

BLOOD COAGULATION FACTOR V: ISOLATION OF BOVINE PLASMA
FACTOR V AND ITS ACTIVATION BY FACTOR Xa, THROMBIN,
AND A PROTEASE FROM RUSSELL'S VIPER VENOM

by

Catherine Margaret Smith

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SIGNED: Catherine Margaret Smith

To Gary and Gerrit

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ABSTRACT

Bovine plasma Factor V was isolated by a procedure which involved adsorption of oxalated bovine plasma with barium sulfate, QAE cellulose extraction of the adsorbed plasma, polyethylene glycol precipitation of the QAE cellulose extract and chromatography of the PEG precipitate on desulphated Sepharose 6B. Factor V was recovered from plasma in yields of 39%, with a specific activity of 60 U/mg, representing an overall purification of 2400-fold. The Factor V preparation appeared heterogeneous when examined by analytical or SDS acrylamide gel electrophoresis, analytical ultracentrifugation and rechromatography on desulphated Sepharose 6B. However, it was free of other clotting activities except for trace amounts of Factor VIII. This preparation contained a single form of Factor V with a molecular weight of 439,000 daltons, determined by Sephadex G-200 gel chromatography. Factor V could be activated by several enzymes, both intrinsic and extrinsic to the coagulation mechanism. Activation was manifest in an increase in the specific activity of Factor V and a concomitant reduction in its molecular weight from 400,000 to 200,000 daltons. The activity of Factor V was stable, and its activatability was reproducible for up to 3 weeks

when the preparation was stored at 4°C, pH 7.5, in the presence of 50 mM CaCl₂ and 10% glycerol.

Specifically, Factor V was activated by thrombin, Factor Xa, a protease from Russell's Viper Venom, and α -chymotrypsin. Thrombin had the greatest specific activity toward Factor V and Factor Xa had the least.

The mode of action of each of these reagents in converting Factor V to a more active form was enzymatic. This was evidenced by chemical and physical modifications of the activators, and analyses of the kinetics of the activation reaction in each case.

Activation of Factor V by thrombin was inhibited by treatment of thrombin with DFP, TLCK, or heat. The extent of the reaction was independent of the thrombin concentration and was maximal at approximately pH 8.0.

Activation of Factor V by Factor Xa was enhanced by both calcium and phospholipid. The rate of activation was independent of the concentration of Factor Xa but the extent of activation was proportional to it. The reaction was inhibited when Factor Xa was pre-treated with DFP or heat, but was not affected when Factor Xa was pre-treated with TLCK or TPCK. Following activation in the presence of calcium only, the Factor V(a) and Factor Xa activities were completely separable on Sephadex G-200 gel chromatography, in the presence of calcium.

The ability of RVV-V to activate Factor V could not be abolished by treatment with DFP or heat. However, from kinetic measurements the activation appeared to be enzymatic. RVV-V, labeled with ^{125}I , could activate Factor V and was separable from the Factor V(a) activity upon Sephadex G-200 chromatography of the activation mixture.

The interaction between α -chymotrypsin and Factor V was inhibited by TPCK.

Activated Factor V was a more reactive form than plasma Factor V in the prothrombinase reaction in which prothrombin is converted to thrombin by a complex of Factor Xa, Factor V, calcium, and phospholipid. Addition of Factor Va resulted in the immediate generation of thrombin, while the addition of plasma Factor V to the same mixture gave a lag period before thrombin activity was evident. The successive addition of calcium, phospholipid and Factor V or Va, to a mixture of Factor Xa and prothrombin resulted in a successively greater rate and extent of thrombin generation. Factor Xa, Factor V or Va and phospholipid, contributed to the production of thrombin in a stoichiometric fashion.

CHAPTER 1

INTRODUCTION

Basic Mechanisms in Blood Coagulation

The ability of blood to coagulate is one of its most important physiological properties in the maintenance of hemostasis in mammals. To the clinician, the failure of blood to coagulate rapidly enough is an acute problem in many disease states. To the research scientist, the phenomenological events occurring when blood clots both normally and with prolonged clotting times, provide an impetus for studying the origins of these mechanisms. The blood clotting scheme provides a most interesting framework for study of protein-protein, protein-metal ion, and protein-lipid interactions, on a molecular as well as on a physiological level.

A great deal of effort has been extended, in the last decade, toward elucidating the interactions involved in the coagulation of blood. Numerous theories have arisen to account for the gelation of blood, some of the earliest being postulated by Hammarsten (1899), and separately by Schmidt (1872), and by Morawitz (1905). These investigators assumed that three components in plasma, and one from the tissues were responsible for the transformation of blood

from a fluid to a solid state. According to this concept, thrombokinase, a lipoprotein liberated upon tissue injury from platelets and damaged cells, acted upon prothrombin in the presence of calcium to generate thrombin. Thrombin in turn, converted soluble fibrinogen to an insoluble fibrin clot.

Since the time of these early observations, many additional plasma proteins have been found which participate in the coagulation mechanism. These proteins were discovered mainly through a study of patients with various bleeding disorders, which were due to the absence of a particular coagulation factor. Current theories have attempted to order the different factors essential to the blood clotting process, and account for the nature of their interactions as well as the nature of the various stimuli initiating clotting. Consequently, two basic pathways of coagulation have been elucidated: first, the intrinsic system, composed of factors solely from plasma, and second, the extrinsic system, composed of both plasma proteins and factors from the tissues. These two pathways are illustrated in Figure 1. They differ in the nature of the initiating stimulus and in the ensuing series of reactions up to the activation of Factor X to Factor Xa, at which point they converge and continue to the ultimate formation of a fibrin clot. Table 1 lists the several plasma proteins which have been shown to participate in these two pathways,

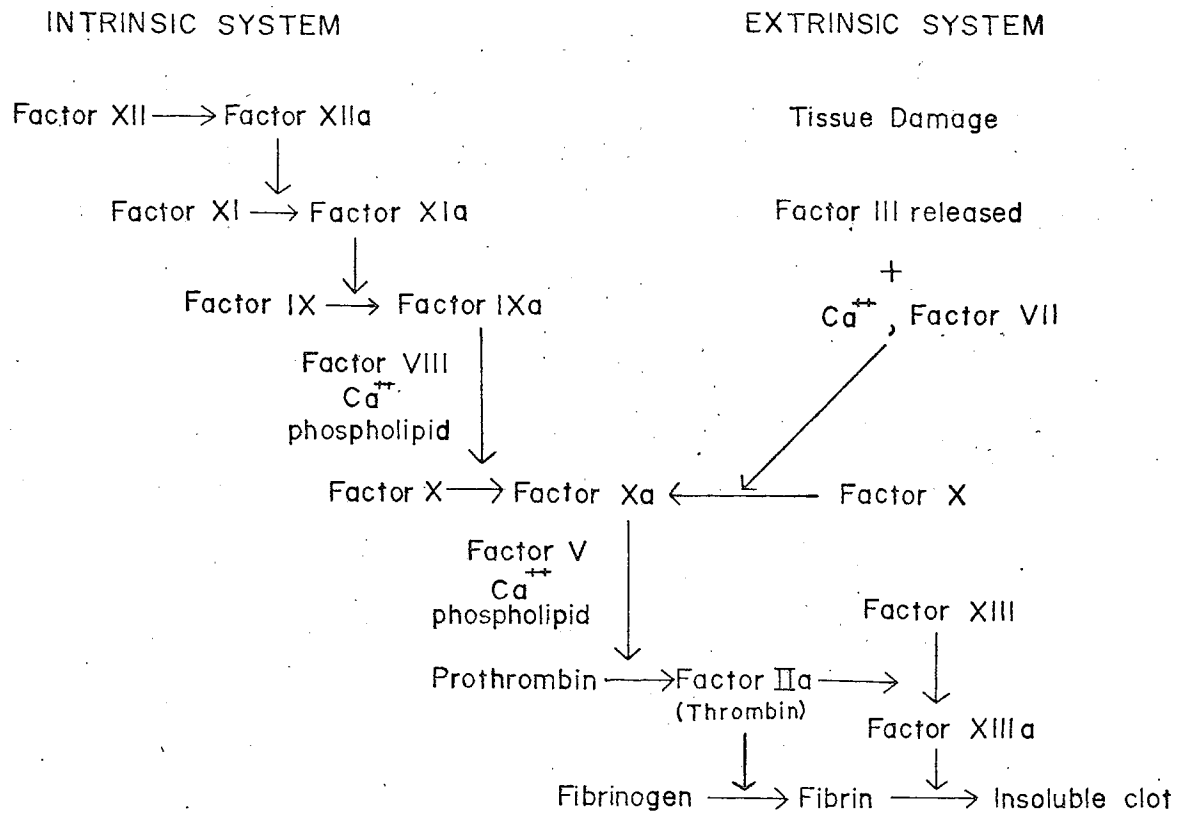


Figure 1. The intrinsic and extrinsic schemes of the reactions of blood coagulation.

Table 1. Properties of some of the well-defined coagulation factors.

Coagulation Factor		Coagulation Pathway		
Roman numeral designation	Common name	Extrinsic system	Intrinsic system	Molecular weight
Factor I	Fibrinogen	+	+	340,000 (human and bovine)
Factor II	Prothrombin	+	+	68,700 (human) 72,000 (bovine)
Factor III	Tissue Factor	+		220,000; 330,000 (bovine)
Factor IV	Calcium ions	+	+	
Factor V	Proaccelerin	+	+	290,000-439,000 (bovine)
Factor VII	Proconvertin	+		63,000 (human)
Factor VIII	Antihemophilic factor		+	1.1 million (human, bovine)
Factor IX	Christmas factor		+	55,400 (bovine)
Factor X	Stuart factor	+	+	55,000 (bovine)
Factor XI	Plasma thromboplastin antecedent		+	160,000 (human, bovine)
Factor XII	Hageman factor		+	90,000 (human) 82,000 (bovine)

Table 1.--Continued

Coagulation Factor		Coagulation Pathway		
Roman numeral designation	Common name	Extrinsic system	Intrinsic system	Molecular weight
Factor XIII	Fibrin stabilizing factor	+	+	300,000 (bovine) 320,000 (human) 146,000 (human platelets)

These factors have been assigned a Roman numeral (Wright, 1959). Most investigators use the roman numeral nomenclature for Factors V through XIII while fibrinogen, prothrombin, calcium ions, and tissue factor are generally referred to by their common names.

and their molecular weights, as determined by various investigators.

The intrinsic system is initiated when Factor XII, or Hageman Factor contacts a foreign surface, which results in the conversion of Factor XII to an enzyme, Factor XIIa. In vivo this "surface" is thought to be collagen, or vascular basement membrane, as described by Niewiarowski, Bankowski, and Rogowicka (1965), and Cochrane et al. (1972), respectively. Also, several plasma enzymes such as kallikrein, Factor XIa, plasmin, and trypsin can activate Factor XII as shown by Wuepper (1972) and Cochrane, Revak, and Wuepper (1973). In vitro this activation occurs by contact with glass (Donaldson and Ratnoff, 1965), celite (Speer, Ridgeway, and Hill, 1965), fatty acids (Ratnoff and Crum, 1964), or other foreign substances. With the activation of Factor XII, a step-wise series of enzyme-substrate interactions is triggered. Factor XIIa activates Factor XI to Factor XIa, also an enzyme, and Factor XIa will activate Factor IX in the presence of calcium to Factor IXa. Factor IXa, together with Factor VIII, calcium, and phospholipid, will convert Factor X to its active form, Factor Xa.

The extrinsic system is initiated by the release of tissue factor from the injured tissues and the formation of a complex between it and Factor VII, in the presence of calcium. This complex then acts directly upon Factor X,

converting it to Factor Xa, thus bypassing the several steps in the intrinsic system which occur prior to the activation of Factor X.

Once formed, Factor Xa can in turn generate the enzyme thrombin, from prothrombin, in the presence of Factor V, calcium, and phospholipid. Thrombin, or Factor IIa, is solely responsible for the conversion of fibrinogen to fibrin. Fibrin monomers are stabilized by Factor XIII, or fibrin-stabilizing Factor, which catalyzes an intermolecular transamidation of adjacent fibrin strands.

The intrinsic system is more complicated and is sluggish compared to the extrinsic system. These two pathways provide alternate mechanisms for the activation of Factor X to Factor Xa, yet both are essential for maintenance of normal hemostasis. The several steps which comprise the overall mechanism provide many points for regulation of the extent of coagulation.

The clotting scheme as comprised of the intrinsic and extrinsic pathways, is outlined in Figure 1, and was called a "cascade" scheme by Macfarlane (1964) and similarly, a "waterfall" scheme by Davie and Ratnoff (1964). These hypotheses viewed the clotting process as a series of enzyme-substrate reactions, the product of each acting as an enzyme on the substrate in the subsequent reaction of the sequence. These viewpoints also served to emphasize the amplifying nature of the clotting sequence since the several

stages in the scheme would serve to give an increased response to the initial stimulus. A few molecules of Factor XIIIa could act on 100 molecules of Factor XI to generate the same number of activated Factor XI molecules and each of these in turn could act on 100 molecules of Factor IX, and so on down the sequence, creating the momentum of an avalanche.

Macfarlane's scheme implied that every factor must be activated prior to participation in the clotting scheme; however, Hougie, Denson, and Biggs (1967) suggested that Factor VIII as it exists in plasma, can participate in the activation of Factor X, and a similar conclusion was reached by Barton, Jackson, and Hanahan (1967) for Factor V. Both factors can be activated by thrombin to form more reactive species in their respective reactions, rather than to be converted to enzymes.

Phospholipid is required in both the intrinsic and the extrinsic pathways. In the extrinsic pathway phospholipid is required for manifestation of Factor VII activity, and is derived from the damaged tissues in the form of a lipoprotein, or tissue factor. In the intrinsic system phospholipid is required for maximal conversion of Factor X to Factor Xa and of prothrombin to thrombin. In vivo this phospholipid comes from the platelets during the "release reaction" (Marcus, 1966). In vitro it has been demonstrated that Factor IXa and Factor VIII form a

macromolecular complex with phospholipid in the presence of calcium (Bergsagel and Hougie, 1956; Spaet and Cintron, 1963; Lundblad and Davie, 1964). Similarly, Papahadjopoulos and Hanahan (1964) and Jobin and Esnouf (1967) demonstrated that Factor Xa and Factor V, in the presence of calcium, adsorb onto phospholipid aggregates. In these two discrete reactions, calcium is an absolute requirement for adsorption of Factors IXa and X as well as Factors II and Xa, to the phospholipid surface. However, both Factor VIII and Factor V can complex with phospholipid in the absence of a divalent metal ion. Complex formation between these several proteins and phospholipid undoubtedly has functional significance since the activation of Factor X to Factor Xa occurs at a maximum rate in the presence of all four components: Factor IXa, Factor VIII, phospholipid, and calcium, as does the conversion of prothrombin to thrombin which involves Factor Xa, Factor V, phospholipid, and calcium.

Current ideas about the interaction of the components of the blood clotting mechanism then, suggest that some steps involve complexation between proteins and lipid. This feature requires considerable modification of Macfarlane's "cascade" theory depicting a sequential transformation of inactive protein precursors to active enzymes products, while maintaining the essential principle

of enzyme-substrate interactions purported by the earliest theorists on blood coagulation.

Review of Other Preparations of Factor V
and Their Properties

In the last decade, much progress has been made toward understanding that reaction, central to the blood coagulation mechanism, in which prothrombin is converted to thrombin. This reaction is catalyzed by the prothrombinase complex, consisting of two proteins, Factor Xa and Factor V, plus calcium ions and phospholipid. Prothrombin, thrombin, and Factor Xa have been purified to homogeneity in recent years and their individual physical characteristics determined, including the molecular requirements for the binding of prothrombin and Factor Xa to phospholipid in the presence of calcium. Also, the mechanism by which prothrombin is converted to thrombin has been well defined.

However, little is known of the biochemical properties of Factor V, or its role in the conversion of prothrombin to thrombin. Factor V was discovered by Quick (1943), who noted the rapidity with which its activity disappeared in stored plasma and called it "labile factor." In 1947, Owren described a patient with an abnormally long prothrombin clotting time which was shortened by the addition of normal plasma freed of prothrombin. Consequently in 1950, Owren postulated a fifth coagulation factor, called Factor V, which was required for the conversion of

prothrombin to thrombin by thromboplastin (a lipoprotein derived from tissues which facilitates coagulation) in the presence of calcium. Fantl and Nance (1946) and Ware, Murphy, and Seegers (1947) also observed that prothrombin was activated more rapidly to thrombin in the presence of Factor V than in its absence. Consequently they labeled Factor V, "accelerator globulin," or briefly, "Ac-globulin."

Since the time of these early observations, all that has been clarified about Factor V is that it has no enzymatic activity toward prothrombin but that it does function as an important cofactor to greatly accelerate the rate of conversion and the yield of thrombin from prothrombin. This paucity of information arises from the difficulties encountered in isolating Factor V from plasma in reasonable yields and in a stable form.

Early attempts to isolate either bovine or human Factor V were mainly attempts to demonstrate that it did exist as a discrete blood clotting factor. Ware and Seegers (1948), and Landaburu and Seegers (1961), working with bovine "Ac-globulin," and Lewis and Ware (1953), Surgenor, Wilson, and Henry (1961), and Blombäck and Blombäck (1963), working with human Factor V, achieved partial purification by similar procedures. These entailed adsorption of bovine or human plasma with barium sulfate, or carbonate, followed by ammonium sulfate, acid, or alcohol precipitation, and finally, either ion-exchange or

gel filtration chromatography. Factor V was recovered in yields ranging from 54% (Blombäck and Blombäck, 1963), to 12% (Lewis and Ware, 1953). Blombäck and Blombäck (1963) noted that calcium stabilized their Factor V preparation during storage.

Cox, Lanchantin, and Ware (1956) isolated human plasma Factor V according to Lewis and Ware (1953), then activated it with thrombin and chromatographed the activation mixture on a column of Amberlite IR-400. Activated Factor V was recovered in a yield of 50% and an overall purification of 100-fold was realized. Protein contamination was revealed upon zone electrophoresis of the preparation.

Aoki, Harmison, and Seegers (1963) also isolated bovine Factor V by a procedure similar to that of Lewis and Ware (1953). They achieved a 100-fold purification in a yield of 15% with a specific activity of 1500 U/mg. Their preparation was contaminated with traces of fibrinogen and Factor VIII. Ultracentrifugal analysis of the product revealed some heterogeneity but the main component had an $s_{20,w} = 4.2$. A molecular weight of 97,400 was calculated on the basis of amino acid analyses. A concentration of 1.4 g hexosamine per 100 g protein was also determined. The stability of this preparation was enhanced when it was stored at -60°C , pH 7.0 in buffer containing 50% glycerol and 0.10 M CaCl_2 .

Papahadjopoulos, Hougie, and Hanahan (1964) isolated Factor V from bovine plasma and from bovine serum and observed by Sephadex G-200 chromatography that there was a difference in the molecular size of Factor V from these two sources. Purification of Factor V entailed barium sulfate adsorption, precipitation with ammonium sulfate between 33 and 50% saturation, and Sephadex G-200 column chromatography. The plasma Factor V preparation separated into three protein peaks on the column and Factor V activity was recovered quantitatively in the first peak with a $K_D = 0.05$, where K_D is defined as that fraction of the internal volume, V_i , of a column accessible to a solute molecule. See methods section for more details. A five-fold purification of plasma Factor V was realized, and when treated with bovine thrombin, its activity increased 10-fold. Thrombin-activated Factor V eluted on Sephadex G-200, with a $K_D = 0.18$, and a 50-fold purification was realized. The serum Factor V preparation also eluted in three protein peaks and the Factor V activity eluted in two peaks, the first with a $K_D = 0.05$, and the second with a $K_D = 0.18$. Serum Factor V was recovered in a yield of 30% and a five-fold purification was realized. Ultracentrifugation of plasma Factor V indicated that it was a high molecular weight protein, not a lipoprotein, since all the Factor V activity was found at the bottom of the tube, at a density of 1.21 g/ml, and contained less than 10% of the lipid phosphorus. The

molecular weight of plasma Factor V was estimated to be at least 400,000, and that of serum Factor V, or thrombin-activated Factor V, to be approximately 200,000.

Esnouf and Jobin (1967) achieved considerable purification of bovine plasma Factor V by a procedure which has subsequently enjoyed widespread use. They isolated Factor V by adsorption of bovine plasma with barium sulfate, batchwise adsorption of the adsorbed plasma with TEAE cellulose (TEAEC), and elution of Factor V from the TEAEC with 0.4 M sodium phosphate, pH 7.0, and finally chromatography of the TEAEC extract on a column of cellulose phosphate. Factor V was recovered in a yield of 50%, with a specific activity of 50, representing a purification overall of 4000-fold. The molecular weight of Factor V, determined by analytical ultracentrifugation was 290,000. The amino acid composition was determined and only 73% of the material analyzed could be accounted for on a weight basis in terms of amino acids and nitrogen, while 94% of the ninhydrin-detectible nitrogen applied was recovered. The composition of the remaining 21% was not determined but these workers speculated that the affinity of Factor V for hydrophobic surfaces may be determined by this residual material. The electrophoretic mobility, immunoelectrophoretic behavior and optical rotary dispersion parameters were also reported. Factor V was unstable to

freezing, treatment with EDTA, or high salt, but its activity was preserved when stored at -20°C in 50% glycerol.

Barton and Hanahan (1967) prepared bovine Factor V according to Esnouf and Jobin (1967) with some modifications, including the addition of a second cellulose phosphate chromatography step, subsequent to the first. They prepared Factor V in a 20-45% yield with an overall purification of 3500-8500-fold. Factor V was free of thrombin, prothrombin, Factor VIII, and Factor X activities and appeared homogeneous by cellulose acetate electrophoresis at pH 8.6. However, when Factor V was chromatographed on a column of Sephadex G-200, it eluted in two peaks of activity: the first and major one had an apparent molecular weight of 350,000 and the second had a molecular weight of 70,000. When it was treated with 8.5 mM CaCl_2 prior to chromatography, Factor V eluted in one peak of activity with a molecular weight of 350,000. Thrombin treatment resulted in a 4-fold increase in Factor V activity and a decrease in the molecular weight of plasma Factor V to 150,000 as determined by Sephadex G-200 gel chromatography. Factor Va, thrombin plus Factor Xa, calcium and phospholipid, generated thrombin activity from prothrombin at a 3-fold faster rate in the first 30 seconds of the reaction than did plasma Factor V. Plasma Factor V or Factor Va, thrombin appeared to be essential to the formation of thrombin from prothrombin by Factor Xa.

Human Factor V was isolated by Kahn and Hemker (1970) using a procedure similar to that of Esnouf and Jobin (1967). Human plasma was adsorbed with $\text{Al}(\text{OH})_3$ in the presence of 50% glycerol followed by adsorption and elution of Factor V from TEAE cellulose. A 15% yield of human Factor V was realized with an overall purification of 30-fold. Glycerol and Mg^{2+} ions helped to stabilize the Factor V.

Colman (1969a) extended and modified the procedure of Esnouf and Jobin (1967), as follows: bovine plasma was adsorbed with barium sulfate, the adsorbed plasma was treated with TEAEC and Factor V was eluted with 0.4 M potassium phosphate buffer, pH 7.0. TEAEC-Factor V was precipitated with ammonium sulfate between 25 and 70% saturation, followed by chromatography on a column of phosphorylated cellulose. The Factor V activity eluted with 0.4 M potassium phosphate, pH 7.0, in a yield of 35%, with a specific activity of 49 U/mg, representing a purification of 2760-fold. This preparation was unstable to freezing but was stable indefinitely at -50°C in 50% glycerol (v/v). Treatment with purified thrombin resulted in a 2-fold increase in Factor V activity within 5 minutes at 17°C which was followed by a significant loss of activity. The rate and extent of activation by thrombin were proportional to the concentration of thrombin added.

Philip, Moran, and Colman (1970) analyzed the above preparation by analytical disc gel electrophoresis as well as by Sephadex G-200 and Sepharose 4B chromatography. Disc gel electrophoresis revealed that this Factor V contained at least seven protein bands. By gel filtration on Sephadex G-200, it contained two Factor V active peaks and three such peaks were observed by chromatography on Sepharose 4B, which were designated form L, with an apparent molecular weight of at least two million, and contained lipid material; form A, molecular weight 310,000 which could be activated two-fold by thrombin; and form C, molecular weight of 160,000, which could not be activated by thrombin. Form A appeared to be an oligomer of form C since the latter, after treatment with 50% glycerol, eluted on a column of Sephadex G-200 with an apparent molecular weight of 310,000. When bovine plasma was collected in several different anticoagulants, and even in the presence of DFP, and subsequently chromatographed on Sephadex G-200, two peaks of Factor V activity eluted from the column, corresponding to forms A and C of the purified Factor V preparation.

Recently, Gumprecht and Colman (1975) prepared bovine Factor V according to Colman (1969a), but with slight modifications, and determined the sialic acid content of different preparations of Factor V, which ranged from 5% to 12%. When sialic acid was removed by treatment with

neuraminidase, the activity of asialo Factor V was 1.6 times greater than native Factor V, which these workers claimed was a statistically significant increase. Asialo Factor V was estimated to be 3.8 times more stable than native Factor V. The activity of native Factor V was enhanced 7-fold by thrombin in 6 minutes at 37°C followed by a gradual decay. Asialo Factor V was also rapidly activated by thrombin but the activation profile was biphasic. The author speculated that this kinetic profile could result from the presence of several forms of asialo Factor V which are both activated and inactivated at different rates.

Evidence that Factor V contains carbohydrate in the form of neutral sugars was reported by Dombrose and Seegers (1973), thus it is not surprising that Factor V also contains sialic acid. However, the observation by the above workers, that the removal of sialic acid positively affects the activity of Factor V is unusual, since such a phenomenon has not been reported for any other coagulation factors, nearly all of which are glycoproteins.

Important observations by Day and Barton (1972) pointed out that some of the techniques used by other workers to purify Factor V produced artifactual forms of Factor V. When Factor V in unfractionated bovine plasma, or in a TEAE extract, prepared according to Esnouf and Jobin (1967), was gel filtered on Bio-Gel P300, the Factor V activity eluted in a single peak with a $V_e/V_0 = 1.11$.

However, when the ammonium sulfate precipitate of a TEAEC extract, or the Factor V fraction obtained after cellulose phosphate chromatography of a TEAEC extract were similarly chromatographed, multiple peaks of Factor V activity were eluted from the column. This indicated that the techniques of ammonium sulfate precipitation and cellulose phosphate chromatography produced dissociation and/or degradation products, which were of lower molecular weight and higher specific activity than that of plasma Factor V. These workers concluded that two of the three forms of Factor V reported by Philip et al. (1970), namely forms L and C, were artifacts of the preparative procedure. In contrast, such techniques as TEAEC adsorption in the presence of sodium acetate buffers at neutral pH, concentration by ultrafiltration, and chromatography on Bio-Gel P300, in the presence of calcium, could be used without altering the native structure of Factor V.

Dombrose et al. (1972) purified bovine Factor V by a relatively simple procedure, resulting in a preparation free of other clotting factors. Bovine plasma was adsorbed with barium carbonate, the adsorbed plasma was recalcified with 10^{-2} M CaCl_2 in the presence of non-ionic cellulose at pH 5.9, and extracted batch-wise with QAE cellulose, in the presence of calcium acetate buffers, pH 7.25. Factor V was recovered in a yield of 180% and a purification of 1300-fold. This preparation was stable at

4°C for one month, and at 25°C for one week, at a protein concentration of 1 mg/ml; however, it was not stable to freezing. The high yield of Factor V suggests that some activation of plasma Factor V may have occurred during the isolation. When Factor V was concentrated by ammonium sulfate precipitation or dialyzed against distilled water and stored in 10% glycerol at pH 5.6, the activity increased, again suggesting activation of Factor V.

Dombrose and Seegers (1973) further reported that Factor V, prepared according to Dombrose et al. (1972) had a molecular weight of $380,000 \pm 7000$, and contained 8.7% neutral sugars, but negligible amounts of phospholipid. Analytical disc gel electrophoresis at pH 4 or 8 revealed from 1-4 bands and SDS electrophoresis revealed four polypeptide constituents. Whether the SDS electrophoresis was carried out under reducing or nonreducing conditions was not indicated. Amino acid analyses was performed but the results were not reported. Only 76% of the composition of Factor V could be accounted for as peptide and neutral sugar.

Recently, preparations of human plasma Factor V have been reported by Giddings (1974) and separately by Rosenberg, Beeler, and Rosenberg (1975). Giddings adsorbed fresh frozen human plasma with aluminum hydroxide followed by ethanol precipitation, acetic acid precipitation, and finally a Rivanol (acridine lactate) precipitation.

Factor V was extracted with NaCl and chromatographed on Bio-Gel A-0.5 m in Michaelis-calcium buffer, pH 7.35. It eluted as a single peak soon after the column void volume, and was recovered in a yield of 18% with a specific activity of 2.5-6.0 U/mg, representing a 400-fold purification. The preparation was free of other clotting factors but analytical disc gel electrophoresis revealed two protein bands. Factor V had a molecular weight of 300,000 determined by gel filtration on Bio-Gel A-1.5 m. This investigator reported that calcium served to stabilize their Factor V preparation.

Rosenberg et al. (1975) isolated human Factor V by adsorbing plasma with barium citrate followed by precipitation at pH 6.5 and then at pH 5.7, batchwise chromatography on hydroxyapatite, precipitation with polyethylene glycol and chromatography on DEAE cellulose. This preparation was free of other clotting factors and when examined by isoelectric focusing gel electrophoresis, a single species was observed at pH 5.1. However, only 5% of the Factor V activity applied to the gel was recovered.

Recently too, preparations of Factor Va have been reported. Day (1975) prepared TEAEC Factor V from bovine plasma according to Esnouf and Jobin (1967), as modified by Day and Barton (1972). TEAEC Factor V was then chromatographed twice on Sephadex G-200, activated with partially purified RVV-V (the Factor V activator from Russell's Viper Venom), or Factor Xa, and subsequently

chromatographed twice again on Sephadex G-200. Factor Va was recovered in a yield of only 3% with a specific activity of 3151, representing a 4500-fold purification. It had a molecular weight of 213,000 determined by Sephadex G-200 gel chromatography. A density of 1.19 ± 0.01 g/ml was determined by sucrose gradient centrifugation for both plasma and activated Factor V. Factor Va was analyzed for both phospho- and neutral lipid and was found to contain only 7.2% neutral lipid. This preparation was extremely unstable, losing 90% of its original activity upon concentration to 0.5 mg/ml by ultrafiltration. This investigator indicated that precipitation of TEAEC Factor V with polyethylene glycol resulted in dissociation of Factor V to a species of molecular weight 276,000, which was no longer subject to thrombin or RVV-V activation. Also, Sephadex G-200 Factor V readily dissociated when concentrated by ultrafiltration. This dissociated form of plasma Factor V is similar to that isolated by Colman (1969a) and that reported by Esnouf and Jobin (1967), in molecular weight and in the inability of these preparations to be activated by thrombin.

