous activation. In contrast, plasmin and trypsin, even at low concentrations, only destroy factor V activity and their first-order rate constants of the decay process are much higher (14.2–20.4 $\times$ 10^{-2} min^{-1}). The presence of trypsin did not interfere with thrombin activation of factor V. Several enzymes, human plasma kallikrein, *Bothrops jaracara* venom, and *Agkistrodon rhodostoma* venom, fail to affect factor V in any concentration. No activation was ever observed and the decay process was the same as that of factor V incubated in buffer.

Interaction of Papain-Treated Factor V with Thrombin Activation of Factor V. Papain had two actions on factor V. In high concentrations (Figure 1, curve A), it behaved like plasmin of trypsin rapidly destroying factor V. At lower concentrations (Figure 1, curve B), activation similar in rate and extent to that produced by thrombin occurred and could be prevented by iodoacetate. The papain digest of factor V apparently inhibited thrombin activation (Figure 1, curve C) but the decline of activity suggested that this inhibition might be due to residual papain activity. In order to ascertain if the products of papain activation of factor V itself were capable of blocking thrombin proteolytic activity on native factor V, the residual papain present in the digest was completely inhibited by iodoacetate. When the iodoacetate-treated digest was incubated with unactivated factor V and the mixture treated with thrombin, there was no interference with the activation process (Figure 1, curve D). Thus, papain-activated factor V was not an inhibitor.

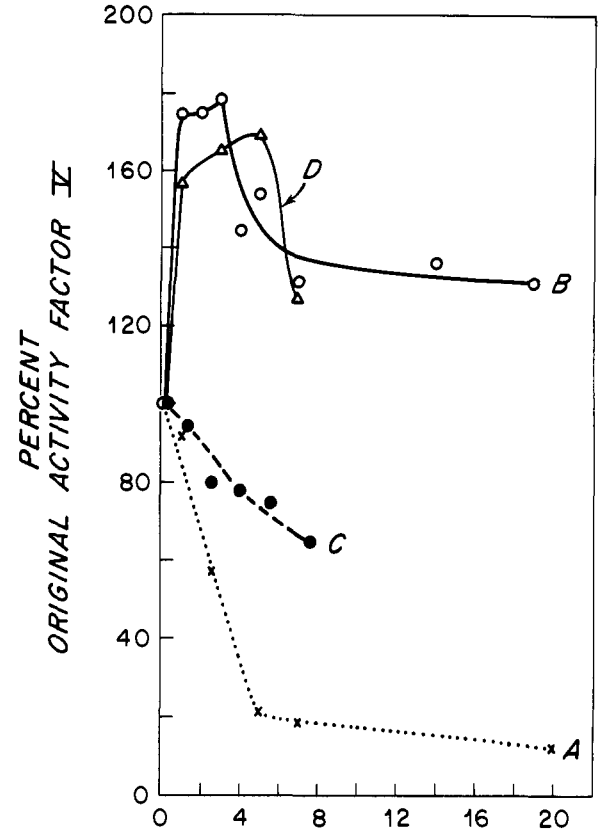

FIGURE 1: The effect of papain on factor V in the presence and absence of thrombin. Purified factor V was used in all experiments at a concentration of 0.70 unit/ml at 17° with thrombin (1 NIH unit/ml) used as indicated. Curve A represents an experiment with the final concentration of papain at 4.9 unit/ml; k of inactivation = 0.144 min^{-1}. In expt B the final concentration of papain was 0.98 unit/ml; k of inactivation = 0.041 min^{-1}. Curve C describes an experiment in which 0.70 unit of factor V was digested with 0.98 unit of papain for 37.5 min at 37°. At that time, an equal volume of native factor V (0.70 unit/ml) was added. The final concentration of papain in the mixture activated by thrombin was 0.49 unit/ml. K of inactivation = 0.058 min^{-1}. D indicates an experiment identical with C except that the papain digest of factor V was treated after 1 min with 2.3 $\times$ 10^{-3} M iodoacetate to inhibit the residual papain