Chulkova and Hernandez (1975) prepared thrombin-activated Factor V by a procedure which involved barium sulfate adsorption of bovine plasma, DEAE-Sephadex chromatography of the adsorbed plasma, thrombin activation of the DEAE-Sephadex eluate, chromatography on Amberlite

CG-50, and finally ammonium sulfate fractionation. Factor Va was recovered in a yield of 160% with a specific activity of 7500 U/mg, representing an overall purification of 4700-fold. By analytical ultracentrifugation, Factor Va in 0.15 M NaCl exhibited an $s_{20,w} = 5.9$ and in 1% SDS, $s_{20,w} = 3.3$. By SDS gel electrophoresis Factor Va appeared as a single component with a molecular weight of 30,000, and analytical disc gel electrophoresis at pH 9.5 revealed two components, both of which were found to contain Factor V activity when eluted from the gel. By gel filtration on Sephadex G-200, Factor Va had an apparent molecular weight of 100,000. The final preparation of Factor Va could be further activated 10-fold by thrombin. This feature does not seem plausible since, once treated with thrombin, Factor V has never been shown to be susceptible to additional activation by thrombin. Furthermore, the extent of purification of Factor Va accomplished by this procedure is difficult to assess since the isolation data were not adjusted to account for activation of Factor V midway through the preparation.

This review of the literature on Factor V illustrates the fact that past attempts to isolate either plasma or activated Factor V have consistently been hampered by the instability of Factor V, which implies either, an absolute loss of activity, or a dissociation and/or degradation of plasma Factor V during its isolation.

Recoveries of greater than 100% of the initial Factor V activity, the presence of multiple molecular weight species of Factor V in partially purified preparations, and the inability of such preparations to be activated by thrombin, are typical features which can be explained in part by the ready dissociability of plasma Factor V. The findings of Day and Barton (1972) and Day (1975), have helped to clarify much of the confusing data reported on previous preparations of Factor V. To date no satisfactory methods have emerged for the isolation of Factor V in good yields and of reasonable stability, let alone a homogeneous preparation. Consequently little information on the composition or properties of native Factor V, or changes in these features that occur when it is converted to a more active form by various proteolytic enzymes, has been reported.

Activation of Factor V

During attempts to isolate Factor V, many investigators also noted that the clotting activity of Factor V could be significantly enhanced by a number of proteolytic enzymes.

Past reports have indicated that principally three enzymes were capable of activating Factor V, namely, thrombin, a procoagulant protein from Russell's Viper Venom, RVV-V, and Factor Xa. Further aspects of these reports are discussed in the following sections.

By Thrombin

It was initially observed by Ware et al. (1947), and later, by Ware and Seegers (1948), that whole serum contained an accelerator globulin which facilitated the formation of thrombin from prothrombin faster than did whole plasma. Addition of thrombin to bovine plasma produced a product indistinguishable from the serum "accelerator globulin." They invoked an inactive precursor-active product relationship for Factor V in plasma versus that in serum. Astrup (1950) provided the names proaccelerin and accelerin to further describe this relationship. Lewis and Ware (1954) demonstrated that citrated human plasma was clotted by thrombin and the Factor V activity of the serum measured 10-fold higher than prior to the addition of thrombin. Addition of thrombin to serum Factor V resulted in only a 3-fold increase in activity. Hjort (1957) likewise observed a 10-fold increase in the Factor V activity of platelet-rich-plasma upon the addition of thrombin.

Bergsagel and Nockolds (1965) concluded that thrombin was the only physiological activator of Factor V and that "activation" was essential for its participation in a complete prothrombin activator. Mixtures of normal serum, calcium and phospholipid were only able to convert prothrombin to thrombin if traces of prothrombin were initially mixed with them, presumably as a source of thrombin for activation of Factor V. They also demonstrated

direct activation of Factor V by thrombin, which was inhibited by treatment with hirudin (a thrombin specific inhibitor), prior to the addition of Factor V. Cox et al. (1956) observed that thrombin altered the retention characteristics of human Factor V on ion-exchange chromatography and took advantage of this behavior to isolate the activated form of Factor V by chromatography on Amberlite IR-400, as discussed in a previous section. Hussain and Newcomb (1963) observed that the elution of Factor V activity from DEAE cellulose varied depending on prior exposure to thrombin, as did its sedimentation behavior on sucrose density gradient centrifugation. Newcomb and Hoshida (1965) achieved partial purification of thrombin activated Factor V by treating a TEAEC extract of barium sulfate adsorbed bovine plasma with thrombin and subsequently chromatographing this mixture on cellulose phosphate. Factor Va eluted thrombin on the column at a different position than native Factor V, and the latter could be activated 2-3-fold by thrombin while Factor Va thrombin could not be further activated after chromatography.

Prentice, Ratnoff, and Breckenridge (1967) observed activation of Factor V in whole plasma and in partially purified Factor V preparations by added thrombin. Thrombin pretreated with hirudin could not activate Factor V but if hirudin was added after mixing Factor V and thrombin, activation of Factor V still occurred. These workers

considered that Factor Xa was a contaminant of their preparation, so in a separate experiment they demonstrated that Factor Xa could not be inhibited by hirudin. Consequently they assumed that thrombin was the exclusive activator of Factor V in these experiments.

These early observations were stimulating yet no attempts to characterize the plasma and more active forms of Factor V on any basis other than specific activity or adsorption characteristics on ion-exchange media were made, until Papahadjopoulos et al. (1964) observed significant differences in the molecular size of plasma and serum Factor V by chromatography on Sephadex G-200, and sucrose density gradient centrifugation. The details of their findings were discussed in a previous section.

Colman (1969a) reported his observations on treating Factor V with highly purified thrombin. After isolating Factor V, Colman observed that thrombin increased the activity of Factor V 2-fold within 5-10 minutes at 17, 27, or 37°C. Activation was followed by a significant decay of activity which was not prevented or retarded by the addition of hirudin. Both the rate and extent of Factor V activation were directly proportional to the concentration of thrombin and inversely proportional to the concentration of Factor V. Colman, Moran, and Philip (1970) observed that thrombin had minimal effects on the activity of forms L, A, or C which comprised the Factor V preparation of Colman

(1969a), as described by Colman et al. (1970). Thrombin enhanced the activity of form L, 1.5-fold, and that of form A to 2.7-fold, while neither form C nor serum Factor V, could be activated by thrombin. To determine whether thrombin altered the molecular size of any of these three forms of Factor V, each was gel filtered on Sepharose 4B or Sephadex G-200. Form L, both before and after exposure to thrombin, eluted in the void volume on Sepharose 4B with a $K_D = 0$. Similarly, form A and form C eluted off both Sepharose 4B and Sephadex G-200 in the same volume regardless of prior treatment with thrombin. In summary, thrombin did not alter the activity or the molecular size of any of these forms of Factor V to a significant extent. These observations are in keeping with those of Day and Barton (1972) and Day (1975) that methods such as ammonium sulfate precipitation and cellulose phosphate chromatography, both of which were used by Colman (1969a), bring about dissociation and/or activation of plasma Factor V to species of lower molecular weight and higher specific activity, which are thereby no longer susceptible to enzymatic activation by thrombin.

Recently, Janssen and vanLeuven (1972) observed that the Factor V activity in plasma increased during clotting of the same, and this was coincident in time with the initial generation of thrombin activity in the same. These activities were measured in citrated human plasma to which

thrombin was added to initiate clotting, or in reclassified human plasma where thrombin was intrinsically generated. They concluded that "activated" Factor V must participate in blood coagulation because no significant activation was observed prior to the initial detection of thrombin activity, at levels of thrombin demonstrated in vitro to activate that amount of Factor V in plasma. They speculated that thrombin was a potential physiological activator of Factor V at least on a temporal basis.

Reports by Surgenor et al. (1961) and Ferguson et al. (1969) have disclaimed any observable activation of Factor V by thrombin. Both of these studies reported only a loss of Factor V activity upon the addition of thrombin, indicating possibly that their Factor V preparation already contained appreciable amounts of activated Factor V and addition of thrombin served only to degrade it, or that the levels of thrombin used were relatively high compared to the amount of Factor V, so initial activation may have been obscured by significant degradation of Factor V.

By RVV-V

Another enzyme that has been observed to activate Factor V is a coagulant protein from Russell's Viper Venom, RVV-V, which is specific for Factor V among the clotting factors. Although it is not a physiological activator of Factor V, it has served as a useful tool in laboratory

studies to generate the activated form of Factor V. Hjort (1957) observed that a preparation of bovine Factor V increased in activity upon incubation with crude Russell's Viper Venom in the absence of calcium ions. Also, Rapaport, Hjort, and Patch (1966) presented some kinetic data which suggested that the extent of activation of Factor V by RVV-V was dependent on the concentration of RVV-V and hence the reaction was not enzymatic but stoichiometric. Subsequently, Schiffman, Theodor, and Rapaport (1969) succeeded in separating two coagulant proteins from Russell's Viper Venom, one which specifically activated Factor X in a calcium-dependent reaction, and the other which specifically activated Factor V with no other additions. Whole RVV was fractionated by chromatography on Sephadex G-200 with a 70-75% recovery of both proteins. The RVV-V was further purified by rechromatography on Sephadex G-25 to yield a total purification of 7-fold over whole venom. Activation of Factor V, as present in $\text{Al}(\text{OH})_3$ -adsorbed human plasma by varying amounts of RVV-V, over a 4-fold concentration range indicated that the yield of activated Factor V increased with increasing RVV-V concentrations. Addition of fresh Factor V to a RVV-V-Factor V mixture saw no further activation of the added Factor V. Varying the Factor V concentration at constant RVV-V concentration resulted in increasing product formation with increasing Factor V concentrations. From such experiments

these workers concluded that the reaction between Factor V and RVV-V was a stoichiometric one in which both were consumed.

More recently, Kahn and Hemker (1972) demonstrated that RVV-V could enhance the activity of both bovine and human Factor V, even in the presence of hirudin. Activation resulted in a reduction in the molecular weight of Factor V, as demonstrated by Sephadex G-200 column chromatography; bovine Factor V decreased from 400,000 to 195,000 daltons while human Factor V decreased from 480,000 to 110,000 daltons. Activation of Factor V from these same sources by thrombin resulted in similar molecular weight changes.

Hanahan, Rolfs, and Day (1972) similarly demonstrated that partially purified RVV-V could activate Factor V in bovine and human plasma approximately 20-fold, while bovine serum Factor V could only be activated 2-4-fold. After treatment with RVV-V, the apparent molecular weight of plasma Factor V decreased from 400,000 to 205,000, while serum Factor V had an apparent molecular weight of 230,000 which did not change after exposure to RVV-V, as determined by Sephadex G-200 gel chromatography. RVV-V activity was stable to heat treatment at 95°C for 15 minutes over a pH range of 1.0 to 7.0.

Esmon and Jackson (1973) noted that the extent to which Factor V, in various preparations, was activated by crude RVV, was exactly the same as that observed with

highly purified bovine thrombin. Factor V, in bovine plasma was activated 14-fold, while TEAEC Factor V was activated 12-fold, by both enzymes. A TEAEC extract, further purified on cellulose phosphate, could not be activated by either enzyme. This last observation is consistent with those of Esnouf and Jobin (1967) and Colman (1969a) who both observed that their preparations of bovine Factor V, isolated by TEAEC extraction and cellulose phosphate chromatography, could not be activated by thrombin. Esmon and Jackson (1973) also claimed that the Factor V activator in RVV could be inhibited by DFP.

By Factor Xa

Breckenridge and Ratnoff (1963) first suggested that Factor V (proaccelerin) was converted into a "prothrombin-converting principle" by Factor Xa, in the presence of phospholipid and calcium ions. This conclusion was based on the effect which a circulating anticoagulant, found in a certain disease state, had on the formation of thrombin from prothrombin in a 2-stage thrombin generating system. When the anticoagulant was added to the first stage, after Factor X was activated by crude RVV but before Factor V was added to form the prothrombin activator, a prolonged clotting time was observed. However, if the anticoagulant was added after Factor Xa and Factor V were mixed together, the thrombin clotting time was not prolonged.

Later, Breckenridge and Ratnoff (1965) repeated essentially the same experiments only using soybean trypsin inhibitor, protamine sulfate, or polybrene, to inhibit the Factor Xa-Factor V interaction. Prolonged clotting times were only observed when a given inhibitor was added to Factor Xa or to Factor V, before the addition of the other factor, so these workers concluded that Factor V was a substrate for the enzymatic activity of Factor Xa and that the activated proaccelerin formed was the substance responsible for the conversion of prothrombin to thrombin.

However, much of this work became questionable when it was demonstrated by Barton et al. (1967), and separately by Jobin and Esnouf (1967), that Factor V alone could not convert prothrombin to thrombin. Each group prepared a complete prothrombinase enzyme consisting of Factor Xa, calcium, phospholipid, and Factor V, then separated out the Factor Xa activity from the Factor V-phospholipid moiety. Jobin and Esnouf (1967) displaced Factor Xa from the complex by treating it with magnesium chloride and recovered the Factor Xa activity in the supernatant after ultracentrifugation. The pellet, containing Factor V bound to phospholipid, could only convert prothrombin to thrombin when Factor Xa was added back. Similarly, Barton et al. (1967) separated out Factor Xa from the prothrombinase complex, treating it with EDTA and by chromatographing the mixture on Sephadex G-200. Factor V eluted with the phospholipid in the void

volume ($V_e/V_o = 1.0$) and Factor Xa eluted later ($V_e/V_o = 2.3$). Addition of the Factor V-phospholipid fraction to prothrombin plus calcium did not generate any thrombin activity, yet when Factor Xa was also added, rapid thrombin activity ensued. Only Factor Xa could convert prothrombin to thrombin without the addition of any of the other prothrombinase components.

Other Enzymes Affecting the Activity of Factor V

Colman (1969b) investigated the effect of several other proteolytic enzymes on the activity of Factor V, and compared this to the effects of thrombin. Both plasmin and trypsin, even at low concentrations did not activate Factor V to any extent, but only produced losses in Factor V activity. It was reported that the presence of trypsin did not interfere with thrombin activation of Factor V. However it seems unlikely that thrombin could activate a degrading protein. Both papain and crude RVV effected activation of Factor V, when added at low concentrations, and inactivated Factor V when added at relatively high concentrations. Also, papain inhibited the activation of Factor V by thrombin, but crude RVV did not interfere with this reaction. The extent of activation of Factor V by RVV or papain was minimal--1.5 to 2.0-fold. This finding again serves to illustrate that this Factor V preparation, reported to

contain dissociated forms of native Factor V, is consequently no longer a substrate for enzymatic activation by thrombin.

Numerous reports attest to the fact that plasma Factor V can be activated by a number of different enzymes. However, the data are confusing in that the degree of activation by any one enzyme varies from 1.0 (no activation) to 14-fold, and reports on the change in molecular size of Factor V upon activation vary from no change to a decrease of approximately one-half. These discrepancies undoubtedly reflect the varied preparations of Factor V which contain one or several forms which are dissociation products of native Factor V, produced as a result of the isolation techniques used. To unequivocally elucidate changes in the activity and molecular weight of Factor V upon activation, a preparation is required which consists only of native or unaltered Factor V, under conditions in which its activity is stable and its ability to be activated is reproducible.

Role of Factor V in the Prothrombinase Reaction

Previous attempts to determine the mode of action of Factor V in the prothrombinase reaction, have demonstrated primarily what it does not do in this reaction. Factor V alone, or even in combination with phospholipid and calcium, cannot convert prothrombin to thrombin (Jobin and Esnouf, 1967; Barton et al., 1967). Factor V has been reported to

interact with Factor Xa, so as to enhance its reactivity toward prothrombin (Colman, 1970), and separately, to bind to prothrombin, presumably rendering it more susceptible to attack by Factor Xa (Esmon, Owen, et al., 1973). Factor V may interact with both of these factors to form a ternary complex on a phospholipid surface, thereby effecting most favorable conditions for rapid proteolysis.

Despite these uncertainties concerning the mode of action of Factor V, there is no doubt that it is an important cofactor in the prothrombinase reaction. Jobin and Esnouf (1967) observed the effect of each component of the prothrombinase complex on the rate of conversion of prothrombin to thrombin. They demonstrated that Factor Xa and separately, prothrombin would bind to a number of different phospholipid emulsions, some active and one inactive in coagulation assays, provided calcium was present. However, Factor V could bind to these same phospholipids in the absence of calcium. When Factor Xa, Factor V, calcium, and/or phospholipid were added in various combinations to prothrombin, significant differences were observed in the rate of thrombin generation. Factor Xa alone slowly converted prothrombin to thrombin. Upon the addition of calcium, the relative rate of thrombin formation doubled and when Factor V was added to this mixture, the relative rate increased 300 times. In a system containing prothrombin, Factor Xa, and phospholipid initially, the

addition of calcium resulted in a 140-fold rate increase and the further addition of Factor V increased the relative rate 1640 times. Although both calcium and phospholipid enhanced the activation of prothrombin by Factor Xa considerably, the contribution of Factor V to this reaction is relatively large.

Esmon, Owen, and Jackson (1974a), using highly purified clotting factors, obtained essentially identical results to those of Jobin and Esnouf (1967). In these studies Factor V was in the form of Factor Va, RVV-V which was purified as described by Esmon (1973). The relative rate of thrombin formation increased 50-fold when phospholipid was added to a mixture of Factor Xa, calcium, and prothrombin, while the separate addition of Factor Va to a mixture of Factor Xa, calcium and phospholipid, produced a 350-fold rate increase. When both phospholipid and Factor Va were added to a mixture of Factor Xa, calcium and prothrombin, a 19,000-fold increase in the rate of thrombin evolution was measured. Once again the critical contribution of Factor Va to a prothrombin activating system was demonstrated.

Plasma Factor V Versus Factor Va in the Prothrombinase Reaction

Many investigators have attempted to distinguish plasma Factor V and Factor Va, in terms of their respective contributions to the rate and extent of conversion of

prothrombin to thrombin by a complete prothrombin converting complex.

Prentice et al. (1967) observed a lag period of 35-45 seconds before thrombin was measurable in a complete prothrombin-converting mixture containing plasma Factor V. However, in an identical mixture but containing Factor Va, thrombin formation occurred immediately after mixing the components. These workers concluded that plasma Factor V was inactive and must be altered by thrombin to become effective in the prothrombinase reaction. When Factor Xa and Factor V were incubated together, in the absence of thrombin, no increase in either their activities was observed so they were confident that the principal activator of Factor V was thrombin.

Kandall, Akinbami, and Colman (1972), using highly purified clotting factors in a two-stage thrombin generating system, indicated that both the rate of formation and yield of thrombin formed from prothrombin increased with increasing concentrations of Factor V. They introduced evidence that the "lag period" reported by Prentice et al. (1967), when plasma Factor V was added initially to the prothrombinase reaction, was an artifact due to linear extrapolation of the thrombin standard curve below 1.0 N.I.H. units/ml thrombin. Using their experimentally derived curve which was non-linear below 1.0 U/ml thrombin, Kandall et al. (1972) saw no time lag in thrombin generation

in the presence of plasma Factor V as compared to activated Factor V. Attempts to quantitate thrombin activity from those non-linear regions of the thrombin calibration curve, probably is not valid. However, a difference in thrombin clotting times of 80 seconds versus 35 seconds is significant, and some qualitative comment about the amount of thrombin activity these times indicate is in order.

Giddings and Bloom (1975), using purified clotting factors from bovine plasma, investigated the effect of thrombin on Factor V and the mechanism by which Factor Va thrombin influences the activation of prothrombin. A complex was prepared of Factor Xa, Factor V, calcium, and phospholipid as demonstrated by chromatography of this mixture on Sephadex G-200. The Factor V and Factor Xa activities co-eluted with phospholipid in the column void volume when the sample was eluted in the presence of calcium. Chromatography in the absence of calcium resulted in co-elution only of Factor V and phospholipid, with the Factor Xa activity eluting later. Kinetic experiments demonstrated that activation of prothrombin to thrombin required all four prothrombinase components. If Factor V was pretreated with trace amounts of thrombin, a steady-state level of thrombin activity was attained one minute after mixing all components, while in the same system but containing unactivated Factor V, 4 minutes lapsed before maximum thrombin activity was measured. Giddings and Bloom (1975) also demonstrated a

difference in the phospholipid binding characteristics of plasma Factor V and Factor Va. A given amount of phospholipid became saturated with increasing concentrations of Factor V, but after conversion of Factor V to Factor Va by thrombin, the same amount of phospholipid became saturated with a lesser amount of Factor Va protein. Consequently activation of Factor V by thrombin resulted in an altered form of Factor V which had an increased affinity for phospholipid. When the concentration of Factor Xa, Factor V was varied, while maintaining the concentrations of the other components constant, both the rate of formation and yield of thrombin correspondingly increased. Varying the concentration of phospholipid in a similar manner also influenced the rate and yield of thrombin generated, but in this instance there appeared to be an optimum reagent concentration. Above this level no further thrombin was formed and there was some inhibition of the reaction.

Rosenberg et al. (1975), using purified Factors V, Xa, and prothrombin from human plasma, also examined the kinetics of thrombin production from prothrombin. They observed that both the rate and extent of conversion of prothrombin to thrombin was a function of the Factor V and separately, the Factor Xa concentrations. The ability of activated human Factor V to participate in this reaction was not explored.

Esmon, Owen, and Jackson (1974b) confirmed the pathway originally set forth by Stenn and Blout (1972) by which prothrombin is converted to thrombin by Factor Xa and by thrombin. Owen, Esmon, and Jackson (1974) further demonstrated that this pathway was unchanged when calcium, phospholipid, and Factor Va were added along with Factor Xa, to prothrombin. This scheme is illustrated in Figure 2. Furthermore, Esmon and Jackson (1974) observed that Intermediate 2, a thrombin precursor lacking a Fragment 2 region, is activated more rapidly by Factor Xa and calcium than either prothrombin or Intermediate I, but, when Factor Va is present, prothrombin or Intermediate I are activated more rapidly than Intermediate II. Addition of Fragment 2, or Fragment 1·2 to Intermediate II, resulted in the rapid activation of Intermediate II by Factor V, Xa, and calcium. Yet, addition of Fragment I to either Intermediate I or to Intermediate 2 plus Fragment 2, had no effect on the ability of Factor V to enhance the generation of thrombin from these prothrombin intermediates. From these experiments they concluded that Factor Va accelerates the conversion of prothrombin to thrombin by interacting with the Fragment 2 region of prothrombin, to facilitate further attack by Factor Xa to ultimately generate thrombin. This would account for continued rapid degradation of prothrombin by Factor Xa when the calcium and lipid binding protein (Fragment 1) is cleaved by thrombin.

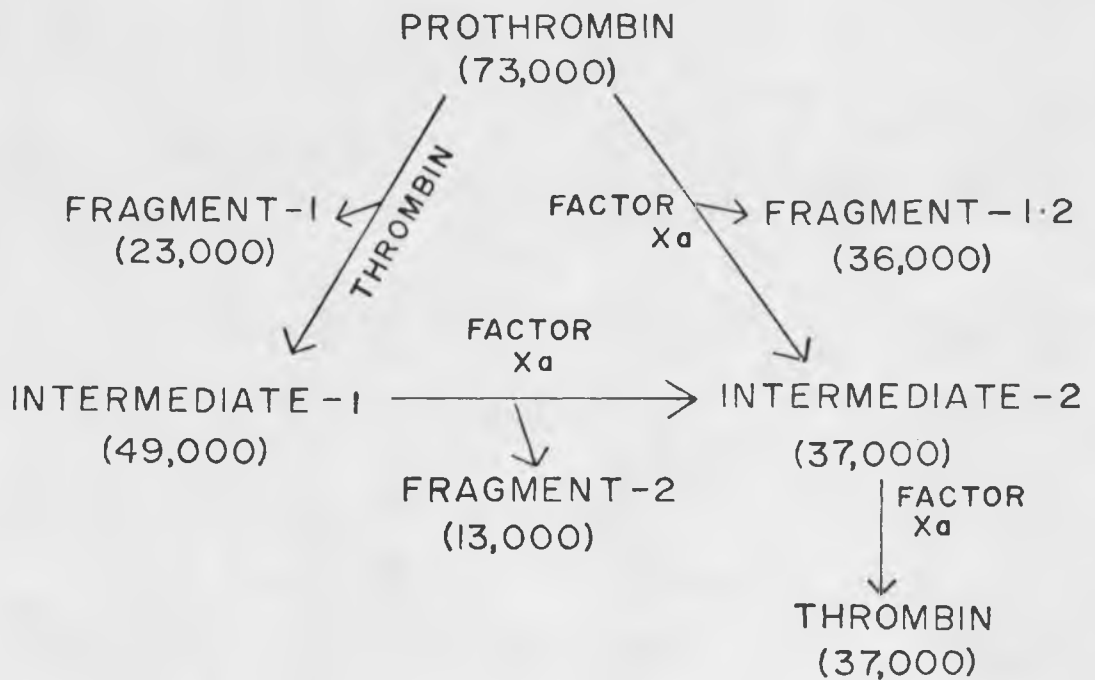


Figure 2. The conversion of prothrombin to thrombin by Factor X_a and thrombin according to Esmon et al. (1974b), as proposed by Stenn and Blout (1972).

Statement of the Problem

From the foregoing literature survey, it is apparent that the isolation of plasma Factor V is a difficult task. This stems from the extreme instability of Factor V, which is reflected in large losses of activity during attempts to isolate it. It is also extremely susceptible to dissociation when subjected to certain techniques of protein fractionation which often involve conditions of high ionic strength and/or removal of calcium from the protein. Dissociation results in the production of multiple forms of Factor V which are of lower molecular weight and higher specific activity than that form of Factor V which exists in plasma, and though these forms are active in the clotting scheme, they cannot be enzymatically converted to Factor Va by thrombin or any of the other enzymes known to activate plasma Factor V.

The objectives of this dissertation then, were primarily to isolate bovine Factor V, in a form that was reasonably stable and typical of that form of Factor V native to plasma so that it could be activated to a significant extent by any one of several enzymes. Secondly, the aim was to determine the extent to which three principal enzymes, thrombin, RVV-V, and Factor Xa, affect the activity and the molecular size of plasma Factor V in converting it to a more active form. Also, it was of interest to determine whether the mode of action of

each of these proteins was enzymatic, since Factor V apparently can be dissociated to a more active form(s) by non-enzymatic means. Finally, it was important to determine the consequence of this activation in terms of the ability of Factor V, or Va, to contribute to the formation of thrombin from prothrombin, by a complex also consisting of Factor Xa, calcium, and phospholipid.

CHAPTER 2

MATERIALS AND METHODS

Materials

QAE cellulose, 0.92 meq/gm, precycled according to the manufacturer's instructions, was a product of Schleicher and Schuell, Inc., Keene, N. H. Sephadex G-25, G-150, G-200, SP-Sephadex, C-50, Sepharose 6B and Dextran Blue 2000 were obtained from Pharmacia Fine Chemicals, Piscataway, N. J.

Sodium lauryl sulfate (SDS), Coomassie Brilliant Blue R, bovine serum albumin, aldolase, γ -globulin, bovine carbonic anhydrase, myoglobin, soybean trypsin inhibitor (Type II-S), heparin (grade I), N- α -p-Tosyl-L-Lysine chloromethyl ketone-HCl (TLCK), L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK), 2-mercaptoethanol, tris (hydroxymethyl) aminomethane, bovine fibrinogen (Cohn Fraction I), immobilized trypsin (polyacrylamide-linked enzyme from bovine pancreas), and phosphorylase-a (from Rabbit Muscle) were all products of Sigma Chemical Co., St. Louis, Mo.

Trypsin (Type III), also from Sigma, was used with no further purification, and was made to 10 mg/ml in Michaelis (Veronal-acetate) buffer, pH 7.35 and stored at 4°C.

α -Chymotrypsin (47 U/mg), three times crystallized from the activation product of the three times crystallized xymogen, was purchased from Worthington Biochemicals, Freehold, N. J. It was used with no further purification and was made to 10 mg/ml in Michaelis buffer, pH 7.35, with storage at 4°C.

Russell's Viper Venom (RVV), lot VRLEF, was purchased from Miami Serpentarium, Miami, Fla. Bovine Topical Thrombin was a Parke, Davis and Co. product (Los Angeles, Calif.). Human thrombin was prepared by Dr. Walter Kisiel of this laboratory by activating human prothrombin with a mixture of human Factor Xa, calcium, phosphatidylserine:phosphatidyl choline (PS:PC) (1:1, w/w), and bovine Factor V. The human thrombin formed was isolated by chromatography on a column of SP-Sephadex, C-50, as described under Methods for the isolation of bovine thrombin. Human thrombin had a specific activity of 180 NIH units/mg, and was homogeneous on SDS electrophoresis under nonreducing conditions.

Apo ferritin was purchased from CalBiochem, LaJolla, Calif. Benzamidine-HCl was an Aldrich Chemical Co. (Milwaukee, Wisc.) product. Barium sulfate was purchased from Matheson, Coleman and Bell, Los Angeles, Calif. Pyronin Y, acrylamide and N, N¹-methylene bisacrylamide, and N, N, N¹, N¹-tetramethylenediamine (TEMED) were obtained from Eastman Chemicals, New York, N. Y. Acrylamide and

bis-acrylamide were recrystallized from chloroform and acetone, respectively, according to Loening (1967). DNP-glycine was obtained from Mann Research Labs, New York, N. Y. Polyethylene Glycol 6000 (Union Carbide Corp.) was recrystallized from acetone-diethyl ether (2:1) according to Albertsson (1962). Dialysis tubing, obtained from Van Waters and Rogers, Los Angeles, Calif., was treated before use according to McPhie (1971). Ortho Coagulation Control plasma was obtained from Ortho Diagnostics, Raritan, N. J.

Phosphatidyl serine and phosphatidyl choline were prepared from bovine brain and hens' eggs, respectively by Dr. M. Gamo (Department of Biochemistry, University of Arizona Medical Center). Diisopropyl fluorophosphate (DFP) and urea were obtained from Swartz-Mann, Van Nuys, Calif. DFP was used as a 1.0 M solution in anhydrous 2-propanol. All other chemicals used were reagent grade and all solutions were made with deionized water.

Factor V deficient plasma was prepared as described by Lewis and Ware (1953). Oxalated human plasma was aged at 37°C until the clotting time in the Factor V assay exceeded 60 seconds. Factor VII and X, and Factors II and VII deficient plasmas were purchased from Sigma Chemical Co., St. Louis, Mo. Factors VII, IX, XII, and XI individual deficient plasmas were obtained from the National Center for Disease Control, Atlanta, Georgia. Normal human pooled plasma was prepared by pooling normal citrated human plasma

from four to five individual donors; it was stored in small aliquots at -20°C .

Human brain thromboplastin was prepared according to Biggs (1972), with a few modifications. An acetone powder of human brain was suspended in 0.15 M NaCl to a concentration of 1.5 g/100 ml. This mixture was warmed between 37°C and 40°C for 20 minutes with constant stirring. The stirring was then stopped to allow the largest particles to settle and the suspension was decanted into glass test tubes in 1.0 to 2.0 ml aliquots and immediately frozen at -20°C . This preparation retained its "thromboplastin" activity for periods of 2-3 weeks.

Cephalin, used in the routine assay of Factor X and Factor II, was prepared as a suspension in Michaelis buffer, pH 7.35 from a chloroform extract of a human brain acetone powder, according to Bell and Alton (1954).

^{125}I as NaI in 0.1 N NaOH was obtained from New England Nuclear, Boston, Mass., and radioactivity was counted using a Nuclear Chicago radiation counter.

Michaelis buffer (Veronal-acetate buffer), pH 7.35 was prepared from sodium acetate trihydrate, 0.0265 M, sodium diethyl barbiturate, 0.0265 M, and NaCl, 0.108 M. Michaelis- CaCl_2 was prepared from Michaelis buffer with the addition of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to 0.025 M. The pH of each of the above solutions was adjusted to 7.35 with 1 N HCl.

Methods

Analytical Methods

Protein Measurement. All protein determinations were made by measuring the absorbance at 280 nm, assuming an $E_{280}^{1\%} = 10.0$ and correcting for Rayleigh light scattering at 320 nm according to Shapiro and Waugh (1966).

Preparation of Desulphated Sepharose 6B. Sepharose 6B was chemically treated to reduce the number of sulfate ester groups, according to the procedure outlined by Porath, Janson, and Laas (1971).

Phospholipid Dispersions. Dispersions of PS:PC (1:1) were prepared in the following manner. Five mg each, of phosphatidylserine (PS) and phosphatidyl choline (PC) in chloroform:methanol (2:1) were taken to dryness under a stream of nitrogen. The residue was re-dissolved in anhydrous ether and again dried under a stream of nitrogen. This latter procedure was repeated twice more to remove all traces of chloroform and methanol. The final residue was taken up in 1.0 ml Michaelis buffer, pH 7.35. This suspension was maintained at 4°C in an ice bath and sonicated at 5 minute intervals for a total of 25-30 minutes at the number 5 setting of a Bronson Model 5110 sonifier equipped with a microtip. The resulting dispersion was then centrifuged at 100,000 x g for one hour

in a Beckman L2-65 ultracentrifuge at 4°C. After centrifugation the solution was kept at 4°C until used in experiments requiring phospholipid. The final phospholipid dispersion was analyzed for total phosphorus by the method of Bartlett (1959). Each preparation contained 9.5-10.2 mg phospholipid per milliliter.

Gel Electrophoresis.

1. Analytical: Analytical polyacrylamide gel electrophoresis was performed according to Davis (1964) in a Buchler Poly-Analyst apparatus. A photopolymerized 2.5% concentrating gel was cast over a chemically polymerized 5% resolving gel. Samples of 20-100 μ l, containing 50-100 μ g protein were electrophoresed for two to three hours at room temperature and subsequently stained and destained according to Fairbanks, Steck, and Wallach (1971).
2. SDS: Electrophoresis in the presence of sodium dodecyl (lauryl) sulfate was carried out according to the procedure of Swank and Munkres (1971), in 7.5% acrylamide gels (1:30, bis acrylamide: acrylamide) containing 0.1% SDS and 8 M urea. Samples containing 0.5-1.0 mg/ml protein were dialyzed at 4°C against 0.02 M Tris phosphate, 0.2% SDS, pH 6.8 for 12-15 hours. The samples were then treated with 5% (v/v) 2-mercaptoethanol, made

8 M in urea and incubated for 1 hour at 37°C. Each sample was made to 20% (w/v) in sucrose and pyronin Y added as a tracking dye. Aliquots of each sample containing 10-15 μ l, were electrophoresed at 25°C at a constant current of 1.5 mA/gel for 13-15 hours. The electrophoresis buffer was 0.1 M Tris phosphate, 0.1% SDS, pH 6.8. Subsequent to electrophoresis the gels were stained and destained according to Fairbanks et al. (1971).

Molecular Weight Determinations.

1. SDS Gel Electrophoresis: The mobility of each protein species on electrophoresis was determined from a densitometry tracing of the gels at 600 nm, made in a Gilford Model 240 spectrophotometer equipped with a Model 2410 linear transport accessory. Corresponding molecular weights were estimated from a linear semi-log plot of molecular weight versus mobility for reduced, standard proteins: bovine serum albumin (68,000), ovalbumin (45,000), bovine carbonic anhydrase (38,000), and myoglobin (17,200).
2. Gel Filtration: All gel filtration experiments were performed according to the procedure of Andrews (1964). The molecular weight of bovine plasma Factor V and of the activity resulting from

thrombin, RVV-V, Factor Xa, or α -chymotrypsin activation, was estimated by gel filtration on a calibrated column of Sephadex G-200, at 4°C using Michaelis-0.025 M CaCl₂, pH 7.35 buffer. A 1.6 x 55 cm column was packed by gravity flow and subsequently maintained at a constant flow rate of 0.18-0.20 ml/min with a Buchler polystaltic pump. The column was calibrated with standard proteins: apoferritin (443,000), γ -globulin (205,000), aldolase (160,000), and bovine serum albumin (68,000). The void volume (V_0), and the internal volume (V_i) were determined with Dextran Blue 2000 and DNP-glycine, respectively. The elution volumes of standards and samples were determined by weighing the eluate obtained from the time of application to the column to the maxima of the protein or activity peak. A calibration curve was constructed by plotting the log of the molecular weight of each standard protein versus the K_D value where $K_D = V_e - V_0/V_i$, and $V_i = V_e$ (DNP-glycine) - V_0 . The apparent molecular weights of the samples were determined by interpolation from the calibration curve.

Analytical Ultracentrifugation.

Examination of bovine Factor V by analytical ultracentrifugation was performed in a Beckman Model E Ultracentrifuge. Sedimentation velocity experiments were carried out at 4°C at a rotor speed of 60,000 RPM using a double sector cell with sapphire windows.

Samples of bovine Factor V were concentrated by ultrafiltration (PM-10 membrane) to 5 mg/ml and dialyzed 12-15 hours against 0.20 M Tris acetate, 50 mM CaCl₂, 10% glycerol, pH 7.5, or against 10⁻³ M Tris acetate, 50 mM CaCl₂, pH 7.5. A single concentration of Factor V was analyzed by this technique, but under several different solvent conditions.

After the Factor V sample(s) had been accelerated to maximum speed, the sample cell was photographed at 10 minute intervals up to 50-60 minutes. The radial distance from the center of rotation to the apex of the boundary pattern at each time interval was measured, and a plot was made of the log of this function versus time, in minutes. The slope of this line was used to calculate the observed sedimentation constant, s_{obs} , according to Equation (1).

$$s_{obs} = \frac{1}{\omega^2 r} \frac{dr}{dt} = \frac{2.303}{60\omega^2} \left(\frac{d \log x}{dt} \right) \quad (1)$$

where ω = angular velocity in radians/second, and $\left(\frac{d \log x}{dt} \right)$ = slope of the line described above.

Assay Methods

Factor V activity was assayed according to the procedure of Kappeler (1955). For each assay, 0.1 ml of Factor V deficient plasma, and 0.1 ml human brain thromboplastin, were preincubated for 30 seconds at 37°C, followed by the addition of 0.1 ml of test sample, diluted in Michaelis buffer, pH 7.35, and finally, 0.1 ml of 0.025 M CaCl_2 . Samples containing calcium were assayed by adding 0.1 ml deionized water to the Factor V deficient plasma-thromboplastin mixture, and the assay initiated by the addition of 0.1 ml of the Factor V sample diluted in Michaelis-0.025 M CaCl_2 , pH 7.35. The assay was standardized with normal human pooled plasma and test solutions were diluted so that clotting times were in the 20-30 second range of the calibration curve. One unit of Factor V activity was considered as the amount present in 1 ml of normal human plasma. Specific activity was defined as units of Factor V per unit of absorbance at 280 nm, after correcting for light-scattering at 320 nm.

Factor Xa was assayed by the procedure of Bachmann, Duckert, and Koller (1958), but with the omission of Russell's Viper Venom (RVV). One unit of Factor Xa activity was defined as that amount present in 1 ml of normal pooled human plasma fully activated by Russell's Viper Venom.

Factor II (prothrombin) was assayed by the one-stage method of Hjort, Rapaport, and Owren (1955). One Ortho unit

of Factor II was defined as the amount of Factor II activity in 1.0 ml of Ortho Coagulation Control plasma, at 37°C.

Factors XII, XI, IX, and VIII were assayed by the procedure of Simone, Vanderheiden, and Abildgaard (1967). Factor VII was assayed according to the method of Nemerson and Clyne (1974). Pooled human plasmas, congenitally deficient in each of the above coagulation factors were used in the assay of each factor. Normal human pooled plasma was used to standardize the assay in each case.

Fibrinogen was detected according to the procedure of Ratnoff and Menzie (1951).

Thrombin activity was assayed by its ability to clot fibrinogen according to a modification of the procedure of Shapiro and Waugh (1966). For this assay, 0.2 ml of fibrinogen, 5 mg/ml in 0.15 M NaCl was pipetted into the reaction cup of an automatic clot timer (nechrolab, Model 202-A or BBL Fibrometer), and prewarmed for 30 seconds at 37°C. Then, 0.1 ml of sample was pipetted into the cup and the clot timer initiated simultaneously. Thrombin clotting times were compared to a log-log plot of clotting times versus thrombin concentration in NIH units/ml, prepared from dilutions of U. S. Standard Thrombin, NIH Lot B-3, 21.7 U/mg.

The fibrinogen, used in the thrombin assay, was purified according to Laki (1951) from Cohn Fraction I, Type I, obtained from Sigma Chemical Co. The purified

fibrinogen, dissolved in 0.3 M KCl, contained 97% clottable protein and was frozen at -20°C in 2-3 ml aliquots at a concentration of 10 mg/ml.

RVV-V activity was assayed according to a modification of the procedure of Schiffman et al. (1969). The RVV-V containing sample was incubated with a Factor V solution for 10 minutes at 25°C . A control, contained buffer in place of the RVV-V sample. Each sample was then assayed for Factor V activity and the RVV-V activity calculated as the Factor V activity in excess of that found in the control. A unit of RVV-V activity was defined as the number of Factor V units which a RVV-V preparation could generate under these conditions.

Preparative Methods

1. Preparation of Bovine Plasma Factor V: In a typical isolation scheme, 21 liters of bovine blood were obtained from a local meat packing house. The blood was collected in 7-liter polyethylene buckets, each containing 1/10 volume of 0.1 M K osalate plus benzamidine-HCl, heparin, and soybean trypsin inhibitor, each at 10 mg/liter blood. After collection, the contents of each bucket were thoroughly mixed by gentle inversion. Within one hour of collection the plasma was separated out by centrifugation at $2300 \times g$ in a Lourdes Clinifuge

for 30 minutes at 4°C. The supernatant was siphoned off and centrifuged for 15 minutes at 4°C in a Sorvall RC-3 centrifuge, using an HG-4 rotor. The final plasma was collected and mixed with BaSO₄, 100 g/liter, for 30 minutes at room temperature with constant stirring. The adsorbed plasma was separated out by centrifugation at 2300 x g for 15 minutes at 4°C. The adsorbed plasma was diluted 1:1 with deionized water and the pH adjusted to 7.0 with 1 N HCl. QAE cellulose (QAEC) was added batchwise in an amount of 9 g per liter original plasma. This mixture was stirred for 20 minutes at room temperature, then the stirring was stopped to allow the QAEC to settle. The supernatant was siphoned off and the QAEC collected by centrifugation at 2300 x g for 15 minutes at 4°C. The cellulose pellet was washed with 0.055 M calcium acetate, pH 7.25, until the absorbance at 280 nm of the wash read 0.10 or less, which usually required 16-18 liters of wash buffer. The Factor V activity was eluted from the QAEC with 0.11 M calcium acetate, pH 7.25. The QAEC cake was suspended in 1/10 volume of the eluting buffer, relative to the original plasma, and stirred intermittently for 10 minutes at room temperature. The final extract was collected on a Buchner funnel (Whatman #4 filter

paper), and the QAEC was washed with a small volume of 0.11 M calcium acetate, pH 7.25 and sucked to dryness. The extract was centrifuged at 5500 x g for 10 minutes at 4°C to remove small amounts of barium sulfate which were carried through the extraction procedure. Protease inhibitors benzamidine-HCl, and soybean trypsin inhibitor, were added to the final extract to a final concentration of 10^{-3} M and 50 mg/liter, respectively. QAEC Factor V was stored at 4°C at a protein concentration of 1.0 to 1.5 mg/ml. For further purification the QAEC Factor V was precipitated with polyethylene glycol 6000 (PEG) and subsequently chromatographed on a column of desulphated Sepharose 6B. Three hundred ml of QAEC factor V were titrated with 1 N acetic acid to pH 6.0 and a 50% (w/v) solution of PEG in deionized water was added dropwise with continuous stirring at 4°C, to a final concentration of 12% in PEG. Addition of PEG results in the formation of a gelatinous precipitate in the QAEC extract. This mixture was allowed to stand undisturbed for 10 minutes at 4°C, then the precipitate was collected by centrifugation at 4000 x g for 15 minutes at 4°C in a Sorvall RC-3 centrifuge. The supernatant was discarded and the pellet was dissolved in 0.20 M Tris acetate, pH 7.5, 50 mM

CaCl₂, 10% glycerol, containing 10⁻³ M, benzamidine-HCl and ε-NH₂ caproic acid, a serine protease and plasmin inhibitor, respectively. The sample was dialyzed at 4°C for at least five hours against the same buffer and applied to a column of desulphated Sepharose 6B, 5 x 90 cm, equilibrated with 0.20 M Tris acetate, 50 mM CaCl₂, 10% glycerol, pH 7.5. The sample was eluted at a flow rate of 0.67 ml/min and fractions of 9-10 ml per tube were collected. The effluent was assayed for Factor V activity and the most active fractions were pooled and stored at 4°C, at a protein concentration of 0.3-0.4 mg/ml. A flow diagram of this preparative procedure is shown in Figure 3.

2. Preparation of Activated Factor V (Factor Va):

Factor Va was prepared by activating Factor V with RVV-V, thrombin or Factor Xa and subsequently isolating the activated Factor V by chromatography on Sephadex G-200. From 10-15 mg (700-1000 total units) of Factor V, prepared as described above, were treated with 15 µg purified bovine thrombin, 55-65 µg purified RVV-V, or 80-90 µg bovine Factor Xa at pH 7.5 for 30-40 minutes and at room temperature. The sample was then concentrated by ultrafiltration (PM-10 membrane) to 1.0 ml and applied to a column of Sephadex G-200, 1.6 x 55 cm at 4°C,

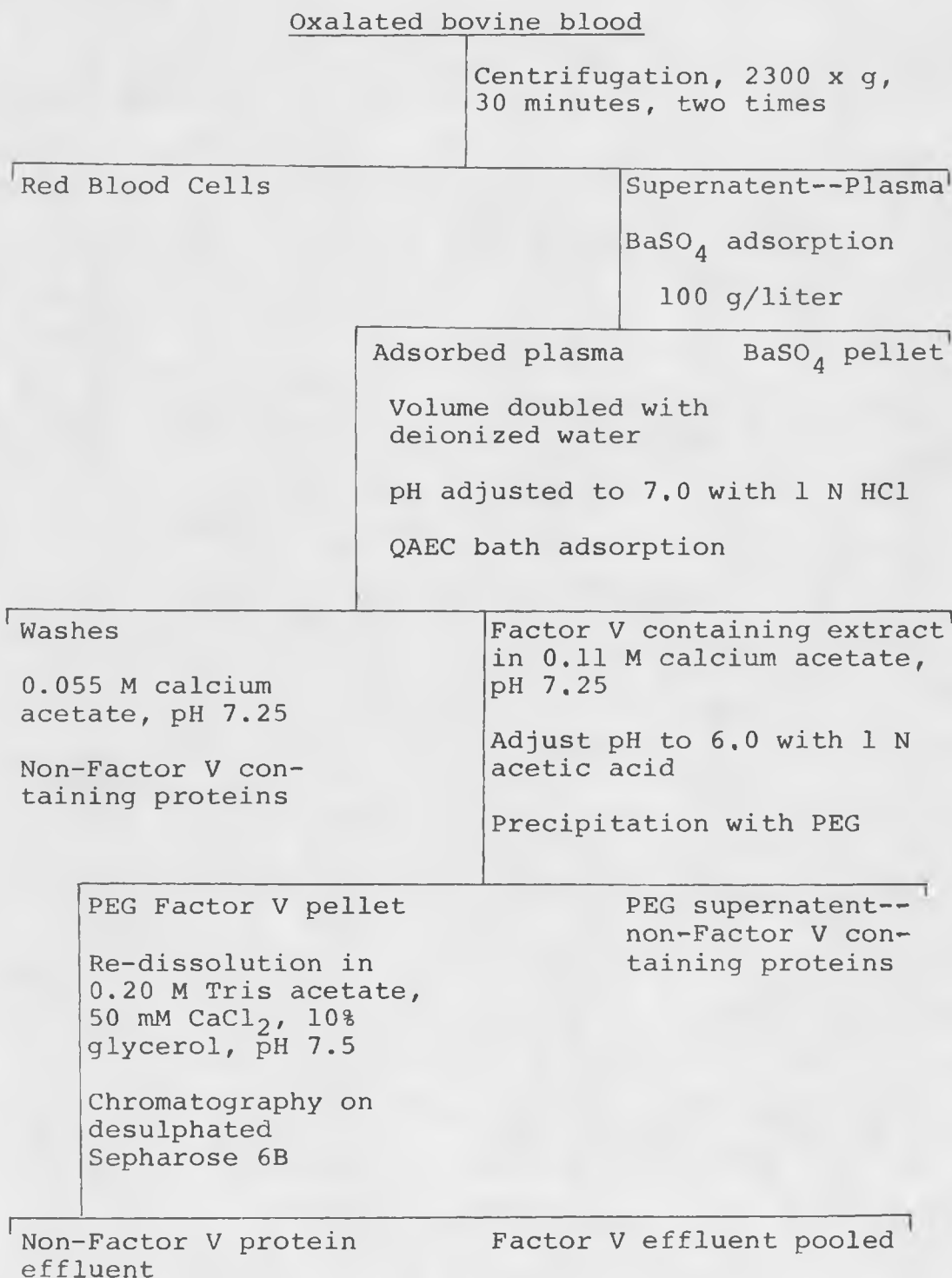


Figure 3. Flow diagram for the preparation of bovine plasma Factor V.

equilibrated with Michaelis-0.025 M CaCl_2 , pH 7.35. The sample was eluted with the same buffer and 1.8-2.0 ml fractions were collected. The effluent was assayed for Factor V activity and for protein. The Factor V activity eluting in a volume corresponding to a molecular weight of 200,000 daltons, when referred to a standard curve, was pooled and concentrated by ultrafiltration (PM-10 membrane) to a protein concentration of 0.2 to 0.4 mg/ml. Factor Va was stored at 4°C for immediate use or made to 50% (v/v) in glycerol and stored at -20°C.

3. Preparation of Bovine Factor II (Prothrombin) and Factor Xa: Bovine Factor II was purified by the procedure developed for the isolation of human prothrombin by Kisiel and Hanahan (1973). This method consisted of barium citrate adsorption, DEAE cellulose chromatography and preparative disc gel electrophoresis. The Factor II preparations had a specific activity of 4 Ortho units/mg and appeared homogeneous when examined by analytical disc electrophoresis.

Bovine Factor Xa was prepared in a similar manner. Factor Xa was generated from DEAE cellulose purified Factor X by the action of RVV-X, the Factor X activator from Russell's Viper Venom, or by immobilized trypsin, and subsequently purified

by preparative disc electrophoresis (Kisiel and Hanahan, 1973). Factor Xa was homogeneous by analytical disc and sodium dodecyl sulfate gel electrophoresis, and the specific activity of repeated preparations ranged from 600-1000 U/mg.

4. Preparation of RVV-V: RVV-V was prepared by a modification of the method of Esmon, Jackson, and Lowery (1973). Five hundred mg crude Russel's Viper Venom (RVV) were dissolved in 2-3 ml of 0.05 M sodium acetate, 0.5 M NaCl, pH 5.0. The sample was centrifuged at 30,000 x g for 15 minutes at 4°C and the supernatant applied to a column of Sephadex G-150, 2.6 x 80 cm, at a flow rate of 0.3 to 0.4 ml/min. Fractions of 4 ml were collected. The effluent was assayed for RVV-V activity and the most active fractions were pooled, concentrated by ultrafiltration (UM-2 membrane) to a volume of 8-10 ml, and dialyzed 4-5 hours against 0.05 M sodium acetate, 0.5 M NaCl, pH 5.0. The retentate was charged on a column of sulfoyl propyl (SP) Sephadex, C-50, 1.6 x 40 cm. The column was washed with 75-100 ml of equilibrating buffer, and then a linear gradient was applied, consisting of 100 ml each of 0.05 M sodium acetate, 0.5 M NaCl, pH 5.0, and 0.05 M sodium acetate, 1.0 M NaCl, pH 5.0. RVV-V eluted in a broad peak of activity starting

at 0.7 M in NaCl, and the most active fractions were pooled, concentrated by ultrafiltration (UM-2 membrane) to 1.0 mg/ml, and dialyzed against 0.1 M Tris-HCl, pH 7.5. The RVV-V concentrate was then portioned in 0.5-1.0 ml aliquots which were stored frozen at -20°C . When examined by SDS electrophoresis under reducing conditions, the RVV-V preparation contained a single band which had a mobility corresponding to a molecular weight of 26-28,000 daltons.

5. Iodination of RVV-V: RVV-V was iodinated according to the procedure of Greenwood and Hunter (1963), with a few modifications. Purified RVV-V, in 0.1 M Tris-HCl, pH 7.5, at a concentration of 6 mg/ml, was treated with 100 μl of Na^{125}I in 0.02 N NaOH (0.4 mCi) followed by 20 μl of chloramine T (2 mg/ml). This mixture was shaken intermittently for one minute at room temperature, then 20 μl of disodium sulfite, 5 mg/ml were added followed by 20 μl KI, 20 mg/ml. The complete mixture was allowed to stand for 2-3 minutes at room temperature and then chromatographed on a column of Sephadex G-25 (Coarse), 0.9 x 20 cm, at 4°C , to separate the ^{125}I labeled RVV-V from the reaction mixture. The sample was eluted with 0.1 M Tris-HCl, pH 7.5, at a flow rate of 0.5 ml/min and 0.5 ml fractions were

collected. Five microliters of selected tubes across the chromatogram were counted for one minute each in a Nuclear Chicago Scintillation Counter. A peak of radioactivity at the column void volume was taken as the ^{125}I -labeled RVV-V and was pooled. The pool was concentrated 3-fold by placing it in a dialysis bag surrounded by solid sucrose for 20 minutes at room temperature. The concentrate was dialyzed against 0.1 M Tris-HCl, pH 7.5 to remove the sucrose and stored frozen at -20°C .

6. Purification of Bovine Thrombin: Bovine thrombin (Factor IIa) was purified according to a modification of the procedure of Lundblad (1971). Two vials of Parke-Davis bovine thrombin, containing 10,000 NIH units each, were reconstituted in 10 ml of 0.1 M sodium phosphate, pH 6.5, containing ϵ -amino caproic acid to 10^{-3} M, to prevent any degradation of thrombin by plasmin. The sample was dialyzed 12-15 hours against the same buffer and then centrifuged for 15 minutes at 4°C and $30,000 \times g$ to clarify the sample. The supernatant was charged on a column of SP-Sephadex, C-50, 2.6×20 cm, equilibrated with 0.1 M sodium phosphate, pH 6.5, at a flow rate of 0.5 ml/min, and 4 ml fractions were collected. The column was washed with 20-50 ml

equilibrating buffer, then the buffer was changed to 0.25 M sodium phosphate, pH 6.5 to elute the thrombin activity. The column effluent was assayed for thrombin clotting activity and protein and the most active thrombin fractions were pooled, concentrated by ultrafiltration (UM-2 membrane) and stored at -20°C in the buffer of isolation at a protein concentration of 0.3 mg/ml, assuming an $E_{280}^{1\%} = 19.5$. The purified thrombin had a specific activity of 2400 U/mg, and on SDS gel electrophoresis this preparation appeared to contain both α and β forms of thrombin with the α form predominate.

Activation of Bovine Factor V

Changes in the activity of Factor V, or formation of activated Factor V, were measured by assaying the activity of Factor V after treatment with several different reagents under the following conditions: From 1-5 units of Factor V, in 0.20 M Tris acetate, pH 7.5, 50 mM CaCl_2 , 10% glycerol were treated with one of the reagents listed below in the presence of 5 mM CaCl_2 at pH 7.5 and 25°C . In each case the final volume of the activation mixture was diluted to 1.0 ml with 0.05 M Tris acetate, pH 7.5, 0.10 M NaCl, bovine serum albumin, 1 mg/ml. The addition of the activator was taken as zero time thereafter which aliquots

were removed at specific time intervals to assay for Factor V activity.

When Factor V was treated with a particular reagent the following additional conditions were met.

Thrombin. Factor V was incubated with 0.36-1.4 μg of bovine thrombin or with 10-15 μg of human thrombin as described above.

RVV-V. From 2.5-10 μg of RVV-V were added to Factor V under the conditions described above.

Factor Xa. Factor V was treated with 12 μg (7.2 Ortho units) Factor Xa, or with 3-4 μg (2.4 Ortho units) Factor Xa plus 0.05 μg PS:PC (1:1, w/w).

α -Chymotrypsin, Trypsin, or Pronase. Factor V was mixed with 3.0-6.2 μg of α -chymotrypsin, or with 0.1-1.0 μg of trypsin, or with 0.1-10 μg pronase according to the conditions described above.

Thrombin Generation Experiments

The formation of thrombin was accomplished by treating bovine prothrombin with various combinations of Factor Xa, Factor V or Factor Va, calcium, and PS:PC (1:1, w/w). A prothrombin activator was prepared by mixing one or more of these reagents at 25°C as follows: Factor V or Va, 0.25 $\mu\text{g}/\text{ml}$ (0.32 units/ml), 0.92-1.84 $\mu\text{g}/\text{ml}$ Factor Xa,

25 $\mu\text{g/ml}$ PS:PC (1:1 2/2) and 10 mM CaCl_2 . To this mixture was immediately added 123 $\mu\text{g/ml}$ (7.4 Ortho units/ml) prothrombin, and the final volume was brought to 1.0 ml with 0.01 M Tris acetate, 0.08 M NaCl, pH 7.5. The addition of prothrombin was taken as zero time thereafter which aliquots were removed for estimation of thrombin activity at specific time intervals up to 30-40 minutes. Samples were diluted for assay in 0.01 M Tris acetate, 0.08 M NaCl, pH 7.5.

Factor Va was prepared by treating plasma Factor V with thrombin, RVV-V or Factor Xa. For some experiments Factor Va was isolated from the activation mixture by Sephadex G-200 gel chromatography as described in a previous section.

CHAPTER 3

RESULTS

Isolation of Bovine Plasma Factor V

The yield, fold purification, and the extent to which Factor V could be activated by RVV-V (activatability) at each step of the isolation procedure are given in Table 2.

During a routine preparation, bovine Factor V was isolated from 14-18 liters bovine blood collected in a 1/7th volume of 0.1 M K oxalate as an anticoagulant, and protease inhibitors, benzamidine-HCl, heparin, and soybean trypsin inhibitor, to prevent any degradation of Factor V early in the work-up.

After the plasma was separated from the cellular components of the blood, it was adsorbed with barium sulfate to remove Factors II, VII, IX, and X, in order to reduce the chances of degradation of Factor V by either Factor Xa, or thrombin, which could be generated from Factor X or prothrombin, respectively, during the isolation. All of the plasma Factor V activity was recovered in the adsorbed plasma.

The adsorbed plasma was deluted 2-fold with deionized water to reduce the protein concentration, and

Table 2. Purification scheme for bovine plasma Factor V.

Fraction	Volume (ml)	Units/ml	Total activity (units)	Specific activity	% recovery	Purif. (fold)	Activatability (fold increase in activity)
plasma	11,205	1.82	20,393	0.0284	100	1.0	20
BaSO ₄ adsorbed plasma	11,000	1.66	18,260	0.0295	90	1.04	--
QAEC extract	1,200	11.7	14,040	7.77	69	274	10
PEG precipitate	42	270	11,340	13.4	56	472	10
Desulphated Sepharose 6B	432	18.2	7,862	66.7	39	2349	10

the pH titrated from approximately 7.7 to 7.0 with 1N HCl, in order to facilitate batchwise adsorption of Factor V to QAE cellulose. Washing of the QAEC with 0.055 M calcium acetate, pH 7.25, served to remove all of the pigmented materials adsorbed to it. However, washing until the A_{280} reading of the wash buffer was nearly zero could lead to losses of 5-10% of the Factor V activity initially adsorbed. Elution of the QAEC with 0.11 M calcium acetate, pH 7.25 recovered 61% of the plasma Factor V activity. The QAEC extract was treated with benzamidine-HCl, and soybean trypsin inhibitor, again to prevent any proteolytic degradation of Factor V. This was important since only part of the QAEC extract was generally worked up immediately through the entire isolation procedure, the remainder being stored at 4°C, up to one month at a protein concentration of 1.0 to 2.0 mg/ml. This preparation was not frozen since losses of 50% or greater resulted. When an aliquot of QAEC Factor V, containing 20-30 units, was chromatographed on a column of Sephadex G-200 in the presence of Michaelis-0.025 M $CaCl_2$, pH 7.35 buffer, the Factor V activity eluted in a single peak close to the column void volume, to give an apparent molecular weight of 439,000 daltons. Also, QAEC Factor V could be activated 10-14-fold by RVV-V.

In order to reduce the volume of the QAEC extract for subsequent gel filtration on a column of desulphated Sepharose 6B, it was precipitated with polyethylene glycol

6000. This provided a rapid means of concentrating QAEC Factor V and achieved a 2-fold purification with a recovery of 56% of the Factor V activity of the starting plasma.

The PEG precipitate was dissolved in 0.20 M Tris acetate, 50 mM CaCl_2 , 10% glycerol, pH 7.5, containing proteolytic inhibitors benzamidine-HCl and ϵ - NH_2 -caproic acid, to eliminate possible proteolytic degradation of Factor V during subsequent dialysis for 4-5 hours prior to gel filtration.

Chromatography of PEG Factor V on a column of desulphated Sepharose 6B resolved this preparation into three protein peaks, as shown in Figure 4. The first peak, eluting at the column void volume, appeared opaque and probably contained a concentration of low and very low density lipoproteins. The second, a relatively smaller and very broad peak, contained the Factor V activity. This was followed by a large protein peak which contained 50% or more of the total protein applied to the column. The Factor V activity was recovered in a yield of 30-40% with a specific activity of 50-70 U/mg, representing a 1000-2000-fold purification overall.