The possibility that the presence of enzymatically active papain was responsible for interference with factor V activation by thrombin was next investigated. The control activation by thrombin is described by curve A of Figure 2. A suboptimal concentration of papain was used for activation (Figure 2B) in order to identify the effects of thrombin and papain. At these concentrations of thrombin and papain, no visible change in the specific activity of factor V was noted in the first 15 sec; yet, exposure to the same concentration of papain for 15 sec is sufficient to markedly inhibit subsequent thrombin activation (Figure 2E). If thrombin is first allowed to interact with factor V and then papain is added (Figure 2C), the activation resembles that with papain alone. The inhibition is not due to continued digestion by papain since adding iodoacetate to papain 15 sec after papain exposure failed to alter the papain-

TABLE I: Effect of Various Enzymes on Activation and Destruction of Bovine Factor V.[a]

Enzyme	Concn (per ml of Incubn Mixture)	Activation Ratio Max Act. to Initial Act.	Rate Constant of Inactivation, K (min^{-1})
Bovine thrombin	1 NIH unit	2.32	0.00,67
Papain[b]	0.95 unit	1.75	0.041
Russell viper venom	5 μg	1.80	0.034
Human plasma kallikrein	0.11 μM tosyl-L-arginine methyl ester Hydrolized/min	1.00	*e*
B. jaracara venom[c]	100 μg	1.00	*e*
A. rhodostoma venom[c]	3 μg	1.00	*e*
Papain[b]	0.25 unit	1.00	*e*
Human plasmin[d]	0.22 casein unit	1.00	0.177
Bovine trypsin[d]	0.1 μg	1.00	0.024
Papain[b]	4.5 units	1.00	0.144

[a] All experiments performed using purified bovine factor V, 26.5 units/mg, at 17° with assay condition as in Methods. The concentration of factor V in all experiments was 0.50–0.70 unit/ml. The decay constant was calculated from the half-life ($k = 0.693/t_{1/2}$) with the time of maximum activity taken as the original activity for this calculation. [b] Different results are noted for these three concentrations of papain. [c] Larger amounts of these enzymes never produced any significant activation. [d] Activation was not noted even at concentrations where no destruction occurred. [e] The $t_{1/2}$ was in excess of 5 hr which is similar to factor V incubated with buffer instead of enzyme.

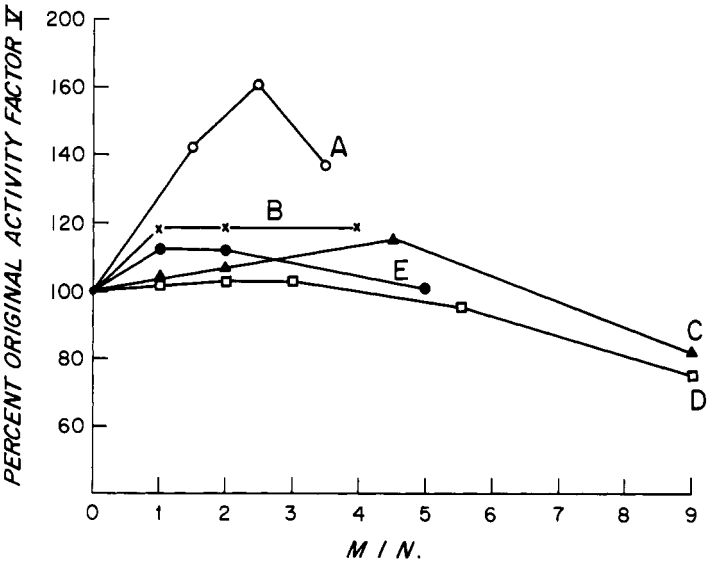

FIGURE 2: The effect of short exposure to papain on the activities of factor V by thrombin. Purified bovine factor V (0.90 unit/ml) was utilized at 17° in the experiments below. In curve A thrombin (1 NIH unit) was added to the incubation mixture, while in curve B papain (0.49 unit/ml) was added. In curve C factor V was incubated with the above concentration of thrombin for 15 sec prior to the addition of papain, while in curve D papain was added 15 sec prior to thrombin. In curve E papain was added, followed 15 sec later by iodoacetate (2×10^{-3} M).

induced inhibition of thrombin activation (Figure 2D). Thus, exposure to papain completely blocked activation by thrombin, but thrombin failed to alter papain activation.

A preliminary study of the decay of papain-activated factor V indicated that once activation of factor V was maximal, addition of iodoacetate failed to alter the decay process similar to the lack of effect of hirudin of the decay of thrombin-activated factor V (Colman, 1969). To ascertain the effect of thrombin on the decay of papain-treated factor V, iodoacetate was added to inhibit residual papain. The activation mixture was then subdivided and thrombin was added to one aliquot with addition of buffer to the other aliquot serving as a control. The results (Figure 3, left) indicate that thrombin fails to alter the decay process. The converse experiment yielded different results. Papain at a low concentration producing a small increase of factor V activity (Figure 3, right, C) caused an immediate and rapid decay of thrombin-activated factor V (Figure 3, right, B) when compared with the control thrombin activation (Figure 3, right, A) without added papain. The decay of thrombin-activated factor V further exposed to papain was comparable to that of papain-activated factor V itself (Figure 3B) with a rapid immediate decay followed by a slower fall. These results indicate that similar to the activation process, the decay of papain-activated factor V is unaffected by thrombin, but thrombin-activated factor V is made more unstable by exposure to papain.

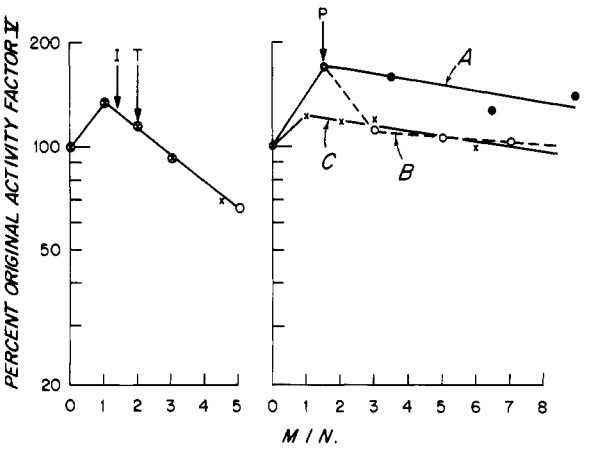

FIGURE 3: The effect of thrombin on the decay of factor V activity following papain activation and the effect of papain on the thrombin-activated factor V. Left: purified bovine factor V (final concentration 0.60 unit/ml) was incubated with papain (final concentration 0.95 unit/ml). At the time indicated by I, the digest was treated with iodoacetate (final concentration 2.3×10^{-3} M) to inhibit residual papain. At the time indicated by T, thrombin (1 NIH unit/ml) was added to an aliquot (x), and buffer was added to another aliquot (○). Right: purified bovine factor V (0.45 unit/ml) was incubated with thrombin (1 NIH unit/ml). At the time indicated by P, papain (0.49 unit/ml) was added to one aliquot, curve B, and buffer to another, curve A. In the experiment indicated by curve C, factor V (0.45 unit/ml) was added to papain (0.49 unit/ml).

Effect of Plasmin Digest of Factor V on Thrombin Activation of Factor V. Plasmin progressively inactivates factor V at a rate proportional to the plasmin concentration and the destruction can be instantaneously blocked by the addition of soybean trypsin inhibitor. At concentrations of 0.55 casein unit/ml, plasmin appeared to interfere with thrombin activation of factor V. This inhibition might, as in the case of papain, be due to a direct effect on the thrombin-catalyzed reaction or to an acceleration of the inactivation of thrombin-activated factor V. To test the former possibility, plasmin was allowed to react with factor V at a concentration producing slow destruction (Figure 4A), but negligible effect in 15 sec. After 15 sec, thrombin was added and the activation (Figure 4C) was compared with a control activation with thrombin alone (Figure 4B). No inhibition occurred if thrombin was allowed to react (4D) first for 15 sec and then plasmin was added. Thus, unlike papain, plasmin does not directly interfere with the activation. However in both of the activation mixtures containing plasmin decay of factor V activity following thrombin appeared more rapid. To test the possibility that plasmin accelerates the decay of thrombin-activated factor V, the pseudo-first-order rate constants were determined for inactivation of thrombin-(1 NIH unit) activated factor V (0.90 unit/ml) in the presence and in the absence of plasmin (0.22 casein unit/ml) at 17°. The rate constants of inactivation were as follows: thrombin-activated factor V = $0.037\ min^{-1}$, plasmin-digested factor V = $0.063\ min^{-1}$, and plasmin digest of thrombin-activated factor V = $0.106\ min^{-1}$. These results indicate that plasmin does accelerate decay of thrombin-activated factor V accounting for the interference with activation. The additive character of the pseudo-first-order rate constants suggests separate inactivation processes.