The preparative Sepharose 6B column was eluted with 0.20 M Tris acetate, 50 mM CaCl_2 , 10% glycerol, pH 7.5. A moderately high ionic strength was chosen in order to minimize any ionic interactions between the sample and the Sepharose matrix. Calcium was included here as well as at

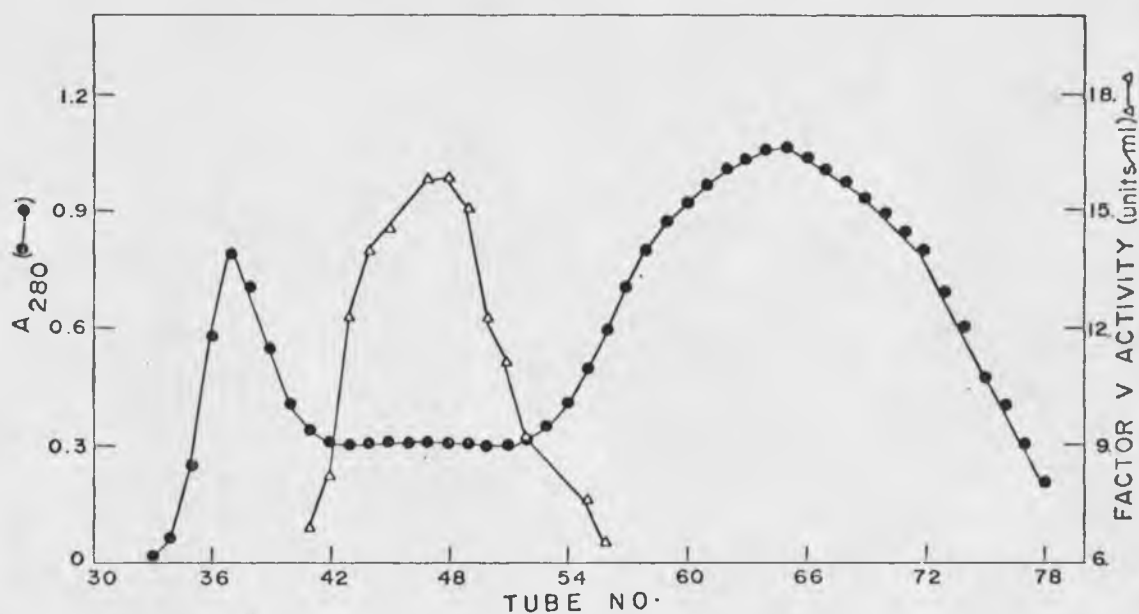


Figure 4. Elution profile of PEG-Factor V on desulphated Sepharose 6B -- Five hundred mg of QAEC Factor V were precipitated with PEG 6000, redissolved in 10 ml of 0.20 M Tris acetate, 50 mM CaCl_2 , 10% glycerol, pH 7.5, and applied to a 5 x 90 cm column of desulphated Sepharose 6B. The column was eluted with the same buffer at a flow rate of 0.7 ml/min and 10.0 ml fractions were collected and analyzed for Factor V activity (Δ — Δ), and protein (\bullet — \bullet).

previous steps of the isolation scheme because of its stabilizing effect on Factor V (Blombäck and Blombäck, 1963; Greenquist, Dolan, and Colman, 1972), as was glycerol because of its stabilizing effect on oligomeric proteins (Bradbury and Jakoby, 1972; Shifrin and Parrott, 1975).

This isolation procedure proved to be highly reproducible and resulted in an extremely stable preparation of bovine Factor V. The Factor V activity from the desulphated Sepharose 6B column was pooled and stored at 4°C for 2-3 weeks at a protein concentration of 0.3-0.4 mg/ml. When stored at -20°C in the buffer of isolation containing glycerol to 50% (v/v), this preparation was stable for several months. However, Factor V lost 50% or more of its activity when frozen at -20°C and thawed after one day.

Physical Characteristics of Bovine Factor V

In order to determine the effects of several proteolytic enzymes on the activity and molecular size of bovine plasma Factor V, it was necessary to prepare Factor V in high yields and in a stable form, while preserving the molecular integrity of the protein. The procedure developed in this study fulfills these requirements. Since this preparation differs from other published preparative schemes, it was necessary to partially characterize the Factor V prepared by this method.

Analyses of Factor V for Other Clotting Activities

The Factor V preparation was examined for the presence of Factors XII, XI, IX, VIII, VII, X, Xa, II and fibrinogen, by classical clotting assays as described in Methods. Factor V was free of other clotting factors except for trace amounts of Factor VIII. Approximately 0.03 units/ml Factor VIII activity were measured which is 3% of that level present in plasma, namely 1.0 unit/ml.

Estimation of the Uniformity of Composition of Factor V

Factor V was assessed for chromatographic homogeneity by rechromatography on an analytical column of desulphated Sepharose 6B. An aliquot of the activity pooled from a preparative column of desulphated Sepharose 6B was chromatographed on an analytical column of the same gel filtration medium. As seen in Figure 5, the protein and Factor V activity profiles were not coincident, and the specific activity (S.A.) across the peak was not constant, indicating that Factor V was not chromatographically homogeneous.

Analytical disc gel electrophoresis of Factor V revealed 6 major staining protein bands. To localize the Factor V activity, two identical Factor V samples were electrophoresed simultaneously; one was stained for protein while the companion gel was sliced in 2-3 mm sections which

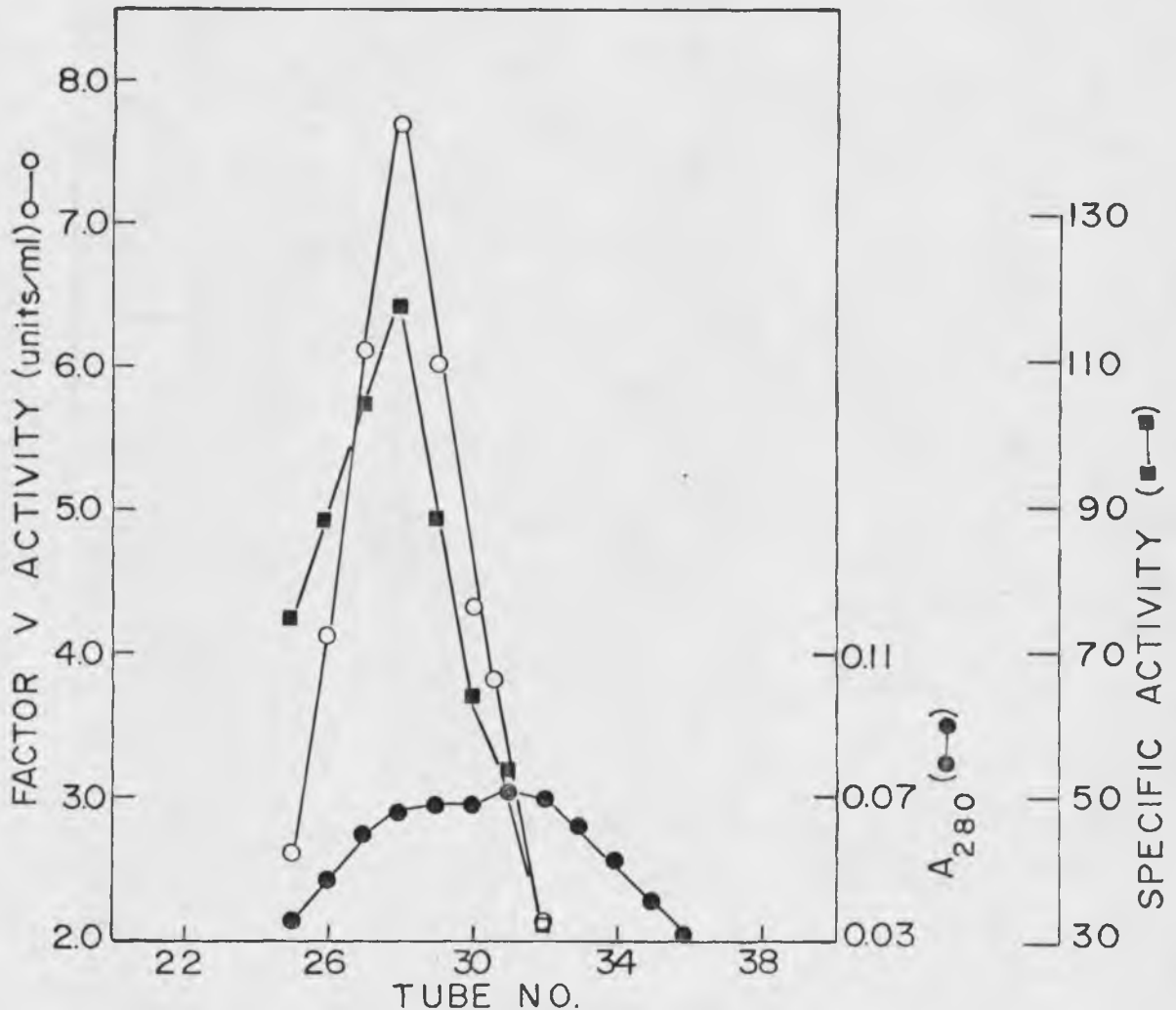


Figure 5. Rechromatography of Factor V on desulphated Sepharose 6B -- Five mg of Factor V were concentrated by ultrafiltration (PM-10 membrane) to 1.0 ml and applied to a 1.6 x 55 cm column of desulphated Sepharose 6B at 4°C at a flow rate of 0.17 ml/min. The column was eluted with 0.20 M Tris acetate, pH 7.5, 50 mM CaCl₂, 10% glycerol and 2.0 ml fractions were collected and analyzed for Factor V activity (o—o) and for protein (●—●).

were individually suspended in 0.4-0.5 ml Michaelis-0.025 M CaCl_2 , pH 7.35 and gently macerated to facilitate diffusion of protein out of the gel. The slices were left to stand at 4°C for 4-8 hours and then assayed for activity. Factor V was located in a moderately staining band near the top of the resolving gel and no activity was detected in the concentrating gel, when the position of the localized activity was referred to the duplicate gel stained for protein (see Figure 6).

The protein banding pattern of Factor V was unchanged when the preparation was subjected to varying conditions of ionic strength prior to analytical disc electrophoresis. Samples of Factor V were dialyzed 12-15 hours at 4°C against 0.01 M Tris acetate, pH 7.5, containing NaCl from 0.0 to 0.14 M. After dialysis each was electrophoresed and subsequently stained for protein with Coomassie Blue. The distribution of stainable protein bands was essentially the same for each sample. In a separate experiment, one sample of Factor V was dialyzed against 0.20 M Tris acetate, 50 mM CaCl_2 , 10% glycerol, pH 7.5 (buffer of isolation), and a second one against the same buffer minus calcium, followed by analytical disc electrophoresis. Again the protein banding pattern was the same in each case. Although calcium is required for the stabilization of Factor V activity, reduction of its concentration by dialysis prior



Figure 6. Analytical disc gel electrophoresis of Factor V -- Fifty μg of Factor V were electrophoresed on the combination of a 2.5% concentrating and a 5% resolving polyacrylamide gel. After electrophoresis the gel was stained with Coomassie Blue. Protein migration is from the top (cathode) to the bottom (anode).

to electrophoresis did not have any perceivable effect on the protein band distribution of the Factor V preparation.

SDS electrophoresis of Factor V under nonreducing conditions revealed 5 major protein bands, with a concentration of stainable material at the very top of the gel indicating several proteins too large to enter the 7.5% acrylamide matrix. Major bands were apparent at 120,000, 86,000, 80,000, 57,500, and 50,000 daltons. Electrophoresis of Factor V treated with 2-mercaptoethanol to reduce disulfide bonds revealed 13-14 stainable protein bands, with major bands at 120,000, 93,500, 80,500, 75,000, and 51,200 daltons. Both gels are pictured in Figure 7.

To further estimate the heterogeneity of the Factor V preparation, sedimentation velocity experiments were conducted under conditions similar to those under which Factor V was isolated. Sedimentation of Factor V in 0.20 M Tris acetate, pH 7.5, 50 mM CaCl_2 , 10% glycerol, saw a sharp peak form, as maximum speed was attained, which spread slowly over the next 50 minutes running time. When the concentration of Tris was reduced to 10^{-3} M and glycerol omitted from the sample buffer, sedimentation of Factor V again saw a single sharp peak when maximum speed was attained, which began to spread over the next 40-50 minutes. A smaller second peak appeared at 40 minutes running time, which sedimented slower than the first.

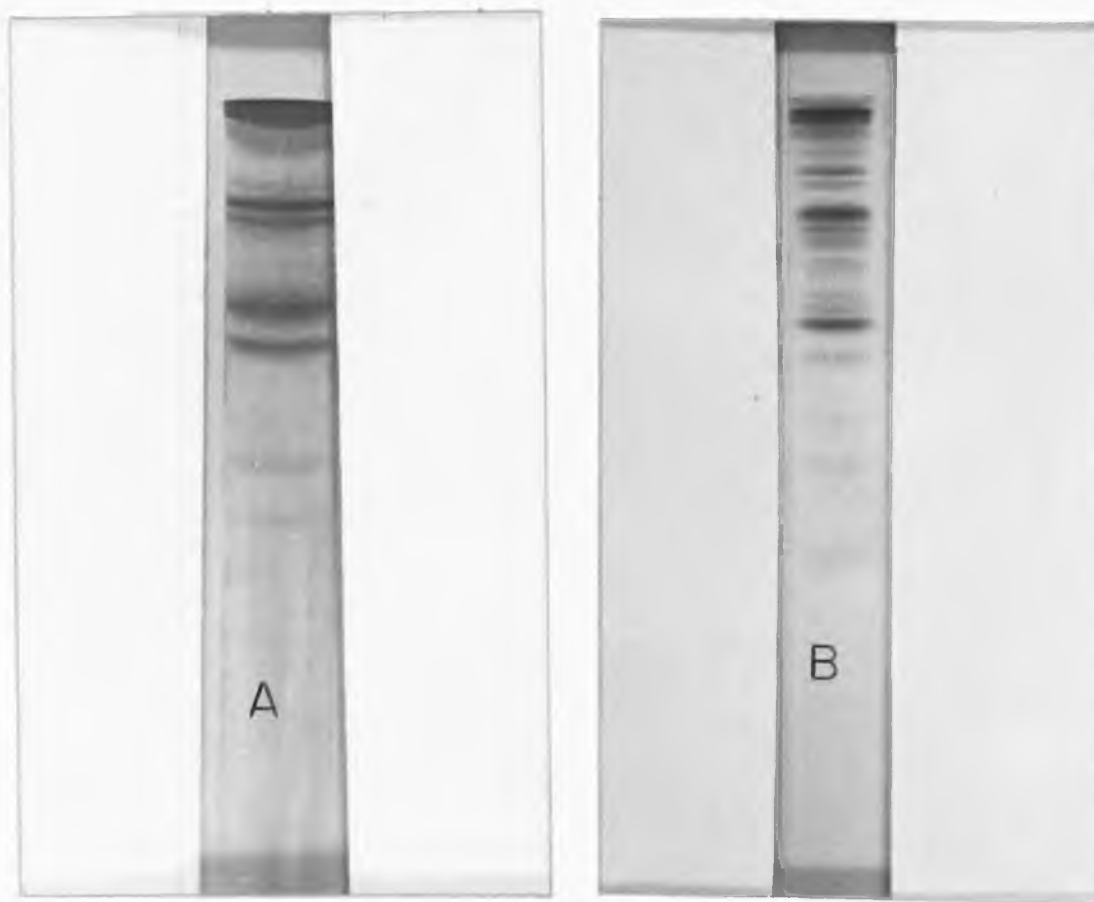


Figure 7. SDS acrylamide gel electrophoresis of bovine Factor V -- Fifty μg of Factor V, in 2% SDS and 8 M urea were electrophoresed under (A) non-reducing conditions and (B) reducing conditions. The gels were stained with Coomassie Blue following electrophoresis. Migration of the bands is from the top (cathode) to the bottom (anode).

From velocity sedimentation analysis of Factor V an observed sedimentation coefficient of 80 was calculated, as described in Methods. This value is quite high indicating a rather large protein or protein aggregate. The observation that the peak spread rapidly after maximum speed was reached could be interpreted as indicating a polydisperse or heterogeneous preparation. On the basis of these observations, a more rigorous analysis of Factor V by this technique was not justified.

Molecular Weight Estimations of Factor V

The molecular weight of Factor V was determined on a calibrated column of Sephadex G-200. Desulphated Sepharose 6B Factor V were concentrated by ultrafiltration (PM-10 membrane) to 1.0 ml and applied to a column of Sephadex G-200. The Factor V eluted as a single activity peak in a volume corresponding to a $K_D = 0.03$, as shown in Figure 8. When interpolated on a calibration curve, prepared as described in Methods, this value corresponded to an apparent molecular weight of 439,000 daltons, similar to QAEC Factor V.

Because Factor V eluted from Sephadex G-200 close to the column void volume, the molecular weight of Factor V was also estimated on a calibrated column of desulphated Sepharose 6B. Eighty units of Factor V were concentrated by ultrafiltration (PM-10 membrane) to 1.0 ml, and applied

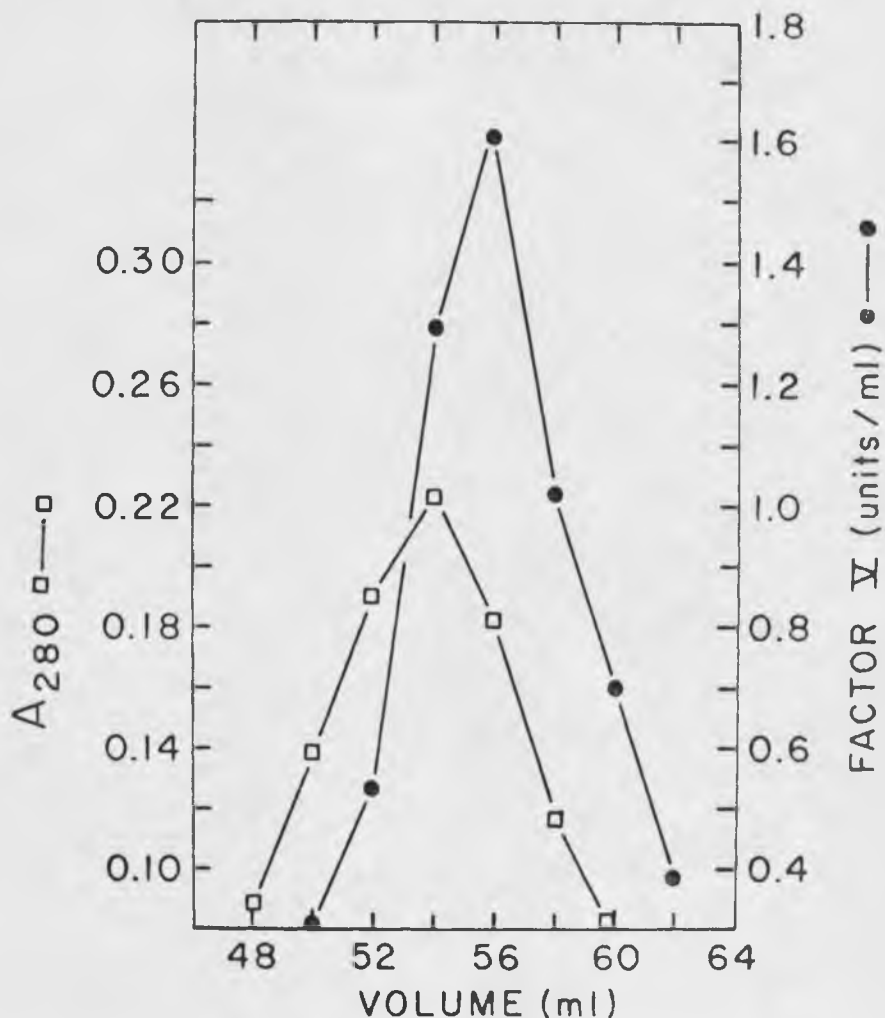


Figure 8. Elution profile of desulphated Sepharose 6B-Factor V on Sephadex G-200 -- Eighty units of the Factor V eluate of Figure 4 was applied to a 1.6 x 55 cm column of Sephadex G-200 at a flow rate of 0.17 ml/min. The column was eluted with Michaelis-0.025 M CaCl_2 , pH 7.35 and 2.0 ml fractions were collected and analyzed for Factor V activity (●—●), and for protein (□—□). The void volume was 54 ml.

to a column of desulphated Sepharose 6B. Also, a sample of Factor V, containing 50 units, was activated with 20 μ g of RVV-V as described in Methods, concentrated to 1.0 ml and separately applied to the same column. The column was calibrated with standard proteins, thyroglobulin, apoferritin and γ -globulin. Factor V eluted from the column in a volume corresponding to a V_e/V_o ratio of 1.40, which when interpolated on a calibration curve gave an apparent molecular weight of 538,000 daltons. Factor Va^{RVV-V} eluted in a volume to give a V_e/V_o ratio of 1.58 and a corresponding molecular weight of 298,000 daltons. The elution profiles of Factor V and Factor Va^{RVV-V} are illustrated in Figure 9. And, in Figure 10 is shown the calibration curve for this column, with the placement of the V_e/V_o ratios for Factor V and Factor Va^{RVV-V}. The apparent molecular weights of Factor V and Va^{RVV-V} on this column are considerably greater than those determined on a calibrated column of Sephadex G-200. Factor V, Factor Va^{RVV-V}, and/or the standard proteins used to calibrate the column, may elute late due to atypically some type of repulsion between the proteins and the Sepharose gel.

Activatability of Factor V

Selected fractions from a preparative column of desulphated Sepharose 6B were tested for activation by RVV-V. Generally all the Factor V activity eluting from the column

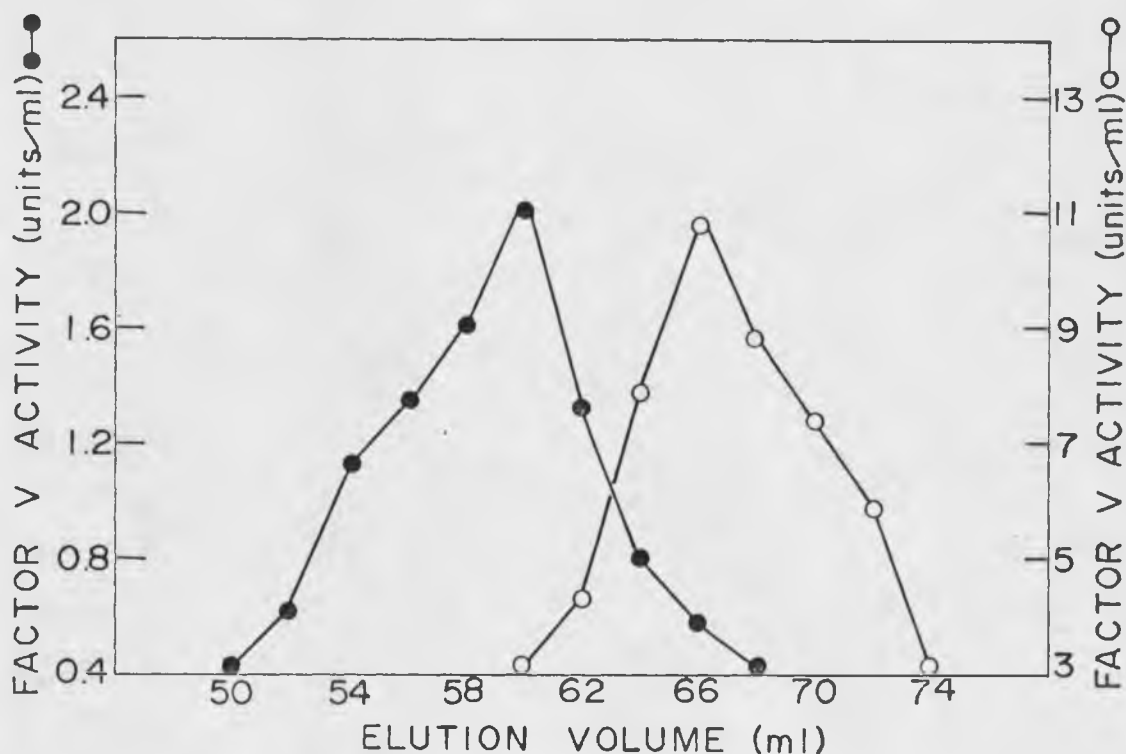


Figure 9. Elution profile of Factor V and Factor Va^{RVV-V} on desulphated Sepharose 6B -- Factor V was applied to a column of desulphated Sepharose 6B, 1.6 x 55 cm at 4°C, at a flow rate of 0.17 ml/min. Four hours later a sample of Factor Va^{RVV-V} was applied to the same column and both samples were eluted with 0.20 M Tris acetate, pH 7.5, 50 mM CaCl₂, 10% glycerol. Two ml fractions were collected and assayed for Factor V activity: Factor V (●—●), and Factor Va^{RVV-V} (o—o).

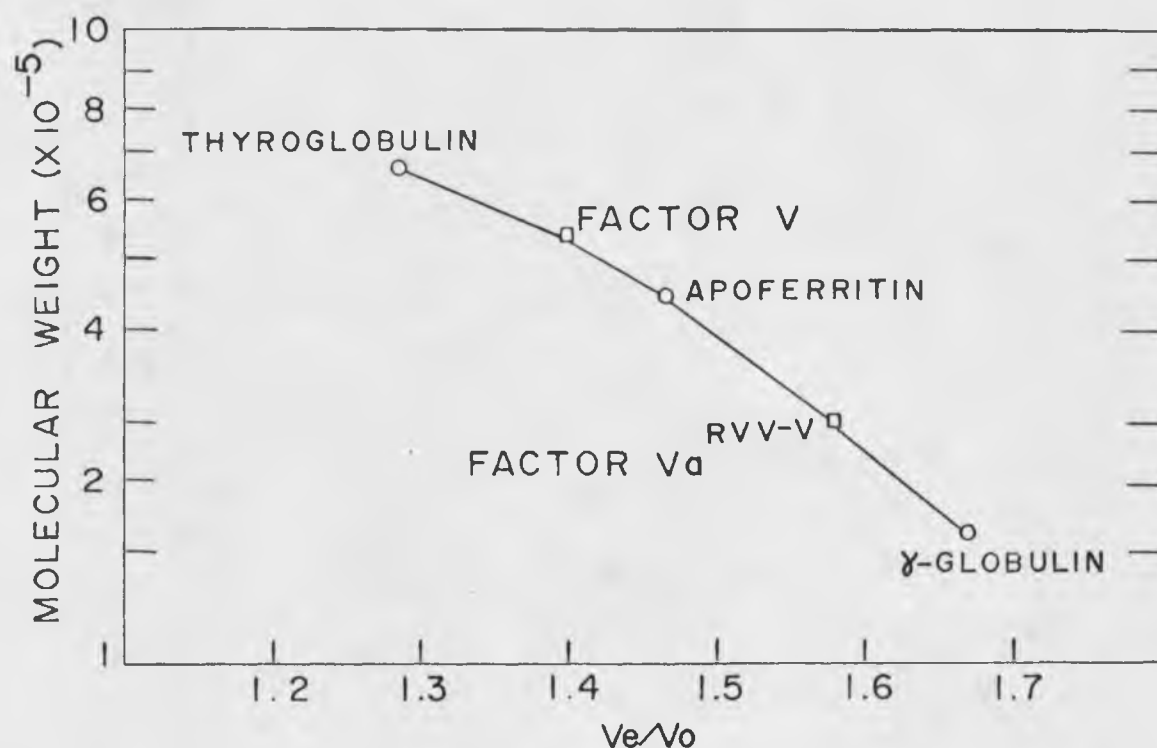


Figure 10. Standard curve for the estimation of the molecular weights of Factor V and Factor Va^{RVV-V} on desulphated Sepharose 6B -- Standard proteins (o) were dissolved in 0.20 M Tris acetate, 50 mM CaCl₂, 10% glycerol, pH 7.5 to a final concentration of 5-10 mg/ml. Factor V samples (□) were in the same buffer. Both samples were applied in a volume of 1.0 ml to a 1.6 x 55 cm column of desulphated Sepharose 6B at a flow rate of 0.17 ml/min and 2.0 ml fractions were collected. The ratio of the elution volume of the proteins to the column void volume (V_e/V_o) was plotted versus the log molecular weight of each standard.

could be activated 8-10-fold by RVV-V, and this was verified by activating an aliquot of the Factor V activity pooled from these same tubes. In addition, Factor V could be activated to the same extent by thrombin and by Factor Xa, as was observed with RVV-V.

Several of the techniques used to physically characterize Factor V following its isolation have indicated that this preparation was heterogeneous. Consequently, a thorough characterization of the physico-chemical properties of bovine Factor V was unwarranted. However, this preparation was essentially free of other clotting factors. Since it consisted of a single molecular weight species upon gel filtration and could be activated reproducibly by several enzymes, it was concluded that Factor V had been isolated essentially unaltered from plasma. Consequently, a study of the changes in activity and molecular size of Factor V by several enzyme activators was feasible, as was an investigation of the role of Factor V in the formation of a prothrombin-converting principle, the prothrombinase complex.

Activation of Bovine Plasma Factor V

Factor V was activated by bovine or human thrombin, bovine Factor Xa, the Factor V activator present in Russell's Viper Venom, RVV-V, or α -chymotrypsin. In each case, this activation took the form of a 7-10-fold increase

in Factor V activity, and a significant decrease in the molecular weight of Factor V as determined by Sephadex G-200 gel chromatography. The "degree of activation" was expressed quantitatively by the ratio of the Factor V activity, in total units, of an "activated" sample, to the Factor V activity present before the activator was added. Some evidence for changes in the subunit structure of Factor V upon activation was obtained by SDS gel electrophoresis of samples before and after activation by thrombin, RVV-V, and Factor Xa. These and other features of these activation studies are detailed below.

By Thrombin

In order to investigate in detail the various aspects of the interaction of Factor V with thrombin, it was necessary to isolate bovine thrombin in a highly purified form. Bovine thrombin was purified from Parke, Davis and Co. Topical Thrombin, to homogeneity on a column of SP Sephadex, as described under Methods. Thrombin eluted in a sharp peak from the column in 0.25 M sodium phosphate, pH 6.5 buffer, as shown in Figure 11. This preparation had a specific activity of 2400 U/mg and when examined by SDS gel electrophoresis under reducing conditions, it appeared to contain both α and β thrombin, with the α form predominate.

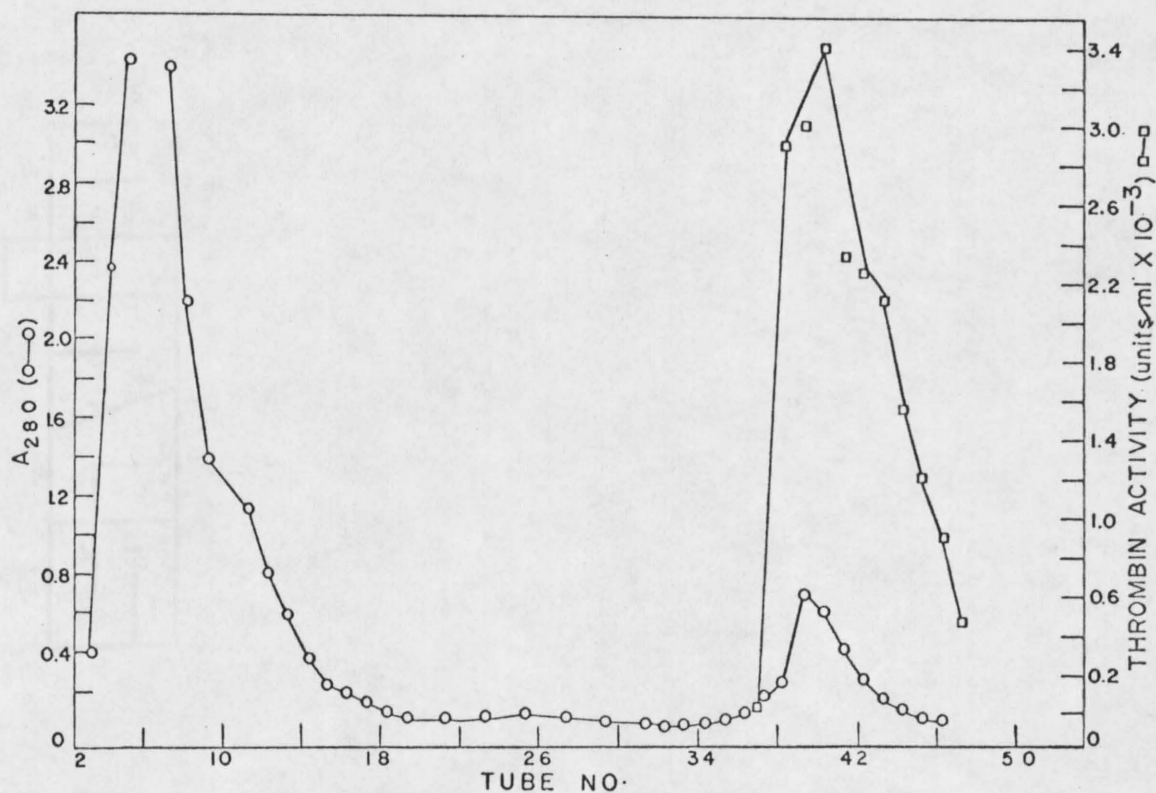


Figure 11. Elution profile of bovine thrombin on SP-Sephadex -- Ten thousand NIH units of bovine thrombin, in 0.1 M sodium phosphate, pH 6.5, were applied to a 2.6 x 20 cm column of SP-Sephadex, C-50. Thrombin was eluted as a single peak (fractions 39-46) with 0.25 M sodium phosphate, pH 6.5. The elution buffer was changed at fraction 20. The column eluate was analyzed for thrombin clotting activity (□—□) and for protein (o—o)

When Factor V was treated with purified bovine thrombin its activity increased 11-12-fold over the basal level in 7-8 minutes, which was followed by a gradual decline in activity over the next 10-15 minutes, as illustrated in Figure 12(A).

Incubation of Factor V with human thrombin resulted in a 12-fold increase in Factor V activity within 15 minutes and was followed by a decline in activity over the next 10-15 minutes, as observed after activation by bovine thrombin, and indicated in Figure 12(A). The activity of the same amount of Factor V, diluted with buffer to a final concentration of 5 mM CaCl_2 in a volume of 1.0 ml, did not change appreciably over the same incubation period.

Activation of Factor V by bovine thrombin did not appear to require calcium. Following its isolation in buffer containing 50 mM CaCl_2 , Factor V was dialyzed against 0.20 M Tris acetate, 10% glycerol, pH 7.5 for 12-15 hours at 4°C, to reduce the calcium concentration in the sample. As a result, approximately 50% of the Factor V activity measured prior to dialysis was lost. However, when 1-5 units of the Factor V remaining activity were treated with 0.7 $\mu\text{g/ml}$ bovine thrombin, activation of Factor V ensued to the same extent and at the same rate as that in the presence of 5 mM CaCl_2 .

Inasmuch as phospholipid has a significant effect on the conversion of prothrombin to thrombin and also on the

Figure 12. Time course of Factor V activation by bovine and human thrombin, RVV-V, and Factor Xa -- (A) Five units of Factor V were incubated at 25°C with 0.6 µg bovine thrombin (□—□), 10 µg human thrombin (Δ—Δ), or buffer alone (o—o) to a final concentration of 5 mM CaCl₂, in 1.0 ml total volume, after the addition of enzyme aliquots of the mixture were withdrawn at the indicated time intervals to assay for Factor V activity. (B) Five units of Factor V were incubated at 25°C with 10 µg RVV-V (□—□) or with buffer (o—o), other conditions were as described in A. (C) Five units of Factor V were incubated at 25°C with 11.4 µg Factor Xa and 5 mM CaCl₂ (□—□) or with 4 µg Factor Xa, 0.05 mg/ml PS:PC (1:1, w/w) and 5 mM CaCl₂ (▲—▲), or with buffer alone (o—o), other conditions are as described in A.

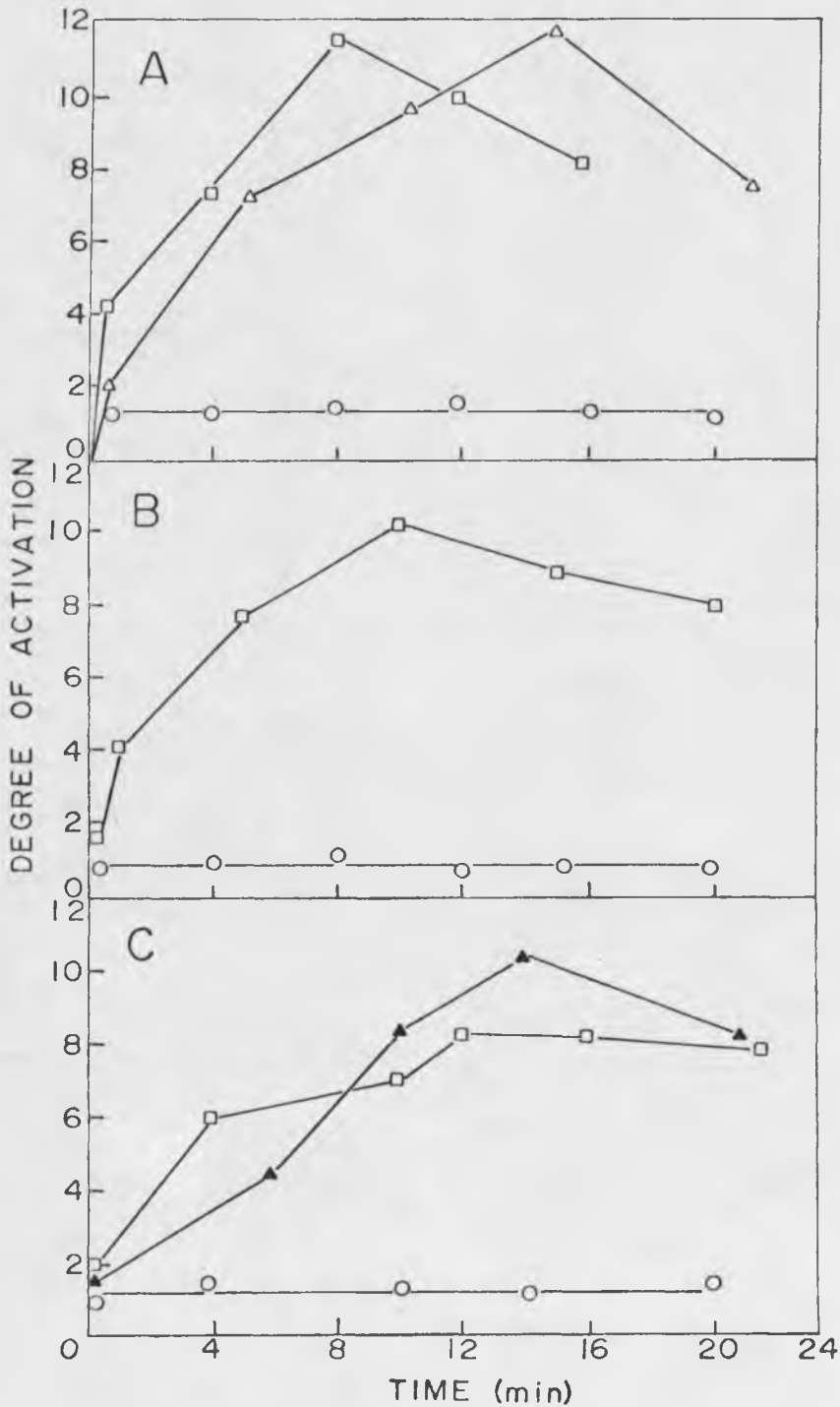


Figure 12. Time course of Factor V activation by bovine and human thrombin, RVV-V, and Factor Xa.

activation of Factor V by Factor Xa, as discussed in a later section, activation by bovine thrombin was also examined in the presence of phospholipid. When Factor V, in amounts of 1-5 units was treated with thrombin in the presence of 0.05 mg/ml PS:PC (1:1, w/w), and 5 mM CaCl_2 , it was activated to the same extent and at the same rate as that in the absence of added phospholipid. Thrombin does not bind to phospholipid in the presence or absence of calcium ions (Papahadjopoulos and Hanahan, 1964; Barton et al., 1967; Jobin and Esnouf, 1967). These facts, together with the observation in this study that the addition of phospholipid to a Factor V-calcium-thrombin activation mixture, has no effect on the rate or the extent of activation, indicates that the complexation of Factor V with phospholipid, per se, does not serve to increase the susceptibility of Factor V to activation by thrombin, at least in vitro.

As noted in this study and also by Colman (1969a), thrombin not only activates but will destroy Factor V activity, especially when thrombin is added in excess of that amount needed for activation only. In order to examine the effect of varying amounts of bovine thrombin on the rate and extent of activation of Factor V, concentrations of thrombin no greater than 0.7 $\mu\text{g/ml}$ were used. At this concentration, a minimal decrease in Factor V activity was observed following activation. However, at greater thrombin

concentrations, significant losses of Factor V activity occurred, and often obscured the extent of the initial activation. So, Factor V, at a constant amount of 3.5 units, was incubated with bovine thrombin from 0.19 to 0.76 ug/ml, in the presence of 5 mM CaCl_2 , pH 7.35 and 25°C, which resulted in a corresponding increase in the rate of activation. However, the extent of activation observed was the same in each case. These results would be predicted for a typical enzyme-substrate reaction.

In another experiment, the addition of a second identical aliquot of Factor V to a thrombin activation mixture after the Factor V activity had maximized, or 10 minutes after the two proteins were mixed, resulted in a slight increase in the Factor V activity of the total mixture, but not to the same extent as observed in the initial activation. Since thrombin can inactivate or degrade Factor V as well as activate it, these observations may be ambiguous in that they reflect opposing actions of thrombin on Factor V. Thus it is difficult to evaluate this second "activation."

Several different experiments were undertaken to determine whether the enzymatic activity of thrombin was a requirement for activation of Factor V. Thrombin was treated with a number of active site inhibitors, previously demonstrated by other workers to abolish its clotting activity.

As a typical serine protease, thrombin is readily inhibited by DFP (Gladner and Laki, 1956). So, an aliquot of thrombin was treated with a 1000-fold molar excess of diisopropylfluorophosphate (DFP) in isopropanol. To a control thrombin was added an equal aliquot of isopropanol alone. Both samples were incubated at 25°C for one hour, then dialyzed for 12-15 hours at 4°C against 0.25 M sodium phosphate, pH 6.5, to remove excess DFP. After dialysis, the samples were assayed for thrombin activity. In the sample treated with DFP, the thrombin activity had been totally inhibited, while the control thrombin retained up to 90% of the activity of the original thrombin preparation.

When Factor V was treated with DFP-thrombin, no activation ensued; treatment of Factor V with the same amount of control-thrombin resulted in at least a 7-fold activation as shown in Figure 13.

Thrombin was also treated with TLCK, which has been shown to modify a histidine residue in the active site of thrombin (Glover and Shaw, 1971). An 0.1 M solution of TLCK in 0.1 M Tris-HCl, pH 7.5 was added to thrombin, at 750 U/ml, to a 1000-fold molar excess. To a control-thrombin was added an identical aliquot of buffer only. Both samples were then processed exactly as described for the treatment of thrombin with DFP. Similar to DFP, TLCK completely abolished all thrombin activity, as well as its ability to activate Factor V.

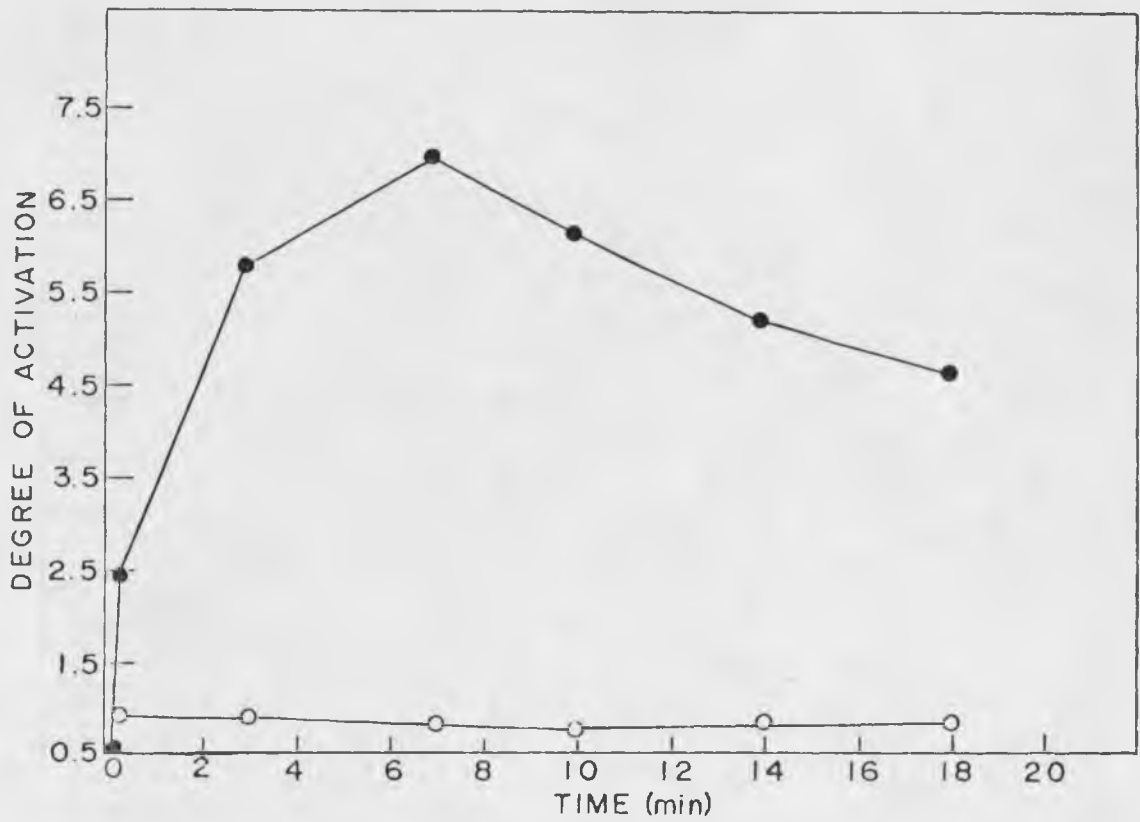


Figure 13. Time course of Factor V activation by DFP treated bovine thrombin -- Five units of Factor V were incubated at 25°C with 1.2 μ g of bovine thrombin (●—●), or 1.2 μ g of thrombin previously treated with DFP (○—○) in the presence of 5 mM CaCl_2 . Factor V activity was assayed in this mixture periodically as described in Methods.

The activity of thrombin was also inhibited when it was physically denatured by heat treatment. A sample of thrombin containing 750 U/ml was heated in a boiling water bath for 10 minutes, while a control-thrombin was maintained at room temperature. After heating, both samples were centrifuged at 2000 RPM in an International Centrifuge, Model HN, for 10 minutes, to collect the condensation which had accumulated on the sides of the tube during heating. Assay of these samples for thrombin activity revealed that heat-treated thrombin lost all of its clotting activity and was able to activate Factor V to only 20% of the level of the control-thrombin, after 8 minutes incubation. In contrast the Factor V activity had increased to greater than 8-fold in 8 minutes when treated with the control-thrombin under identical conditions.

The activation of Factor V by bovine thrombin appeared to be both a temperature and a pH dependent reaction. When Factor V was incubated with thrombin, in the presence of 5 mM CaCl_2 and 37°C , or under identical conditions except at 25°C , its activity increased at a greater rate at 37°C , yet the extent of activation at both temperatures eventually increased to the same level. When the same reaction was conducted at different pH values, with other conditions maintained the same, a marked difference in the extent of activation by thrombin was observed. Factor V was mixed with thrombin and the volume of the

mixture was adjusted to 1.0 ml with 0.05 M Tris acetate, 0.1 M NaCl, bovine serum albumin, 1 mg/ml, at pH 6, 7, 8, or 9. At each pH studied, the extent of Factor V activation was determined relative to the activity of the same amount of Factor V diluted to 1.0 ml in buffer alone at that pH. This control Factor V was maintained at 25°C for a period of 20-30 minutes, similar to the thrombin treated sample. Consequently, activation of Factor V was markedly reduced at pH 6.0 and at pH 9.0, but was significantly great at pH 7.0 and 8.0. When the maximum activation in units was plotted against the pH at which that amount of activation was measured, a bell-shaped curve resulted. As seen in Figure 14, activation of Factor V by thrombin was optimum at pH 8.0, approximately.

Activation of Factor V by thrombin also resulted in a significant reduction in the molecular weight of Factor V. This was observed by treating Factor V bovine thrombin for 20-30 minutes at room temperature or until 10-fold activation was measured. The sample was then concentrated by ultrafiltration (PM-10 membrane), to 1.0 ml, and applied to a calibrated column of Sephadex G-200. A control or untreated sample of Factor V was applied in 1.0 ml to the same column approximately four hours previous. The elution profiles of Factor V and Factor Va^{thrombin} are shown in Figure 15(A). Factor Va^{thrombin} eluted in a volume to give a $K_D = 0.139$ while untreated Factor V gave a $K_D = 0.03$.

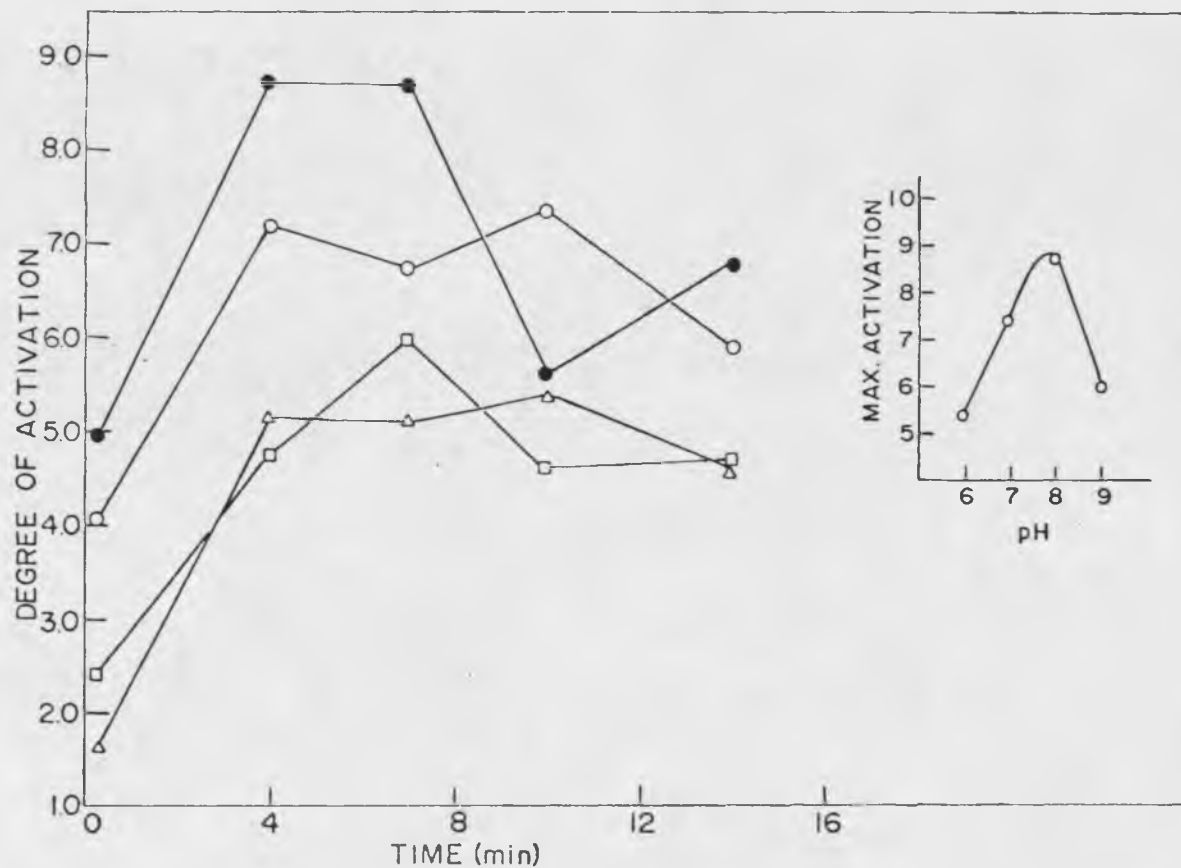


Figure 14. Time course of Factor V activation by bovine thrombin at pH 6.0, 7.0, 8.0, and 9.0 -- 3.5 units of Factor V were incubated at 25°C with 0.3 μg bovine thrombin. The sample was brought to a final concentration of 5 mM CaCl_2 , with 0.15 M Tris acetate, bovine serum albumin, 1 mg/ml, at pH 6.0 (Δ — Δ), pH 7.0 (o—o), pH 8.0 (●—●), or pH 9.0 (□—□). The inset shows a plot of the maximum degree of activation versus the pH at which that activation was measured.

Figure 15. Elution profile of plasma Factor V, Factor Va^{thrombin}, and Factor Va^{RVV-V} on Sephadex G-200 -- (A) Fifty units of Factor V were incubated with 14 μ g of bovine thrombin (●—●), or with buffer alone (o—o), for 20 minutes at 25°C and pH 7.5, in a final volume of 1.0 ml. Each sample was applied separately to a column of Sephadex G-200, and eluted with Michaelis-0.025 M CaCl₂, pH 7.35, at a flow rate of 0.17 ml/min. Two ml fractions were collected for estimation of Factor V activity. (B) Forty units of Factor V were incubated with 20 μ g RVV-V (●—●) before being applied to a column of Sephadex G-200 as described in A.

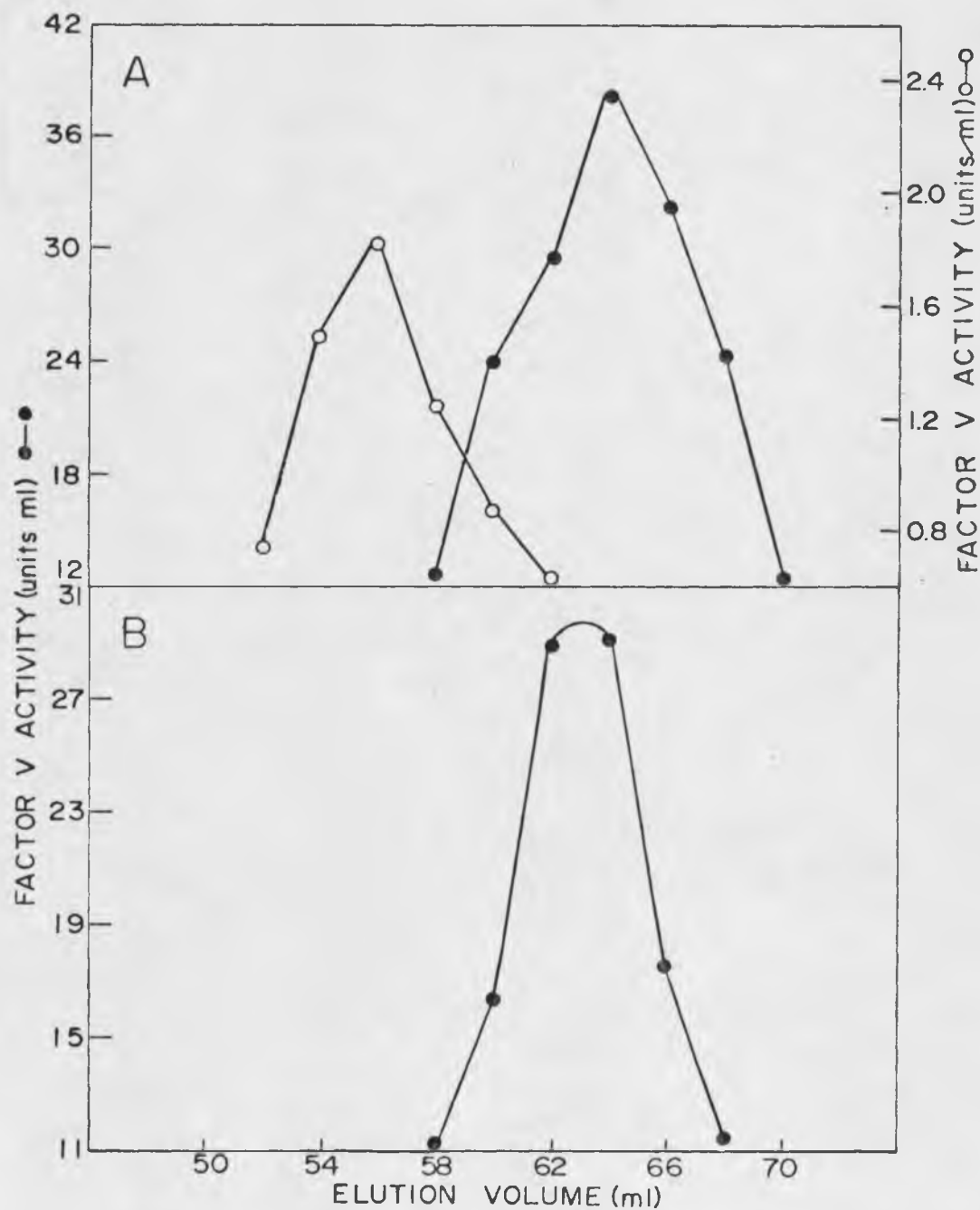


Figure 15. Elution profile of plasma Factor V, Factor vathrombin, and Factor Va^{RVV-V} on Sephadex G-200.

When these values were interpolated on a standard curve as shown in Figure 16 an apparent molecular weight of 443,000 and 239,099 daltons were determined for Factor V and Factor Va^{thrombin}, respectively.

By RVV-V

Prior to an investigation of that reaction in which Factor V is converted to a more active form by RVV-V, it was necessary to purify the venom protein. Esmon, Jackson, and Lowery (1973) described a purification scheme for this enzyme and the RVV-V used in the current study was prepared by a slight modification of this method.

RVV-V was purified to homogeneity by gel filtration on Sephadex G-150 followed by ion-exchange chromatography on SP-Sephadex. The RVV-V activity was recovered quantitatively in a sharp peak from the Sephadex column. However, RVV-V eluted from the SP-Sephadex column as a very broad activity peak, beginning at 0.7 M NaCl in 0.05 sodium acetate, pH 5.0, which was coincident with a spreading protein peak, as shown in Figure 17. Desorption of RVV-V required a higher ionic strength than reported by Esmon, Jackson, and Lowery (1973). They eluted RVV-V activity beginning at 0.25 M NaCl and in a sharp peak. However, they used sulfoyl ethyl (SE) Sephadex, and the results in this report may reflect a difference in the adsorptive properties between SE and SP Sephadex. A small pool of the RVV-V

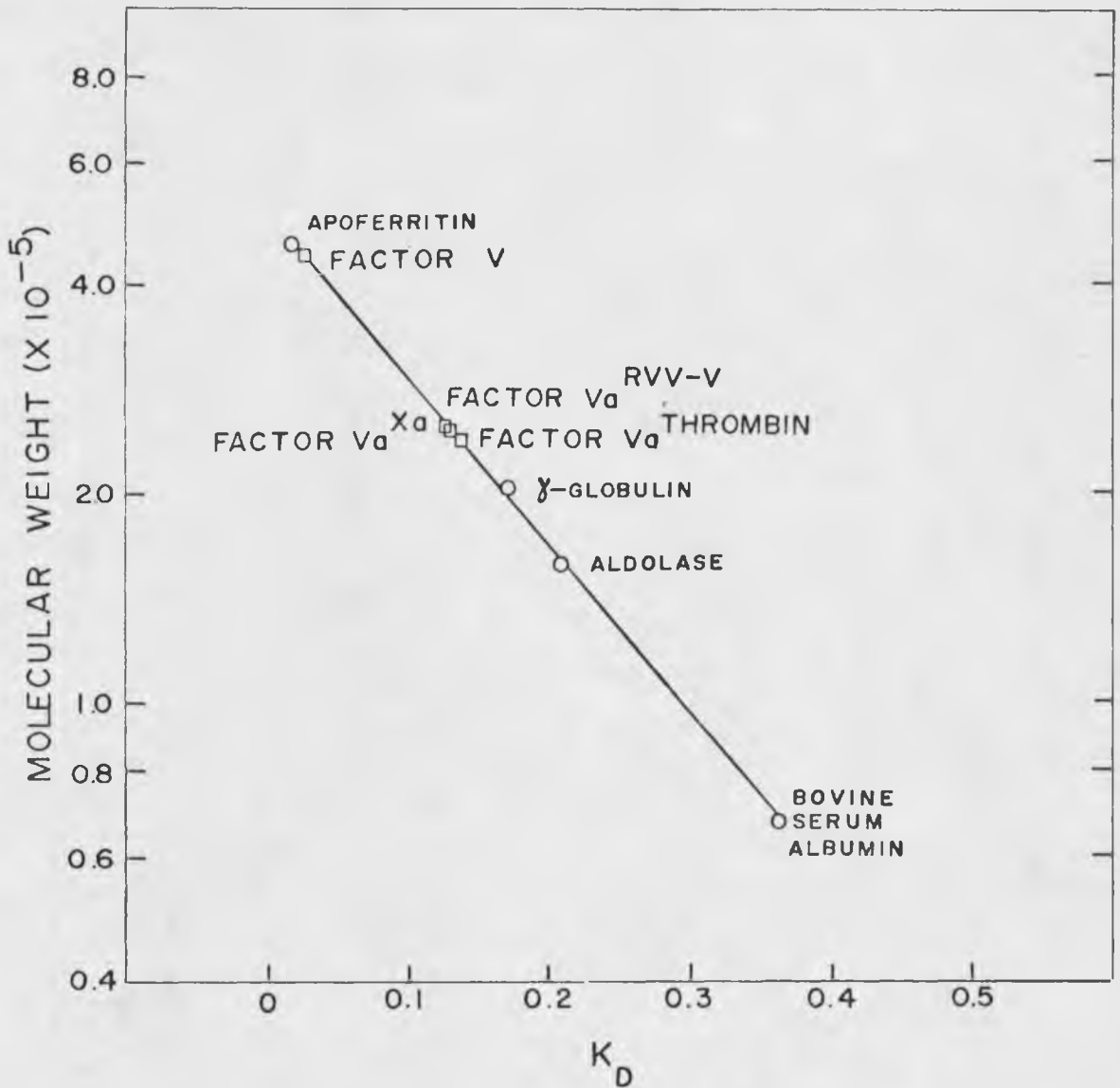


Figure 16. Standard curve for the estimation of the molecular weights of Factor V and Factor Va, as formed by thrombin, RVV-V, or Factor Xa on Sephadex G-200 -- Standard proteins (o) were dissolved in Michaelis-0.025 M CaCl_2 , pH 7.35, to a final concentration of 5-10 mg/ml. Plasma and activated Factor V samples were in the same buffer. Both samples (□) were applied in a volume of 1.0 ml to a 1.6 x 55 cm column of Sephadex G-200, at a flow rate of 0.17 ml/min and 2.0 ml fractions were collected. The K_D was plotted versus the log molecular weight for each protein standard.