Discussion

To ascertain the specificity of the effects of thrombin on factor V, a variety of enzymes known to have similar substrate specificity to thrombin were tested as to their ability to activate or destroy factor V. The enzymes tested either exhibited preference for basic amino acid amide or ester bonds (trypsin, kallikrein, plasmin, and papain) or shared with thrombin the ability to clot fibrinogen (papain, *Bothrops jaracara* venom, and *Agkistrodon rhodostoma* venom). Russell viper venom was tested because of its known activation of factor X which in turn interacts with factor V. The enzymes tested fell into several categories: those which only activated factor V even in high concentrations, thrombin and Russell viper venom; those which have no effect on factor V in any concentration (kallikrein, *Bothrops jaracara* venom, and *Agkistrodon rhodostoma* venom), and those which only destroy factor V even in low concentrations (plasmin and trypsin). Papain is unique in that at low concentrations it activates factor V and at high concentrations destroys it.

Several enzymes were tested which proved to have no effect on factor V. The venom from *Agkistrodon rhodostoma* which clots fibrinogen does not activate or destroy factor V in agreement with the results of Sharp *et al.* (1968) who found no decrease in factor V following defibrination with a preparation purified from this venom. *Bothrops jaracara* venom (reptilase), which like thrombin clots fibrinogen but only releases one of the two fibrinopeptides liberated by thrombin, also failed to effect factor V activation perhaps because it has even greater specificity than thrombin. This is in contrast to results reported by Bersagel and Nockolds (1965) who found activation with this venom. Since a crude factor V preparation was used in that study, this effect may have been produced indirectly. Human plasma kallikrein (Colman *et al.*, 1969) is an arginine esterase and a proteolytic enzyme releasing the nonapeptide bradykinin from its plasma precursor. At three times the concentration as the latter reaction, it was totally inert toward factor V.

Russell viper venom was studied because it is a known activator of factor X, a protein which interacts with factor V. In concentrations as low as 2 μg/ml, activation of factor V was achieved. In contrast to papain, no interference with thrombin activation was produced by Russell viper venom. Russell viper venom, unlike thrombin or papain, has no direct effect on fibrinogen. Its mode of action on factor V and the final products of the reaction may be quite different from thrombin. The direct effect of Russell viper venom on factor V has important implications in interpreting experiments involving the formation of the agent that converts prothrombin to thrombin. Breckenridge and Ratnoff (1965) performed experiments purporting to show that