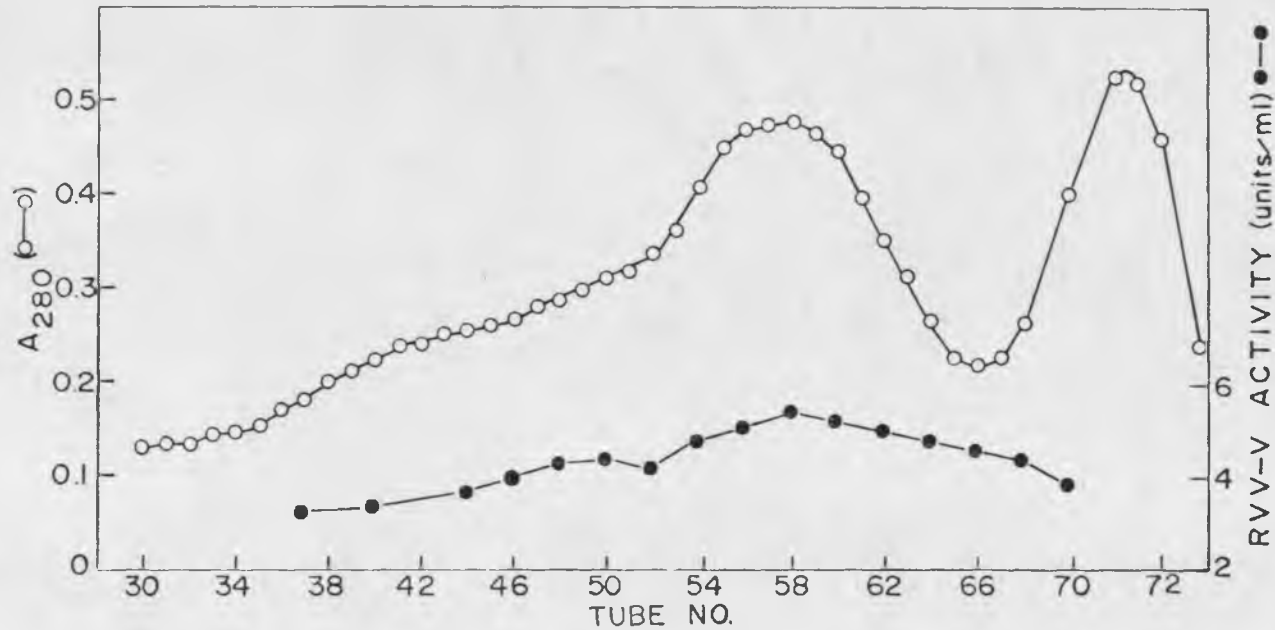


Figure 17. Elution profile of RVV-V on SP-Sephadex, C-50 -- Approximately 140 mg of Sephadex G-150 purified RVV-V, in 0.05 M sodium acetate, 0.5 M NaCl, pH 5.0 were applied to a 1.6 x 50 cm column of SP-Sephadex, C-50, equilibrated with the same buffer at 4°C. A gradient from 0.50 to 1.0 M NaCl, in 0.05 M sodium acetate, pH 5.0 was applied to the column at tube 25. The column eluate was assayed for RVV-V activity (●—●) and for protein (o—o). RVV-V active fractions, 52 through 62 were pooled.

activity from the SP-Sephadex column was collected and when the preparation was examined by SDS gel disc electrophoresis, under reducing conditions, a single protein band was observed. When the migration distance of this band was interpolated on a standard plot as described in Methods, it corresponded to a molecular weight of 25,000 daltons. This RVV-V preparation was stable for at least 6 months when stored frozen at -20°C .

When Factor V was incubated with RVV-V, in the presence of 5 mM CaCl_2 a 10-fold increase in its activity occurred in 10-15 minutes and was generally followed by a gradual decline in activity, as shown in Figure 12(B) and when Factor V was incubated with buffer alone, in the presence of 5 mM CaCl_2 it was observed to maintain a stable level of activity for upwards of 25-30 minutes.

The activation of Factor V by RVV-V was not absolutely dependent on the presence of calcium ions. Factor V, originally prepared in the presence of 50 mM CaCl_2 , was dialyzed against 0.20 M Tris acetate, 10% glycerol, pH 7.5 to reduce the concentration of calcium. As a result 50% of the original Factor V activity was lost but when the remaining activity was treated with RVV-V, its activity increased 7-10 fold in 10-15 minutes incubation time.

The effect of phospholipid on the activation of Factor V by RVV-V was also investigated. Factor V were

mixed with 0.05 mg/ml PS:PC (1:1, w/w) and with RVV-V, in the presence of 5 mM CaCl_2 . A Factor V control was prepared similarly, only an aliquot of buffer, 0.05 M Tris acetate, 0.1 M NaCl, bovine serum albumin, 1 mg/ml, pH 7.5, was added in place of the phospholipid. There was no significant difference in the rate or the extent of activation, whether the reaction mixture contained phospholipid or not.

Activation of Factor V by varying amounts of RVV-V, from 1.2 to 9.6 μg , resulted in a corresponding increase in the final extent of activation after 10-15 minutes incubation time, yet the observed rate of activation was essentially the same in each case. When a plot was made of the maximum degree of activation versus the concentration of RVV-V added, a linear relationship resulted, as seen in Figure 18. Schiffman et al. (1969) observed the same phenomenon when Factor V was activated with similar amounts of a partially purified RVV-V preparation. These investigators interpreted these results as indicating that RVV-V was consumed in the reaction.

Activation of varying amounts of Factor V, from 5.5 to 22.0 U/ml by the same quantity of RVV-V, namely 9.6 $\mu\text{g/ml}$ resulted in a corresponding increase in the extent of activation, but the rate of activation was the same in each case. These experiments suggest that RVV-V was acting in an enzymatic manner. This conclusion was supported by a separate experiment in which a second aliquot of Factor V

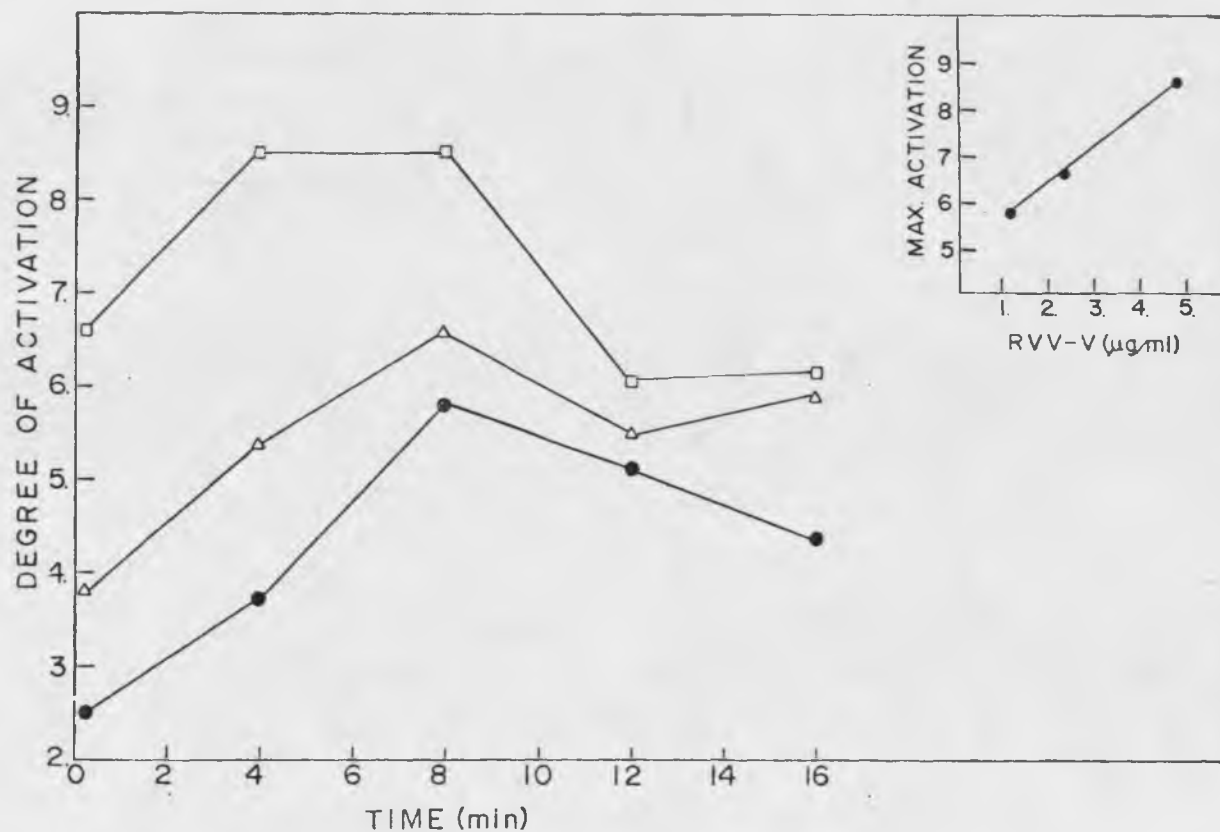


Figure 18. Time course of Factor V activation by varying amounts of RVV-V -- Two units of Factor V were incubated at 25°C with 1.2 µg (●—●), 2.4 µg (Δ—Δ), or 4.8 µg of RVV-V (□—□), in the presence of 5 mM CaCl₂, in a final volume of 1.0 ml. The incubation mixture was assayed for Factor V activity as described in Methods. The inset shows a plot of the maximum degree of activation measured versus the concentration of RVV-V used to achieve that amount of activation.

was added to a mixture consisting of Factor V, calcium, and RVV-V, at that time when the initial activation had maximized with the result that a similar increase in activity was subsequently observed, as illustrated in Figure 19.

Following activation by RVV-V, a gradual decline in Factor V activity resulted. In order to determine whether this was due to further proteolysis by RVV-V, a second aliquot of RVV-V was added to the activation mixture at that time when the Factor V activity had peaked, or 10-15 minutes incubation time. No accelerated decrease in the Factor V activity of the total mixture occurred, but instead, it remained essentially unchanged. Perhaps the observed losses in Factor V activity after activation are due to an inherent instability of Factor Va^{RVV-V} relative to plasma Factor V, under these conditions.

To ascertain whether the action of RVV-V on Factor V was enzymatic in nature, an additional series of experiments was undertaken. In confirmation of a previous report by Hanahan et al. (1972), RVV-V was stable to heat treatment in its ability to activate Factor V. Stock RVV-V, at a concentration of 0.96 mg/ml in 0.1 M Tris-HCl, pH 7.5 was placed in a boiling water bath for 10 minutes, in a polycarbonate tube capped with a marble to minimize evaporation. After heating, the sample was placed in an ice bath for 5-10 minutes to cool and then centrifuged at 2000 RPM for 10 minutes to collect the condensation formed

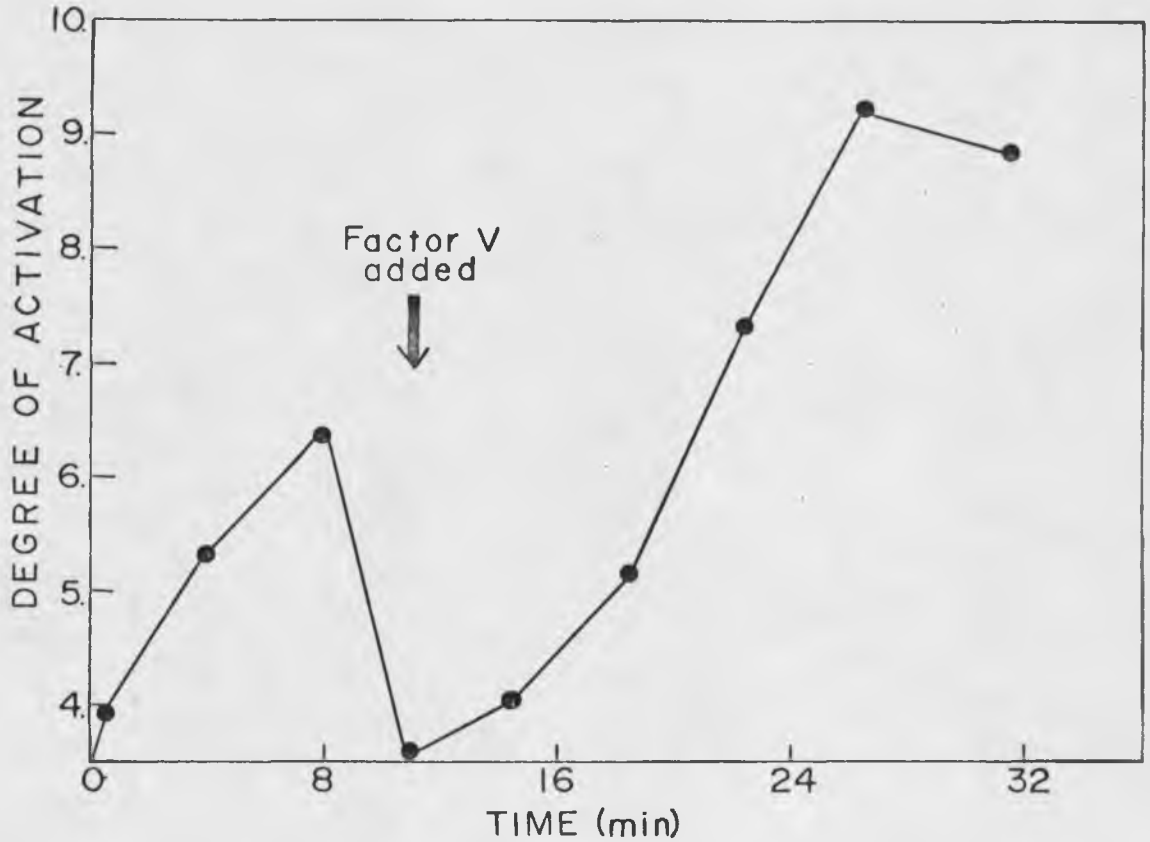


Figure 19. Time course of the activation of two samples of Factor V added successively to RVV-V -- Three units of Factor V were incubated at 25°C with 2.7 μ g of RVV-V for 10 minutes. At 11 minutes incubation time, a second aliquot containing 3 units of Factor V, was added to the Factor V-RVV-V mixture and incubation was continued. Factor V activity of the mixture was assayed periodically as described in Methods.

during heating. An RVV-V control was maintained at 4°C during the heating process. Heat-treated RVV-V was able to activate Factor V to 76% of the level of the control RVV-V.

Also enhancement of Factor V activity by RVV-V appeared to be a temperature dependent process. When 1-5 units of Factor V were treated with 10 µg/ml RVV-V in the presence of 5 mM CaCl₂ at 37°C, or at 25°C, activation proceeded at a faster rate, at 37°C. However, the same final extent of activation was attained at both temperatures.

Esmon and Jackson (1973) reported that RVV-V has properties of a serine protease since it could be inhibited by DFP. However, efforts in this current study to destroy ability of RVV-V to activate Factor V with DFP were unsuccessful. When RVV-V was treated with DFP, at the levels reported by Esmon and Jackson (1973) to be sufficient to inactivate RVV-V, namely 10⁻³ M, or up to 10⁻¹ M, for 24 hours at 37°C, it could still activate Factor V to 78% of the level of a control RVV-V. The control was treated with an aliquot of isopropanol alone, and subsequently maintained under conditions similar to the DFP-treated sample.

After Factor V was activated with RVV-V the subsequent addition of a second Factor V activator other than RVV-V did not result in any further increases in Factor V activity. Factor V was incubated initially with RVV-V, in the presence of 5 mM CaCl₂, and after maximum Factor V

activity was measured in 10-15 minutes, an aliquot of Factor Xa was added to the mixture. Factor V activity was monitored for an additional 25-30 minutes and as shown in Figure 20, no significant changes were observed. When the same experiment was repeated except that Factor Xa was added first, no further changes were measured in the Factor V activity upon the subsequent addition of RVV-V. Both RVV-V and Factor Xa seem to effect the same changes in Factor V since the successive addition of these enzymes did not produce any increase or decrease in Factor V activity.

In addition to modifying the activity of Factor V, RVV-V also brought about a change in the apparent molecular weight of Factor V. Factor V was activated with RVV-V, concentrated to 1.0 ml and applied to a calibrated column of Sephadex G-200. Figure 15(B) depicts the elution profile of Factor Va^{RVV-V} and that of a control or untreated Factor V sample on Sephadex G-200. Factor Va^{RVV-V} eluted in a volume to give a $K_D = 0.13$ and when this value was interpolated to a standard curve of the log molecular weight versus K_D for a series of standard proteins, as illustrated in Figure 16, it gave an apparent molecular weight of $250,000 \pm 4000$ daltons. As described in a previous section, the molecular weight of Factor Va^{RVV-V} was also estimated on a column of desulphated Sepharose 6B. As indicated in Figure 10, Factor Va^{RVV-V} gave an apparent

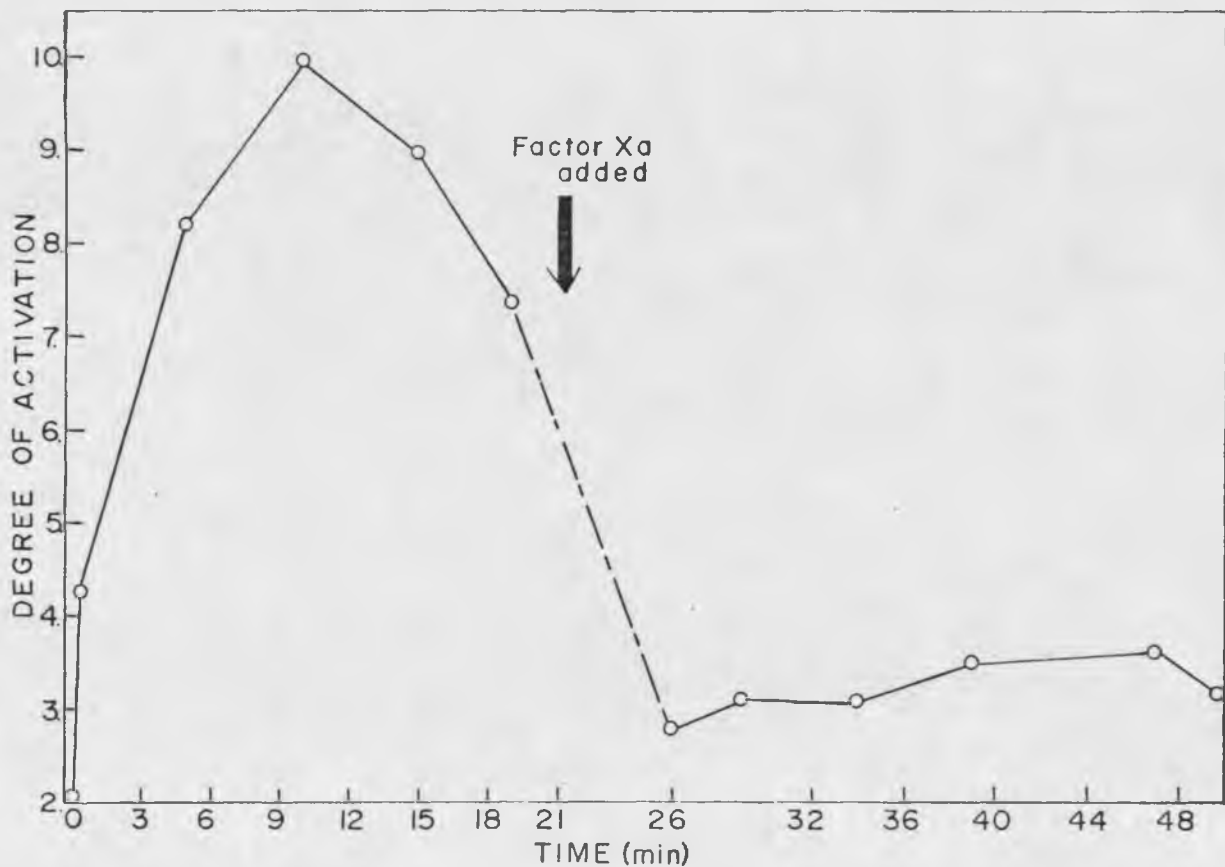


Figure 20. Time course of the activation of Factor V by RVV-V followed by Factor Xa -- Two units of Factor V were incubated at 25°C with 10 μ g RVV-V for 19 minutes; at 21 minutes incubation time, 40 μ g of bovine Factor Xa was added to the Factor V-RVV-V mixture and the total mixture was incubated an additional 26 minutes.

molecular weight of 298,000 daltons on this gel filtration medium.

Efforts to locate RVV-V activity in the effluent from the Sephadex G-200 column after elution of the Factor V-RVV-V activation mixture were unsuccessful. No protein peak appeared in the effluent in a volume corresponding to a molecular weight of 25,000 daltons, as expected for RVV-V. Attempts to assay for RVV-V activity were also unsuccessful, since no peak of such activity was localized outside that volume in which the Factor Va eluted. In those fractions containing Factor Va activity, RVV-V could not be assayed since apparent increases in the activity of native Factor V to which aliquots of such fractions were added, were obscured by the Factor V activity also present in these aliquots.

Consequently, in order to localize the RVV-V activity after activation of Factor V, RVV-V was labeled with ^{125}I although the iodination procedure decreased the Factor V activating ability of RVV-V by 20-30%. Significant enhancement of Factor V activity was still observed upon incubation with ^{125}I -RVV-V, and, as shown in Figure 21, after Factor V was treated with ^{125}I -RVV-V and the activation mixture chromatographed on a column of Sephadex G-200, the Factor V activity and ^{125}I -RVV-V eluted in separate volumes. ^{125}I -RVV-V eluted in asymmetrical near the inclusion volume which was the same elution volume in which

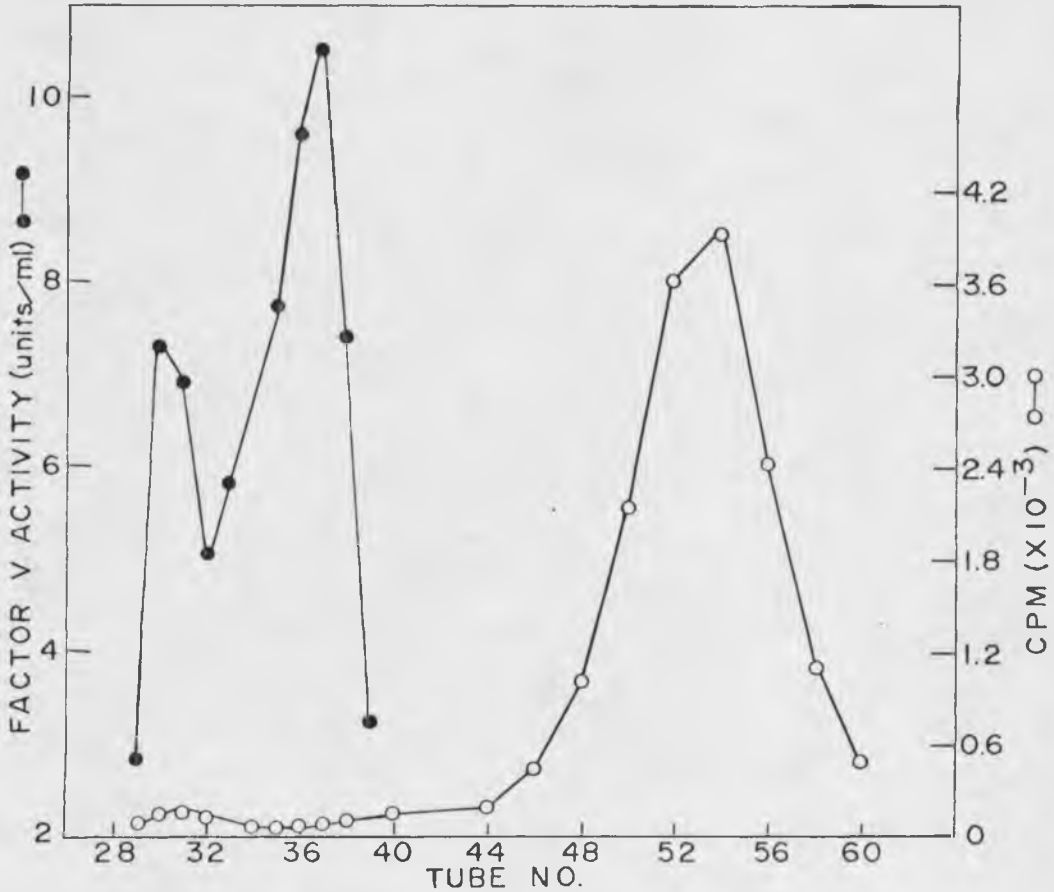


Figure 21. Elution profile of Factor V activity and ^{125}I -RVV-V on Sephadex G-200 -- Twenty units of Factor V were treated with 20 μg of ^{125}I -RVV-V for 20 minutes at 25°C and pH 7.5, and the sample was applied to a column of Sephadex G-200, 1.6 x 55 cm, at a flow rate of 0.17 ml/min at 4°C. Two ml fractions were collected and analyzed for Factor V activity (●—●) and radioactivity (o—o).

a sample of non-labeled RVV-V eluted on the same column. Approximately 90% of the radioactivity applied as ^{125}I -RVV-V was recovered in a single peak (tubes 48-58) from the column. No significant radioactivity was detected in the region where Factor V or Factor Va eluted from the column. The Factor V activity eluted in two peaks, one coming near the void volume and the second in a volume corresponding to Factor Va. RVV-V and Factor V (or Va), then, do not bind together as a result of their interaction and thus RVV-V is not "used up" in the activation of Factor V, at least in the sense of forming an inactive complex with its substrate, or with the product of the reaction. Further evidence to support this came from the observation that the ^{125}I -RVV-V activity pooled from the G-200 column was able to activate a fresh aliquot of Factor V. Four units of native Factor V were incubated with an aliquot of the G-200- ^{125}I -RVV-V pool and after 10-15 minutes activity had increased 4-5-fold.

Since it was observed by Jackson and Hanahan (1968) that the conversion of Factor X to Factor Xa was autocatalytic under certain conditions, the possibility was explored that Factor Va can perpetuate its own formation from plasma Factor V under conditions similar to those under which the activation of Factor V was observed. Three samples of Factor V, each containing 2-3 units, were incubated with different amounts of Factor Va^{RVV-V}, in the

presence of 5 mM CaCl_2 . Factor $\text{Va}^{\text{RVV-V}}$ was prepared as described in Methods. In each case the average level of Factor V activity assayed was only 1.5-fold greater than could be accounted for on the basis of the amount of Factor V plus Factor $\text{Va}^{\text{RVV-V}}$ activity initially mixed together. However, as shown in Figure 22, no obvious trend in the activity in any of the samples was observed over the total incubation time. It was concluded that Factor Va did not alter the activity of plasma Factor V when the two were incubated together under those conditions where activation of Factor V by thrombin, Factor Xa, or RVV-V was usually observed.

However, activation of plasma Factor V by Factor Xa, in the presence of added Factor $\text{Va}^{\text{RVV-V}}$ did not proceed to the same extent as that observed in the absence of added Factor $\text{Va}^{\text{RVV-V}}$. Factor V was treated with Factor Xa alone, or with Factor Xa plus Factor $\text{Va}^{\text{RVV-V}}$, in a final volume of 1.0 ml and in the presence of 5 mM CaCl_2 . The Factor V activity was monitored over a period of 20 minutes and as shown in Figure 23, activation of Factor V proceeded to only 45% of that extent observed in the absence of Factor Va.

By Factor Xa

Previous to this current study no direct effect of Factor Xa on the activity and/or structure of Factor V, per se, has been elucidated. In an investigation of the

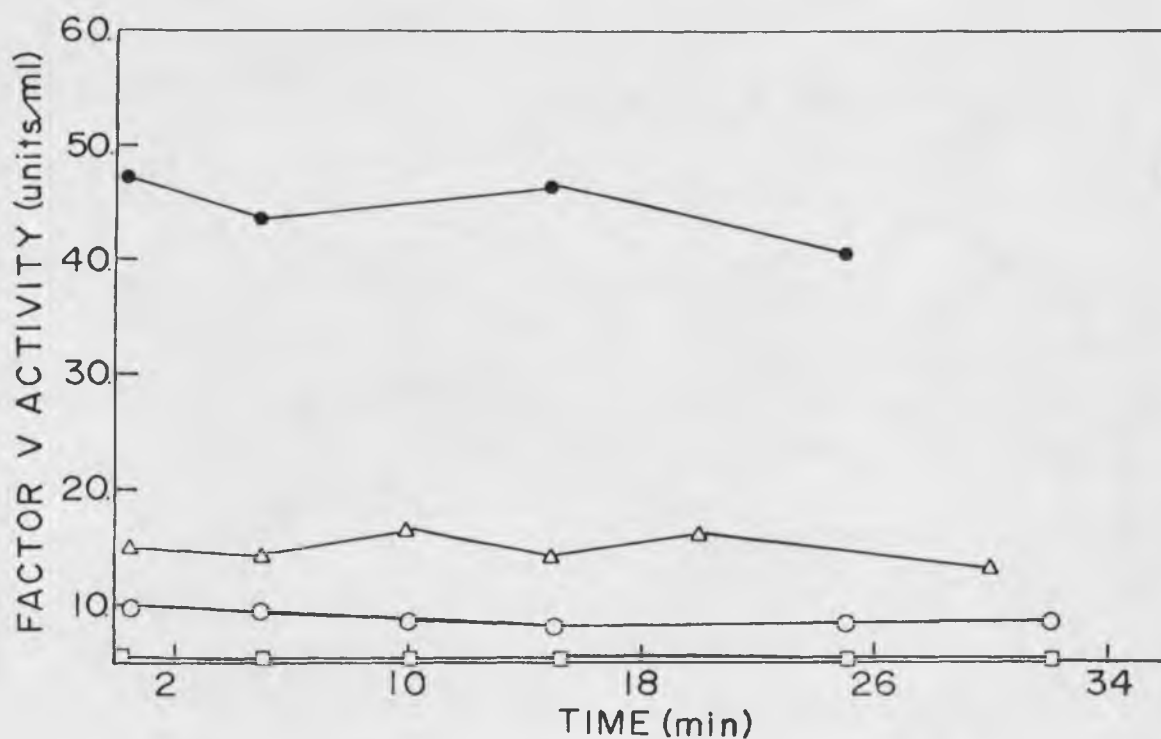


Figure 22. Time course of Factor V activity in the presence of Factor Va^{RVV-V} -- 2.2 units of Factor V were incubated with 3.6 units (○—○), 5.22 units (Δ—Δ), or 26.1 units of Factor Va^{RVV-V} (●—●) or with buffer alone (□—□). After addition of Factor Va^{RVV-V}, aliquots of the mixture were removed periodically to assay for Factor V activity.