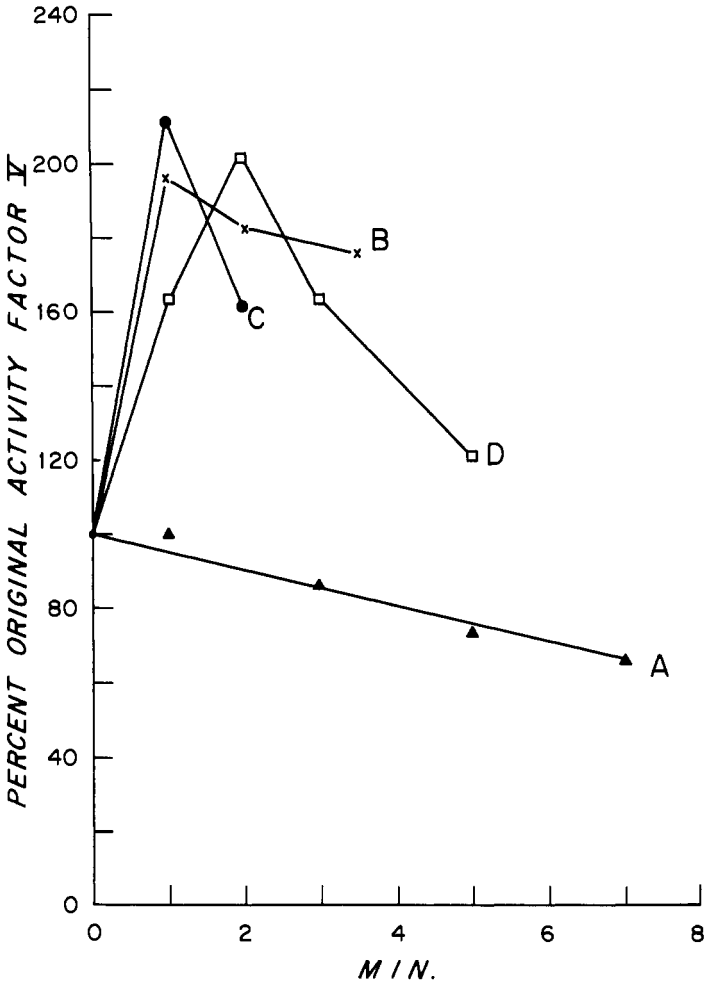

FIGURE 4: The effect of short exposure to plasmin on the activation of factor V by thrombin. Purified bovine factor V (0.60 unit/ml) was utilized at 17° in the experiments below. Curve A indicates an experiment in which plasmin (0.22 casein unit) was added to factor V whereas curve B thrombin (1 NIH unit) was added. In curve D thrombin was added for 15 sec followed by plasmin and in curve C plasmin was added for 15 sec followed by thrombin.

factor X activated by Russell viper venom in the presence of calcium and phospholipid converts factor V into an active form capable of converting prothrombin into thrombin. They were unable to document a role for thrombin. However, the result of the present investigation indicate the direct activation of factor V by Russell viper venom would have rendered it unsusceptible to thrombin activation, and accounted for an increase in factor V activity. Prentice *et al.* (1967) have observed that activated factor X does not catalyze an increase in factor V activity in contrast to their previous results (Breckenridge and Ratnoff, 1965). Schiffman *et al.* (1968) have succeeded in separating two fractions from crude Russell viper venom, one of which catalytically activates factor X and another which increased the activity of crude human factor V.

Three enzymes tested apparently degraded factor V without prior activation. Trypsin, in low concentrations, rapidly inactivated factor V at rates proportional to its concentration and without activation even in amounts too low to inactivate factor V. No activation was apparent and the trypsin digest did not inhibit thrombin action on factor V. Apparently, trypsin affects bonds which inactivate factor V prior to hydrolysis of the bonds responsible for its activation with thrombin.

No previous studies on the effect of papain hydrolysis of factor V have been attempted. Papain, which exhibits a preference for peptide bonds involving basic amino acids, showed a dual effect of factor V. At low concentrations (0.49–0.98 unit/ml) activation occurred leading to an unstable form of factor V similar to thrombin-activated factor V. At higher concentrations (2.5–4.9 unit/ml) only destruction was noted. The papain digest appeared to block thrombin activation of unactivated factor V, but that was due to the residual papain in the digest since the digest failed to inhibit thrombin activation once the residual papain has been inhibited by iodoacetate. However, even brief exposure to papain renders unactivated factor V inert to thrombin even if the papain is inhibited shortly after it reacts with factor V.

Superficially, it appears that the decay following papain activation is similar to that following thrombin activation (Colman, 1969). That further proteolysis is not necessary for loss of activity is clear from the observation that the inhibitor iodoacetate fails to alter the rate of papain-activated factor V. However, examination of the character of the decay indicates that there is an immediate sharp decrease of factor V activity following maximal activation followed by a slower component similar to loss of activity after thrombin. When papain is added to thrombin-activated factor V, no additional activation is observed, but the fast component of the decay observed following papain is

exactly mimicked. In contrast, thrombin does not affect the decay of activity noted after papain activation.

These series of observations of the interaction of papain and thrombin can be explained by postulating different classes of peptide bonds hydrolyzed by papain. At low concentrations, papain cleaves not only the bonds also susceptible to thrombin which results in an increase in specific activity but simultaneously other bonds which result in a more rapid decay of activity. The additional bonds cleaved by papain explain why papain interferes with thrombin-activated factor V while thrombin fails to alter activation of factor V by papain or its decay. At high concentrations, activation by papain is clearly masked by nonspecific rapid destruction presumably due to hydrolysis of additional bonds.

The behavior of these two enzymes activating factor V is similar to their ability to clot fibrinogen. Thrombin initiates a limited proteolysis which hydrolyzes four arginylglycine bonds which allows subsequent polymerization of the fibrin monomers to form the fibrin clot. Papain also is capable of clotting fibrinogen, presumably by hydrolysis of the same four bonds, but further proteolysis ensues with eventual lysis of the fibrin clot (Eagle and Harris, 1937).

Plasmin, which also degrades factor V without apparent activation, presents still another variation. Unlike trypsin, plasmin appears to prevent thrombin activation. It appeared possible that plasmin attacked those bonds responsible for inactivation prior to those responsible for activation and this prevents thrombin from acting on these bonds and activating factor V. Such a mechanism has been shown to be operative for the effect of plasmin on fibrinogen by Pechet and Alexander (1962) where as little as 15-sec exposure to plasmin prevented thrombin-catalyzed proteolysis of fibrinogen from occurring. In experiments with factor V, however, brief exposure to plasmin had no effect on the course of thrombin activation; thus it appeared that the plasmin-induced loss of activity may have merely masked simultaneous thrombin activation. That this mechanism is operative is strongly suggested by the additive effects of thrombin and plasmin on the rate of inactivation of factor V. Thus, it appears that plasmin, unlike papain, does not attack similar bonds to thrombin but degrades factor V rapidly by hydrolyzing other bonds.

The implications of these findings for regulation of the rate and extent of blood coagulation in the organism are manifold. Of the enzymes studied for effect on factor V, only kallikrein, thrombin, and plasmin are in the plasma. Plasmin destroys factor V without previous activation and appears to act independently of thrombin action on factor V. Kallikrein has no effect. Thus, only thrombin, among the major plasma proteases, is capable of activating factor V. Papain and Russell viper venom are interesting models but not physiologic agents.

Acknowledgments

The author gratefully acknowledges the excellent technical assistance rendered by Miss Julieta Moran. Some of the plasmin used in these experiments was a gift from Dr. Lawrence E. Sherman, Washington University School of Medicine, St. Louis, Mo.

References

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

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

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

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

Colman, R. W. (1969), *Biochemistry 8*, 1438 (this issue; preceding paper).

Colman, R. W., Sherry, S., and Mattler, L. (1969), *J. Clin. Invest. 48*, 23.

Eagle, H., and Harris, T. N. (1937), *J. Gen. Physiol. 20*, 543.

Kline, D. L., and Fishman, J. B. (1961), *J. Biol. Chem. 236*, 3232.

Pechet, L., and Alexander, B. (1962), *Biochemistry 1*, 875.

Prentice, C. R. M., Ratnoff, O. D., and Breckenridge, R. T. (1967), *Brit. J. Haematol. 13*, 898.

Schiffman, S., Rappaport, S. I., and Theodor, I. (1968), *XII Congr. Intern. Soc. Hematol. 0*, 179.

Sharp, A. A., Warren, B. A., Paxton, A. M., and Allington, M. J. (1968), *Lancet 1*, 493.

Sherry, S., Alkjaersig, N., and Fletcher, A. P. (1965), *Am. J. Physiol. 209*, 577.

Siegelman, A. M., Carlson, A. S., and Robertson, T. (1962), *Arch. Biochem. Biophys. 97*, 159.

Troll, W. E., and Sherry, S. (1955), *J. Biol. Chem. 213*, 881.