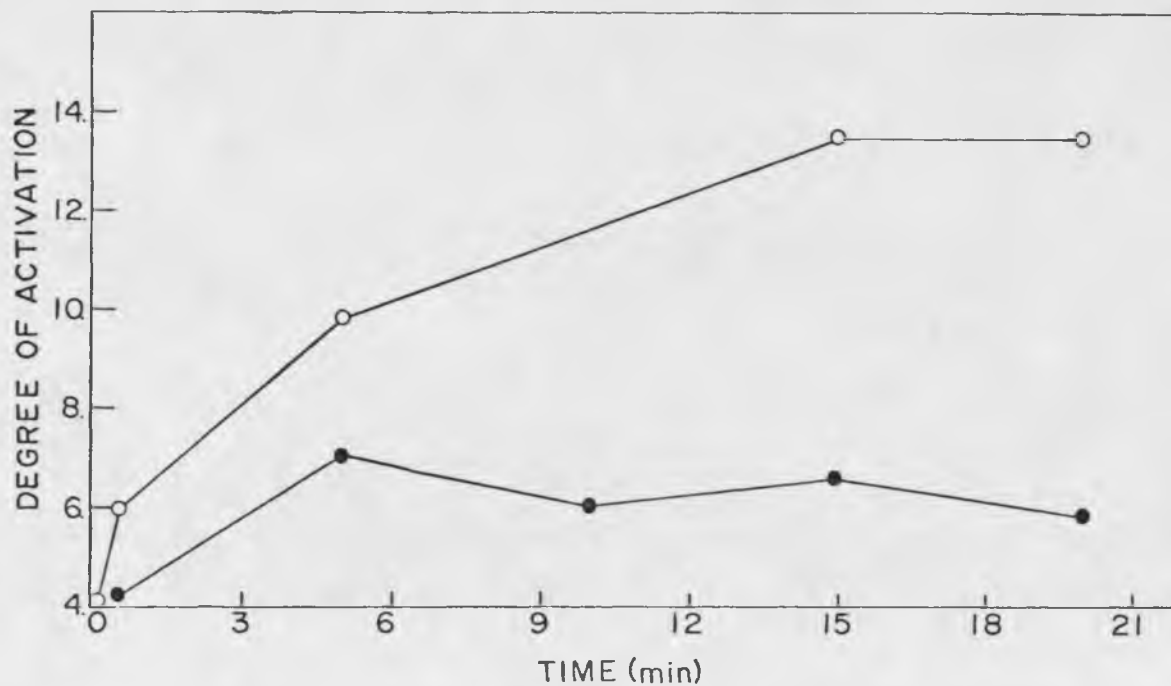


Figure 23. Time course of Factor V activation by Factor Xa in the presence of Factor Va^{RVV-V} -- 2.7 units of Factor V were incubated with 5.2 μ g of bovine Factor Xa (o—o), or with 5.2 μ g of Factor Xa and 5.22 units of Factor Va^{RVV-V} (●—●), in the presence of 5 mM CaCl₂. After the addition of Factor Xa, the mixture was periodically estimated for Factor V activity.

mechanism by which prothrombin is converted to thrombin, by Factor Xa, calcium, phospholipid and Factor V, it was observed here that some enhancement of the activity of Factor V occurred in mixtures with Factor Xa and calcium. At this time Factor V was isolated by a modification of the procedure of Esnouf and Jobin (1967) through the TEAEC extraction step. However, activation by Factor Xa was also observed with more purified Factor V preparations as described below.

As shown in Figure 12(C) treatment of Factor V with bovine Factor Xa, in the presence of calcium, resulted in a 10-fold increase in activity in 12-14 minutes. In the presence of calcium and PS:PC (1:1, w/w), the activity of Factor V was enhanced 8-fold by Factor Xa in 10-12 minutes. The activity of Factor V in the presence of the same amount of calcium and phospholipid but no Factor Xa did not change appreciably over the same time period. When Factor V was activated by Factor Xa and calcium, approximately three times more Factor Xa were required to achieve activation at the same rate as that observed in the presence of phospholipid. If the level of calcium in the Factor V sample was reduced by dialysis prior to the addition of Factor Xa, no activation occurred over a period of 30 minutes incubation time.

The concentration of Factor Xa, calcium and PS:PC (1:1, w/w) were varied individually in this reaction to

determine their effect on the rate and the extent of activation of Factor V. When the concentration of calcium was varied from 5-20 mM in a mixture containing Factor V and Factor Xa, both the apparent rate and the extent of the activation of Factor V were greatest at 5-10 mM CaCl_2 . This reaction was not carried out at concentrations of calcium less than 5 mM so it can only be concluded that levels of calcium greater than 10 mM did not maximally enhance activation by Factor Xa. The effect of varying amounts of calcium in a Factor V-phospholipid-Factor Xa activation mixture, was not examined. In a similar set of experiments, the concentration of PS:PC (1:1, w/w) was varied over a range of 0.04-0.12 mg/ml phospholipid. Optimum activation of Factor V by Factor Xa was observed at 0.05 mg/ml phospholipid in the presence of 5 mM CaCl_2 .

Although PS:PC (1:1, w/w) was used almost exclusively in these reactions, the ability of cephalin to substitute for PS:PC was explored. When cephalin in amounts from 0.06 mg/ml to 0.175 mg/ml was used in place of PS:PC, no appreciable activation of Factor V was observed at any concentration in this range.

The concentration of Factor Xa was also varied, from 6-24 U/ml, or 10-40 $\mu\text{g/ml}$, at a constant level of 5 mM CaCl_2 , 0.05 mg/ml PS:PC, and 1-5 units of Factor V. Under these conditions the apparent rate of activation of Factor V was nearly the same at each concentration, yet the final

extent of activation increased with increasing amounts of Factor Xa. These observations would not be predicted for an enzyme acting on a substrate in a totally aqueous environment. However, this reaction occurs at a phospholipid surface, similar to the activation of prothrombin by the complex of Factor Xa, calcium, phospholipid and Factor V, in which it has been observed that the amount of any one component, be it the actual enzyme, Factor Xa, or a "cofactor" such as calcium, phospholipid or Factor V, affects not only the initial rate of the reaction but the ultimate yield as well.

However, Factor Xa did not appear to be consumed in the process of activating Factor V. When a second aliquot of Factor V was added to an activation mixture consisting of Factor V, calcium and Factor Xa, after the initial activation of Factor V had reached a maximum level, a second burst of Factor V activity occurred, to the same extent as measured for the initial activation. This is illustrated in Figure 24. Although it is not illustrated, this same phenomenon was also observed in an activation mixture containing Factor V, Factor Xa, calcium, and PS:PC (1:1, w/w).

In contrast to thrombin, Factor Xa did not appear to degrade or inactivate Factor V. After an initial activation was observed, in the presence of Factor Xa and calcium, addition of a second aliquot of Factor Xa,

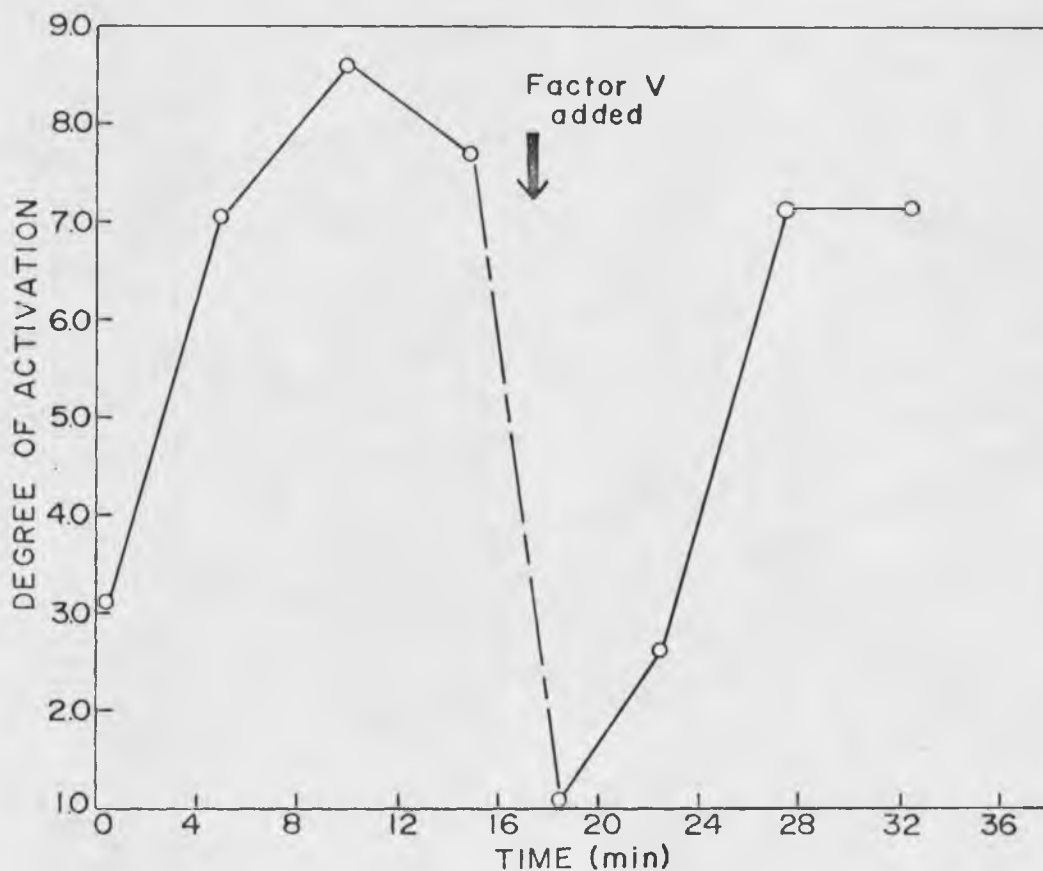


Figure 24. Time course of the activation of two samples of Factor V, added successively to Factor Xa -- Two units of Factor V were incubated at 25°C with 12 μ g of Factor Xa for 15 minutes. At 17 minutes incubation time a second aliquot, containing 2.0 units of Factor V, was added to the Factor V-Factor Xa mixture, and the total mixture was incubated an additional 15 minutes.

equivalent to that used to initiate the activation, did not produce any significant increase, or decrease in the Factor V activity of the total mixture, as seen in Figure 25.

Similar to those studies conducted on the activation of Factor V by thrombin, Factor Xa was chemically or physically modified so as to eliminate its clotting activity, and subsequently tested for its ability to activate Factor V.

It has been well documented that Factor Xa is inhibited by DFP (Titani et al., 1972; Fujikawa, Legaz, and Davie, 1972), although not as readily as thrombin (Magnusson, 1971). A sample of Factor Xa containing 600 U/ml, was treated with a 1000-fold molar excess of DFP in isopropanol, and a Factor Xa control was treated with the same aliquot of isopropanol alone. Both samples were incubated at 37°C for two hours. After the incubation period, both were dialyzed for 12-15 hours at 4°C to remove excess DFP. Assay of DFP-Factor Xa revealed that at least 90% of the control-Factor Xa activity was lost. Subsequent treatment of Factor V with DFP-Factor Xa, in the presence of 5 mM CaCl₂, did not produce any measurable increase in Factor V activity, upwards of 18 minutes. The control-Factor Xa however, as shown in Figure 26, activated Factor V nearly 8-fold after the same period of time.

Inasmuch as Factor Xa is a serine protease, an experiment was conducted to determine whether its clotting

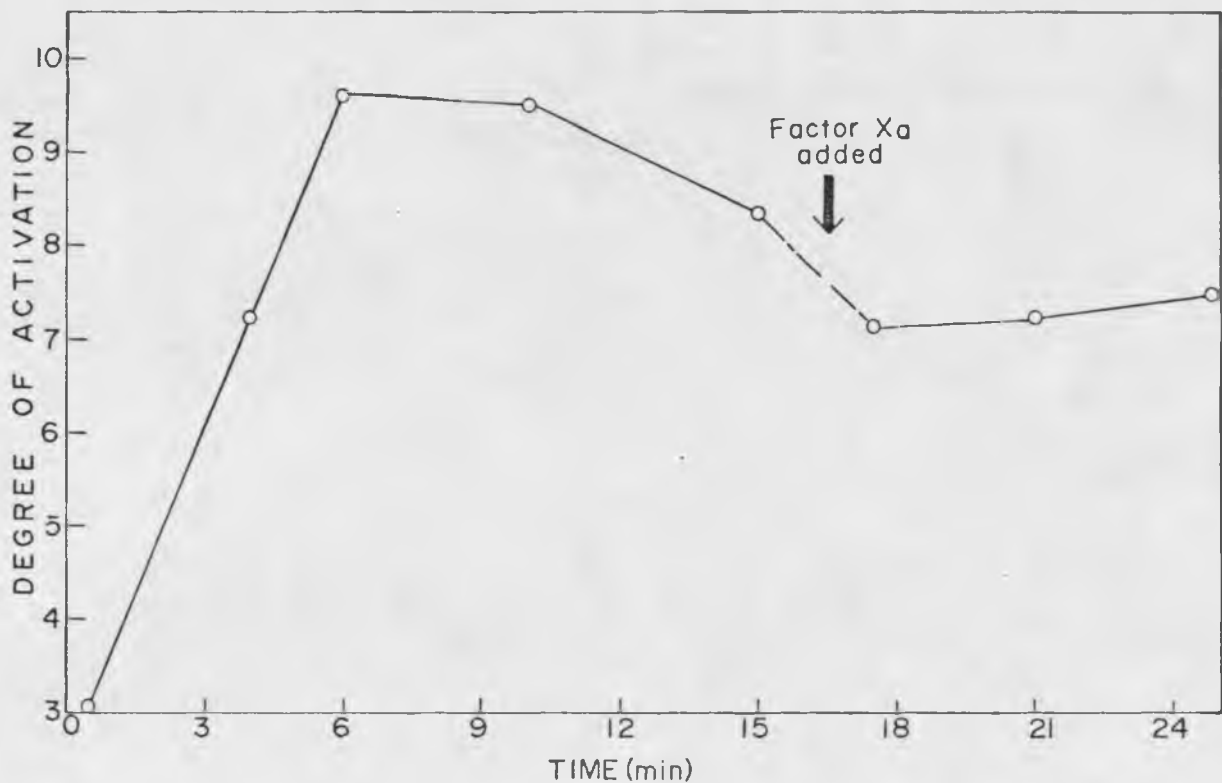


Figure 25. Time course of Factor V activation by two samples of Factor Xa added in succession -- Three units of Factor V were incubated at 25°C with 10 μ g of Factor Xa for 15 minutes. At 16 minutes incubation time, a second aliquot, containing 10 μ g of Factor Xa was added to the Factor V-Factor Xa mixture and incubation was continued for 10 minutes.

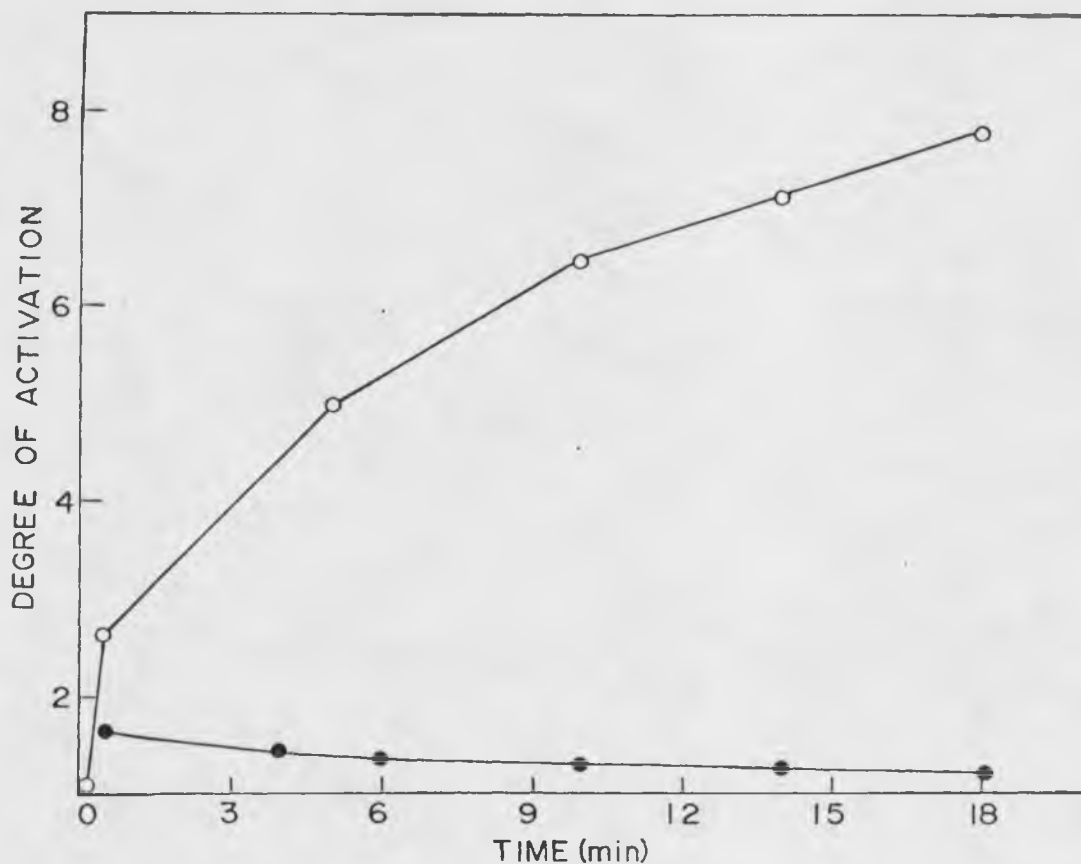


Figure 26. Time course of Factor V activation by DFP-Factor Xa -- 3.8 units of Factor V were incubated at 25°C with 11 μ g of Factor Xa (o—o), or with 11 μ g of Factor Xa previously treated with DFP (●—●) in the presence of 5 mM CaCl_2 . Each sample was separately assayed periodically for Factor V activity.

activity could be inhibited by TLCK, as had been observed with thrombin. Factor Xa, at 600 U/ml, was incubated with a 1000-fold molar excess of TLCK in 0.1 M Tris-HCl, pH 7.5, or with an equal aliquot of buffer alone, under the same conditions as described above for DFP treatment of Factor Xa. The TLCK and control-Factor Xa samples assayed at the same level of clotting activity. Consequently, TLCK-Factor Xa did not differ significantly from the control-Factor Xa in its ability to activate Factor V. Both samples enhanced the activity of Factor V, 8-10-fold in the presence of 5 mM CaCl_2 , after 20 minutes.

Similarly, TPCK, normally an inhibitor specific for α -chymotrypsin (Ryan and Feeney, 1974), had no effect on the clotting activity of Factor Xa or on its ability to activate Factor V. TPCK was made in methanol to 10^{-1} M and Factor Xa was treated with it under the same conditions described for the DFP and TLCK treatments.

Similar to thrombin, Factor Xa was sensitive to heat treatment. Four hundred units of Factor Xa were heated exactly as described for heat treatment of bovine thrombin. A control-Factor Xa was maintained at 25°C during the heating period. Consequently, all of the Factor Xa clotting activity was abolished and when 10 μg of heat-treated Factor Xa were added to a sample of Factor V, containing 4 units, in the presence of 5 mM CaCl_2 , no activation occurred. In contrast, 10 μg of control-Factor Xa were able

to activate the same amount of Factor V, 8-10-fold after 10 minutes.

When the activation of Factor V by Factor Xa was carried out at different temperatures, the apparent rate varied as a function of temperature. Activation at 37°C in the presence of 5 mM CaCl₂ proceeded at a faster rate but eventually to the same extent as that normally conducted at 25°C.

The actual role of Factor V, or Factor Va, in the conversion of prothrombin to thrombin, has not been clearly determined. Colman (1970) indicated that Factor V can affect the reactivity of Factor Xa toward its substrate, prothrombin, since the rate of ester hydrolysis by Factor Xa in the presence of Factor V, calcium, and phospholipid, but in the absence of prothrombin, was 3-fold greater than that in the presence of calcium and phospholipid, only. If Factor V has a role in modifying the activity of Factor Xa, perhaps one consequence of its activation is to generate a species more reactive toward Factor Xa and this might be reflected in an increase in the Factor Xa activity of a Factor V activation mixture, as well as an increase in the activity of Factor V. To examine this possibility, Factor Xa clotting activity in a mixture of Factor Xa, Factor V and calcium, was monitored over the same period of time and under the same conditions under which activation of Factor V had been observed. Although the basal Factor Xa activity

assayed was greater than the calculated amount added initially, the Factor Xa activity did not increase or decrease substantially over a 20-30 minute incubation period.

As observed with thrombin, activation by Factor Xa resulted in a decrease in the molecular weight of Factor V. Factor V was treated with Factor Xa in the presence of 50 mM CaCl_2 for 30-40 minutes. After 30-40 minutes an 8-10-fold activity increase was measured, and the sample was then concentrated by ultrafiltration (PM-10 membrane) to 1.0 ml and applied to a calibrated column of Sephadex G-200. Factor V activity eluted in a volume corresponding to a $K_D = 0.131$, which interpolated on a standard curve to an apparent molecular weight of $246,000 \pm 4000$ daltons, as shown in Figure 16. The Factor Xa and Factor Va activities were completely separated on the column as illustrated in Figure 27. When 5 units of native Factor V, in the presence of 5 mM CaCl_2 , were incubated with a fresh aliquot of Factor Xa, in an amount of 5.88 units per ml, or, with 3.88 units/ml of the Factor Xa activity recovered from the G-200 column, a measurable activation occurred in each case; with G-200 Factor Xa the activity of Factor V increased to at least 65% of that level observed with a fresh or "unused" amount of the same Factor Xa preparation, after 15 minutes at 25°C and pH 7.35.

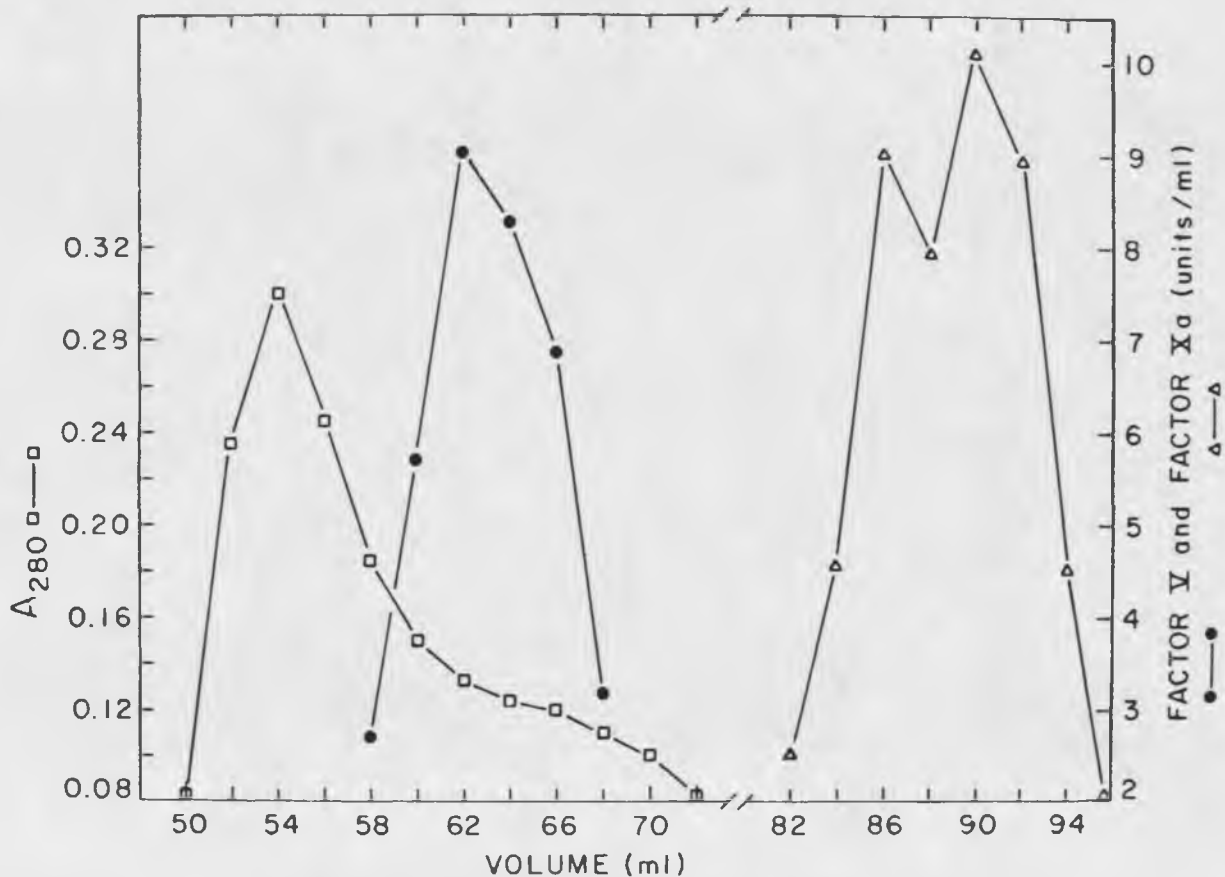


Figure 27. Elution profile of Factor Va^{Xa} and Factor Xa on Sephadex G-200 -- Forty units of Factor V were incubated with 40 μ g of Factor Xa for twenty minutes in the presence of 25 mM CaCl₂. The sample was applied to a Sephadex G-200 column, 1.6 x 55 cm at 4°C at a flow rate of 0.17 ml/min. The sample was eluted with Michaelis-0.025 M CaCl₂, pH 7.35, and 2.0 ml fractions were collected and analyzed for Factor V activity (●—●), Factor Xa activity (Δ—Δ), and protein (□—□).

Activation of Factor V by Factor Xa in the presence of both calcium ions and phospholipid followed by chromatography on the Sephadex G-200 column described above, resulted in a different elution profile of clotting activities. Factor V and Factor Xa eluted together with phospholipid, in the void volume in this case. Gel filtration of the same mixture in the absence of calcium or after treatment of the complex with EDTA, resulted in the separation of Factor Xa from the Factor V-phospholipid complex. Similar observations were made by Barton et al. (1967). As a result, chromatography of this type of Factor V activation mixture did not indicate any changes in the molecular weight of Factor V, since both Factor V and Va bind to phospholipid, in the absence of calcium and these complexes elute in the void volume of a Sephadex G-200 column.

SDS Gel Electrophoresis of Factor V and Factor Va-
Thrombin, Factor Xa and RVV-V

In order to determine whether any changes in the subunit composition of Factor V occurred upon activation by Factor Xa, thrombin or RVV-V, samples of Factor V were treated with one of these activators and then subjected to SDS electrophoresis under reducing conditions, together with a control or untreated sample of Factor V. Factor V was incubated with 5-10 μ g of either Factor Xa, thrombin or RVV-V at 25°C and pH 7.5 for 10-15 minutes along with a Factor V control to which no additions were made. Also a

second sample of Factor Va^{RVV-V} was prepared as described, but after the incubation period Factor Va was isolated from the activation by Sephadex G-200 gel chromatography. The samples were then heated in a boiling water bath for 10 minutes to inactivate the enzyme(s) and subsequently prepared for electrophoresis in the presence of SDS and urea as described in Methods.

After staining and destaining the gels, a multiplicity of bands, ranging in molecular weight from 100,000 to 25,000 daltons, were observed in the gels. When a semi-quantitative assessment was made of the relative amounts of each protein species in the gel by integrating the area of each peak, those gels containing both Factor Va^{RVV-V} samples showed a quantitative increase in two protein components of molecular weight 85,000 and 30,000 daltons, with a decrease in a protein band of approximately 105,000 daltons, when compared to the electrophoretic pattern of native Factor V, as shown in Figure 28. The banding pattern observed in those samples activated by Factor Xa prior to electrophoresis appeared essentially the same as the control. And the electrophoretic pattern of Factor Va^{thrombin} showed several bands of molecular weight 20-50,000 daltons and a considerable diminution of all bands greater than 80,000 molecular weight, compared to the control.

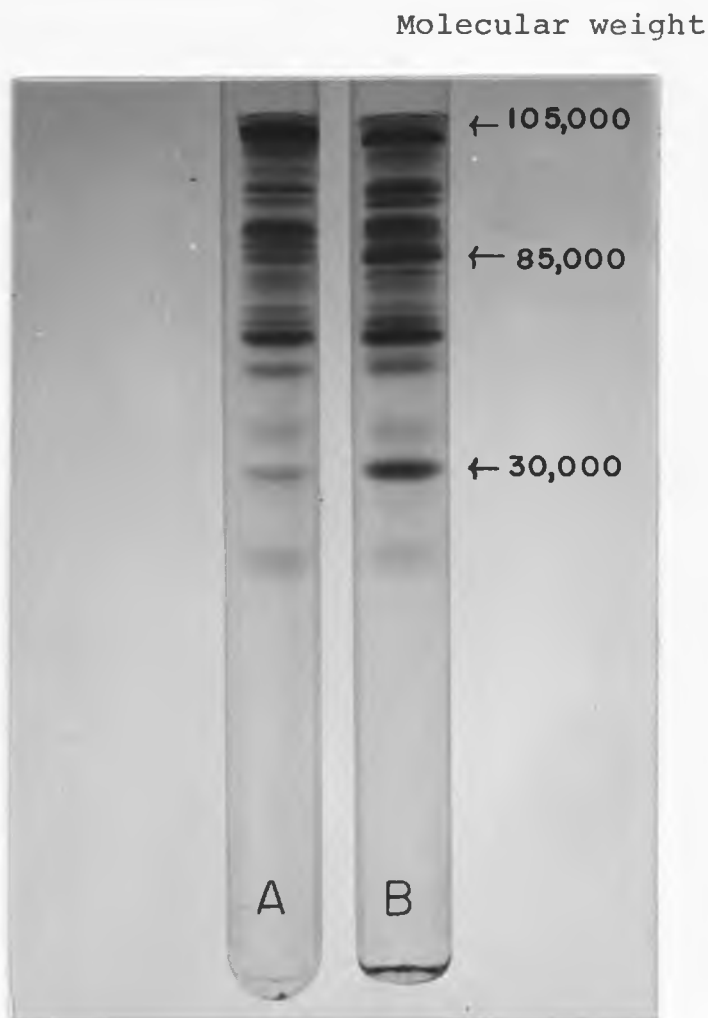


Figure 28. SDS acrylamide gel electrophoresis of Factor V and Factor Va^{RVV-V} -- Fifty μ g of (A) Factor V, and (B) Factor Va^{RVV-V}, purified by Sephadex G-200 column chromatography, both in 2% SDS, and 8 M urea, were electrophoresed on 7.5% polyacrylamide gels containing 0.1% SDS. The samples were reduced with 2-mercaptoethanol (5%) prior to electrophoresis. The gels were stained with Coomassie Blue after electrophoresis. Migration of the bands is from the top (cathode) to the bottom (anode).

Activation of Factor V by Other Proteases:
 α -Chymotrypsin, Trypsin, and Pronase

It was found in this study that Factor V could be modified in its activity by α -chymotrypsin, with results similar to those observed for activation by thrombin, RVV-V, or Factor Xa. Treatment of Factor V with α -chymotrypsin saw a 6-fold increase in Factor V activity over a period of 15 minutes, activation was generally followed by a decided decrease in activity as illustrated in Figure 29.

In order to determine whether the proteolytic activity of α -chymotrypsin was required for it to effect activation of Factor V, the enzyme was treated with TPCK, a specific active site inhibitor (Ryan and Feeney, 1974). A solution of TPCK in methanol was added to a sample of α -chymotrypsin to a 1000-fold molar excess. A control enzyme received an identical aliquot of methanol alone. Both samples were incubated at 37°C for one hour, after which time both were dialyzed against a 1000-fold volume excess of Michaelis buffer, pH 7.35, at 4°C for 12-15 hours to remove excess TPCK. As a result, TPCK- α -chymotrypsin, in an amount of 3 μ g/ml, was unable to activate Factor V when incubated together for at least 20 minutes.

Activation by α -chymotrypsin also resulted in a change in the molecular weight of plasma Factor V. Factor V were treated with α -chymotrypsin at 25°C and pH 7.35, for 25-30 minutes, and a 13-15-fold increase in Factor V

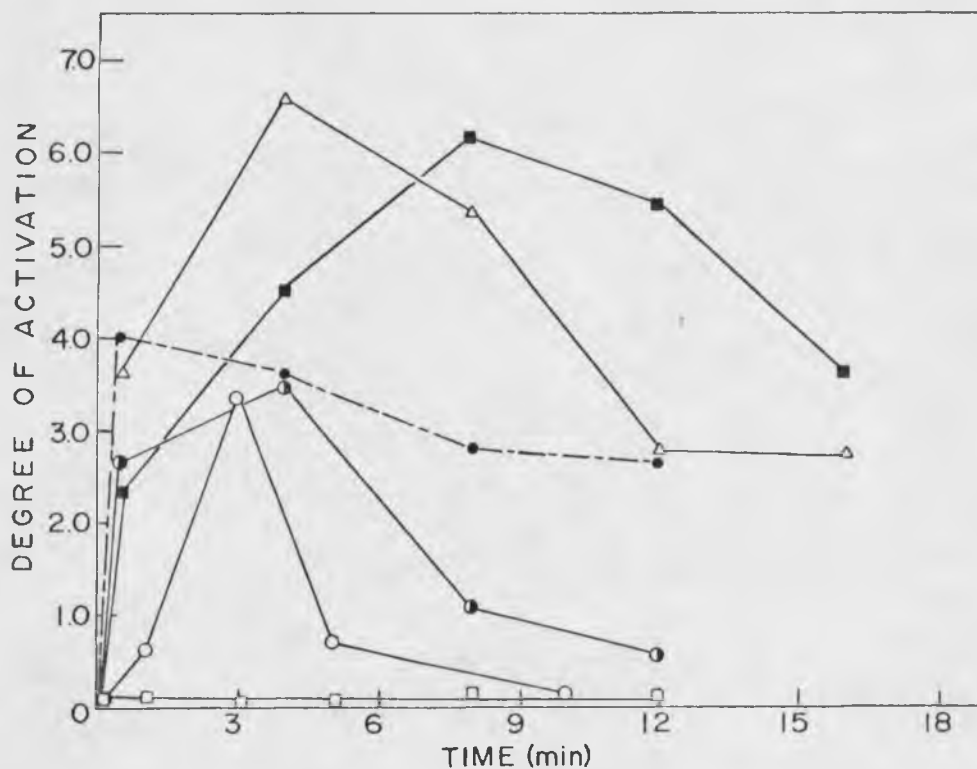


Figure 29. Time course of Factor V activation by α -chymotrypsin -- 2.5 units of Factor V were incubated at 25°C with 3.13 μg (■—■), 6.25 μg (Δ—Δ), 12.5 μg (●—●), 25 μg (○—○), 50 μg (□—□), of α -chymotrypsin, or with buffer alone (□—□), in the presence of 5 mM CaCl_2 . Each sample was assayed periodically for Factor V activity.

activity was observed. The sample was concentrated to 1.0 ml (PM-10 membrane) and applied to a calibrated column of Sephadex G-200. An identical sample of Factor V was applied, without further treatment, to the same column, but four hours previous to the Factor V activation mixture. The elution profile of the control Factor V activity and that treated with α -chymotrypsin are shown in Figure 30. The control Factor V eluted in a volume to give a $K_D = 0.04$ and a corresponding molecular weight of 439,000 daltons, while Factor Va (α -chymotrypsin) eluted to give a $K_D = 0.17$ indicating a molecular weight of 238,000 daltons, when compared to a calibration curve.

Colman (1969b) observed that bovine trypsin only inactivated bovine Factor V. And when Factor V was treated with trypsin, in this study, the results were similar. Treatment of 3-5 units of Factor V, with trypsin in amounts ranging from 0.1 to 1.0 μg , resulted in either no change or a decided loss of Factor V activity over a period of 15-30 minutes.

Pronase had effects similar to trypsin on the activity of Factor V. Incubation of Factor V with 0.1 to 0.5 $\mu\text{g/ml}$ pronase saw essentially no change in activity over a period of 20-30 minutes. At greater concentrations of pronase, up to 10 $\mu\text{g/ml}$, the Factor V activity rapidly declined within 8-10 minutes after addition of the enzyme.

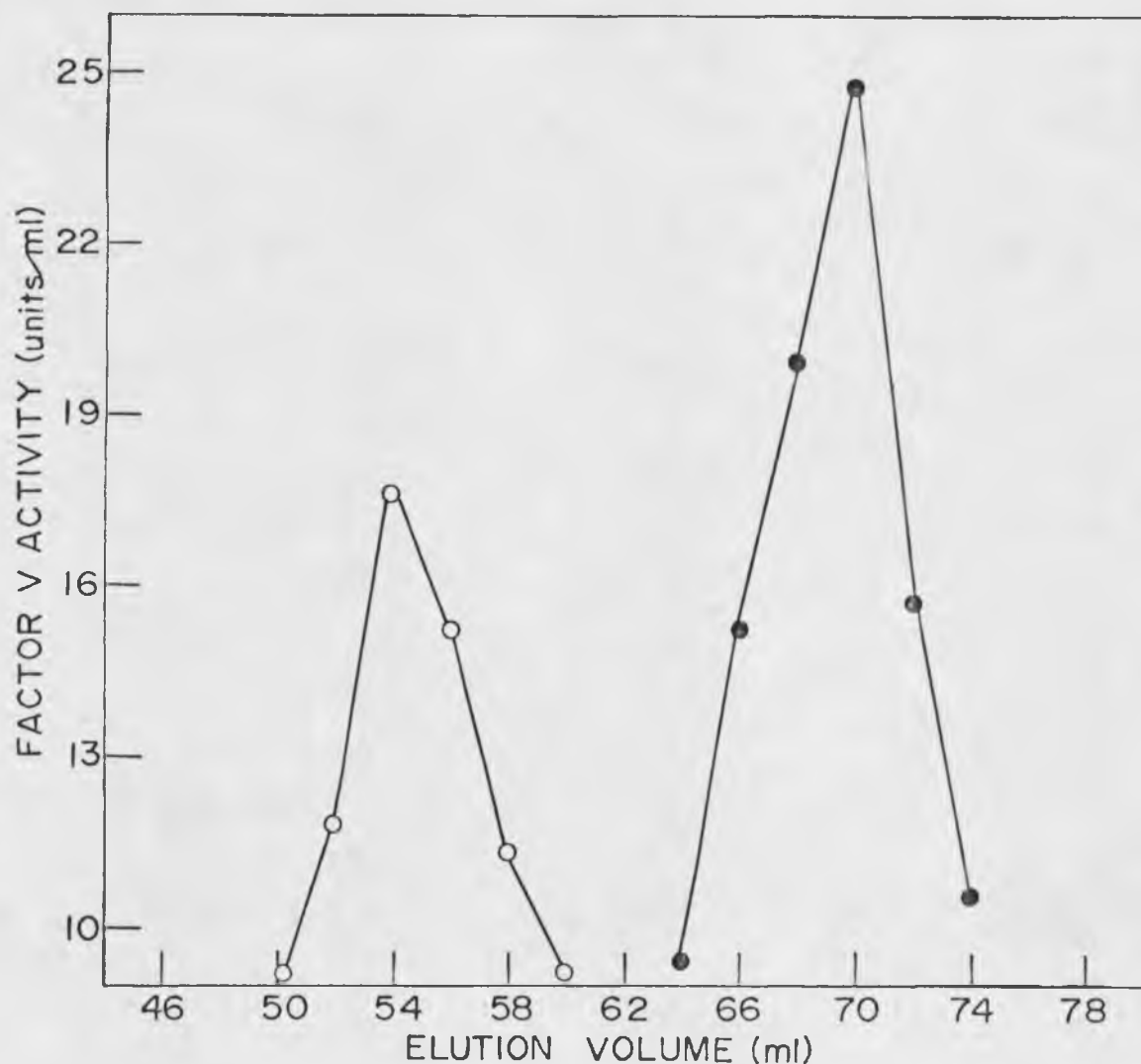


Figure 30. Elution profiles of Factor V and Factor Va (α -chymotrypsin) on Sephadex G-200.-- Twenty units of Factor V (o—o) were applied with no further treatment, or after incubation at 25°C with 6.25 μ g of α -chymotrypsin in the presence of 25 mM CaCl_2 (●—●), to a column of Sephadex G-200, 1.6 x 55 cm. The samples were separately eluted with Michaelis-0.025 M CaCl_2 , pH 7.35 and the eluate assayed for Factor V activity.

Generation of Thrombin from Prothrombin in the Presence
of Factor Xa, Calcium, Phospholipid and Either
Factor V or Va

Optimum thrombin generation, in terms of both the apparent rate and the final yield, was observed when prothrombin was treated with the complete prothrombinase enzyme, consisting of Factor Xa, Factor V, calcium, and phospholipid. When taken individually, only Factor Xa was capable of converting some prothrombin to thrombin; addition of either calcium, or calcium plus phospholipid, or calcium, phospholipid, and Factor V (or Va) markedly increased both the initial rate of conversion as well as the maximum amount of thrombin generated from prothrombin.

As shown in Figure 31, minimal thrombin activity developed when prothrombin was incubated with Factor Xa and calcium, over a period of 36 minutes incubation time. However, thrombin generation was slightly enhanced over the same time period by the further addition of PS:PC (1:1, w/w). And, when either Factor V, or Va, which was formed by the action of RVV-V, thrombin or Factor Xa, were also added to such a mixture, thrombin activity was generated very rapidly, reaching a maximum level in only 20-30 minutes. In this case the yield of thrombin activity was 60-65 times the amount observed in any of the other thrombin generating mixtures described above. Factor V, Factor Va alone, or each in combination with calcium and phospholipid could not

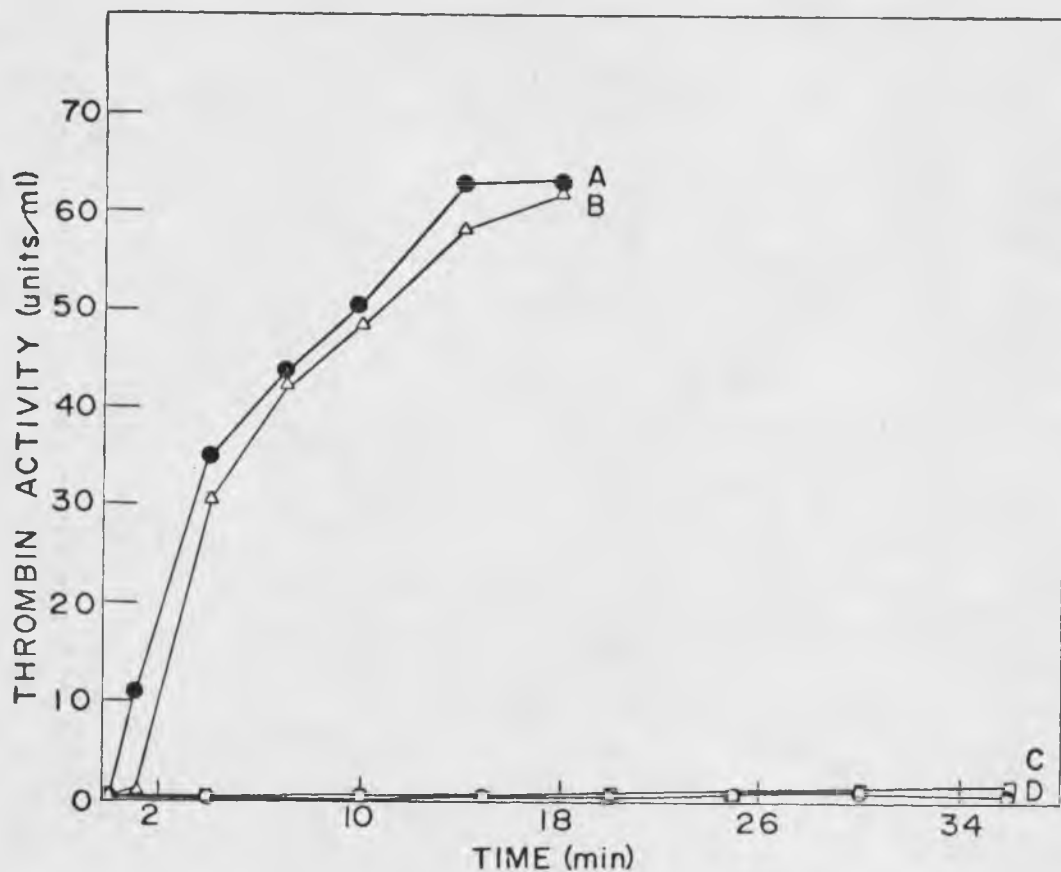


Figure 31. Time course of thrombin generation by various mixtures of Factor Xa, calcium, PS:PC and Factor V or Va -- (A) 123 μ g of prothrombin were incubated with 1.84 μ g of Factor Xa, 0.05 mg/ml PS:PC (1:1, w/w), 10 mM CaCl_2 and 0.25 μ g Factor Va^{RVV-V} (●—●); (B) As (A) except 0.25 μ g Factor Va^{RVV-V} (Δ — Δ) in place of Factor V; (C) As (B) except buffer in place of Factor V; (D) As (C) only buffer in place of PS:PC. After the addition of prothrombin, in each case aliquots of the mixture were removed to assay for thrombin activity.

generate any measurable thrombin activity from prothrombin over 30-40 minutes time at 25°C and pH 7.5.

A difference in the rate of thrombin generation was observed when prothrombin was treated with a complete prothrombinase complex containing Factor V, compared with that containing the same amount of Factor Va protein. In the presence of Factor Va, an immediate burst of thrombin activity occurred within 15 seconds of mixing all the components. This was the shortest time in which an assay for thrombin activity could be initiated. However, in the presence of native Factor V a brief "lag time" of 30-60 seconds was observed before measurable thrombin activity was detected. After the "lag period," however, the rate of formation of thrombin approached that observed in a similar mixture but initially containing Factor Va instead of native Factor V. These observations are shown in Figure 32, where Factor Va was generated by RVV-V activation. Identical results were obtained in this reaction when Factor Va was formed by the action of thrombin or Factor Xa. This difference in the initial rate of thrombin generation was also observed when only phospholipid was omitted from the thrombin forming system. When prothrombin was treated with Factor Xa, calcium and either Factor V or Va, a more obvious difference was observed in the rate as well as the extent of thrombin generation. As shown in Figure 33, thrombin activity was detectible within 4-5 minutes after prothrombin

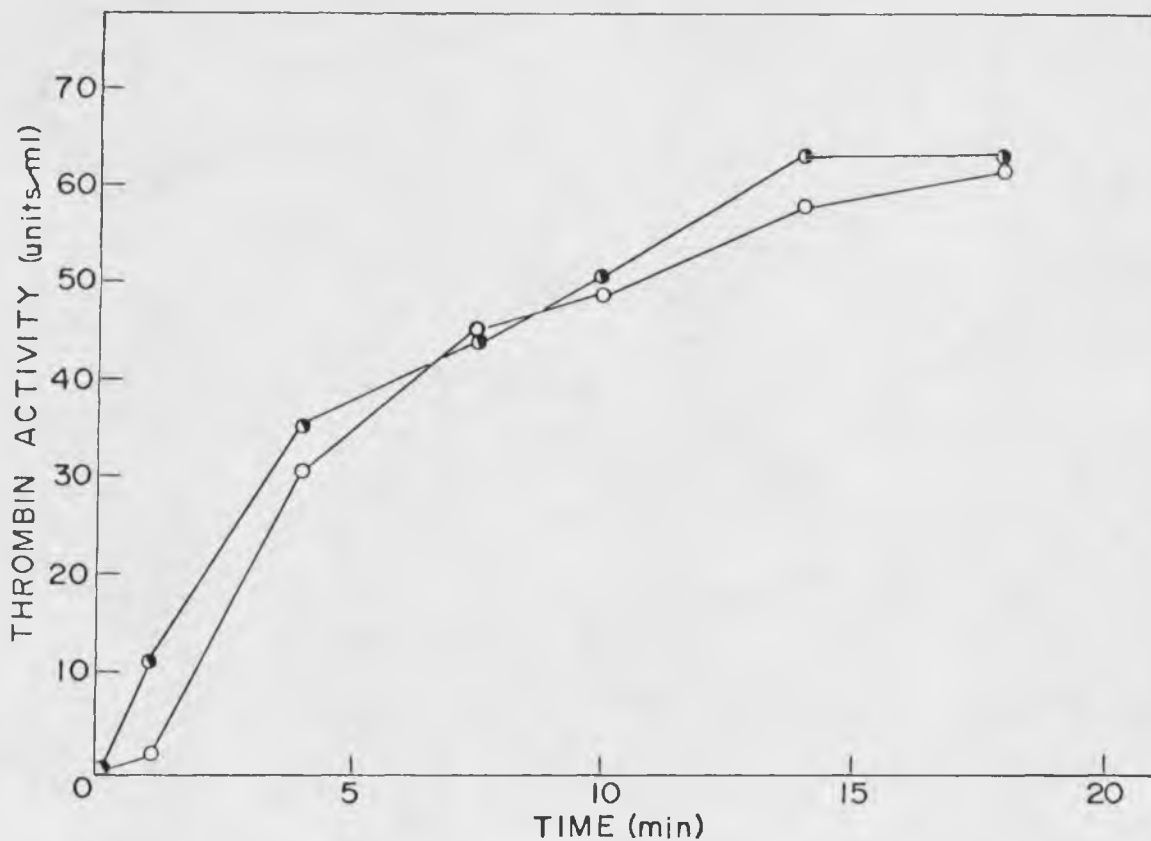


Figure 32. Time course of thrombin generation by Factor Xa, calcium, PS:PC, and either Factor V or Factor Va^{RVV-V} -- 123 μ g of prothrombin were incubated with 1.84 μ g Factor Xa, 10 mM CaCl₂, 0.05 mg/ml PS:PC (1:1, w/w), and either 0.25 μ g Factor V (o—o) or 0.25 μ g Factor Va^{RVV-V} (●—●). Further details are described in Figure 31.

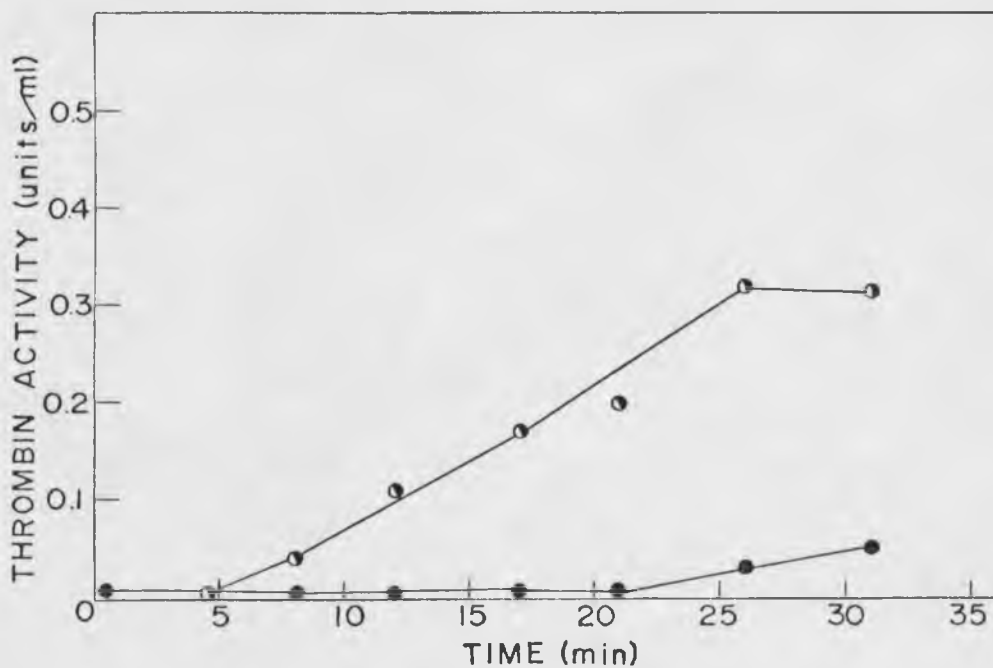


Figure 33. Time course of thrombin generation by Factor Xa, calcium and either Factor V, or Factor Va thrombin -- 100 μ g of prothrombin were incubated with 1.84 μ g Factor Xa, 10 mM CaCl_2 , and either 0.25 μ g Factor V (●—●), or 0.25 μ g Factor Va thrombin (○—○).

was treated with Factor Va plus calcium and Factor Xa, whereas in the same system, but containing Factor V, thrombin activity appeared only after 20 minutes incubation. The yield of thrombin after 31 minutes incubation time was approximately 6.5 times greater in the reaction mixture containing Factor Va, relative to that containing native Factor V. However, if the reactions had been followed for a longer period of time, the final yield of thrombin may have been the same in both.

Conversion of prothrombin to thrombin by a complete prothrombinase enzyme containing Factor Va proceeded at essentially the same rate and to the same extent regardless of how the activated Factor V was prepared. In the current study, Factor V was activated by RVV-V, thrombin or Factor Xa and the Factor Va was subsequently isolated from the activation mixture by gel filtration on Sephadex G-200. Previous workers exploring thrombin formation in the presence of Factor Va, have generated the active form of Factor V by adding the activator, namely thrombin, to the complete prothrombin-forming system, but in small enough quantities so as not to shorten the clotting time of the mixture in the thrombin assay. Consequently, a comparison was made of prothrombin activation by a prothrombinase complex containing Factor Va, either activated by thrombin, which was subsequently separated from the activation mixture by gel filtration, or that activated by a minimal

amount of thrombin which was not separated from the activation mixture before being added to the prothrombinase reaction. When prothrombin was incubated with Factor Xa, calcium, PS:PC (1:1, w/w) and 0.25 µg/ml G-200 purified Factor Va^{thrombin}, or with the same quantity of Factor Va plus thrombin, thrombin activity developed rapidly over a period of 20-30 minutes, at essentially the same rate and to the same extent in each case. The calculatable amount of thrombin actually added to the thrombin-forming mixture as part of the Factor Va sample, was < 0.003 N.I.H. units/ml. This activity was essentially undetectable in the thrombin clotting assay since it did not clot a solution of fibrinogen in less than three minutes.

When the conversion of prothrombin to thrombin was accomplished by a complete prothrombinase containing Factor V, or an equivalent amount of protein which had been converted to Factor Va by one of the above mentioned activators, only a difference in the rate of thrombin generation was observed; the yield of thrombin was the same in each case. When the same reaction mixtures were prepared, only with Factor V and Va present at the same level of Factor V activity, a large difference in both the rate and the extent of activation of prothrombin was observed. In this case the rate and the extent of thrombin generation were considerably depressed in the presence of Factor Va, at approximately 1/20th the protein concentration but the same

amount of activity as native Factor V and, as shown in Figure 34, when the initial amount of Factor Va protein was varied upwards from 12.5 $\mu\text{g/ml}$ to 0.25 $\mu\text{g/ml}$, both the initial rate and extent of thrombin formation were progressively increased. Apparently native Factor V and Va are not equivalent in the total prothrombinase reaction at an equal level of Factor V activity, and the total amount of Factor V protein, whether it consists of Factor V or Va, affects the overall yield as well as the rate of thrombin formation.

In addition, the initial concentration of Factor Xa or of phospholipid each determined both the rate and the extent of prothrombin conversion to thrombin.

When prothrombin was treated with increasing amounts of Factor Xa, at a constant amount of Factor Va^{thrombin}, calcium, and PS:PC, the initial rate of formation and the final yield of thrombin varied in the same direction, as shown in Figure 35.

Likewise, when a single concentration of prothrombin was treated with a constant amount of Factor Xa, calcium and Factor Va^{thrombin}, but with increasing amounts of phospholipid, namely PS:PC (1:1, w/w), both the initial rate of formation and the final yield of thrombin again varied accordingly, as shown in Figure 36.

Only the amount of calcium in the reaction mixture did not contribute to the reaction in a stoichiometric

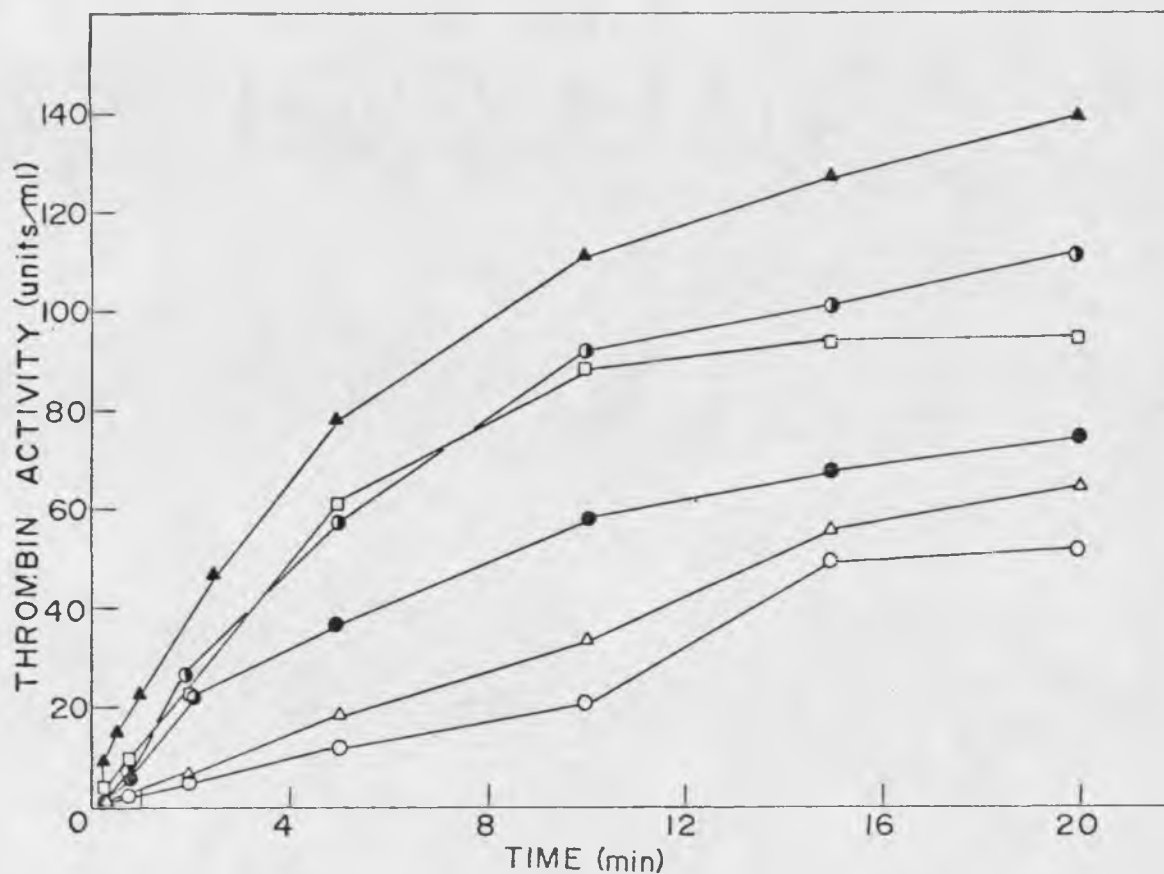


Figure 34. Time course of thrombin generation from prothrombin by Factor Xa, calcium, PS:PC, and either Factor V or varying amounts of Factor vathrombin -- 123 µg of prothrombin were incubated with 1.84 µg Factor Xa, 10 mM CaCl₂, 0.05 mg PS:PC (1:1, w/w), and 0.25 µg Factor V (●—●), or with 0.25 µg (▲—▲), 0.125 µg (□—□), 0.0625 µg (●—●), 0.025 µg (Δ—Δ), 0.0125 µg Factor Va^{thrombin} (○—○). See text for further details.

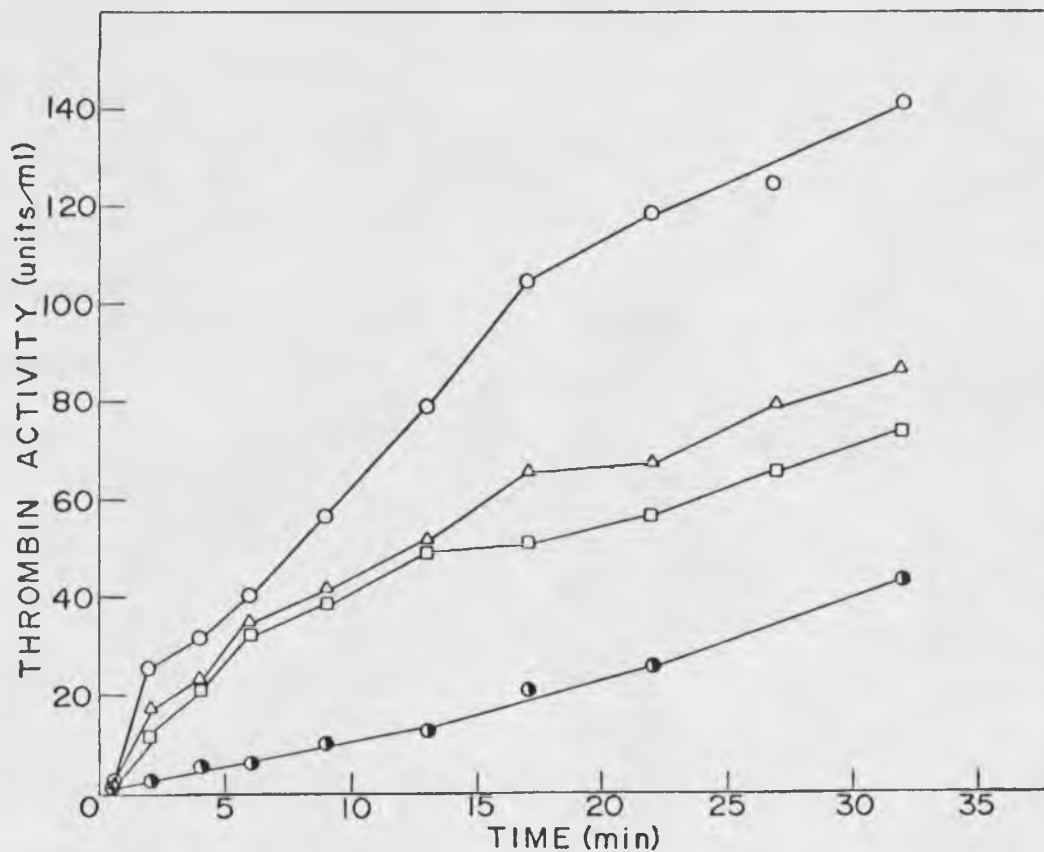


Figure 35. Time course of thrombin generation by Factor $Va^{thrombin}$, calcium, PS:PC, and varying amounts of Factor Xa -- 123 μ g of prothrombin were incubated with 0.25 μ g Factor $Va^{thrombin}$ 10 mM $CaCl_2$, 0.05 mg PS:PC (1:1, w/w) and 9.2 μ g (o—o), 1.84 μ g (Δ — Δ), 0.92 μ g (\square — \square), or 0.46 μ g (\bullet — \bullet) Factor Xa. Further details are exactly as described in Figure 31.

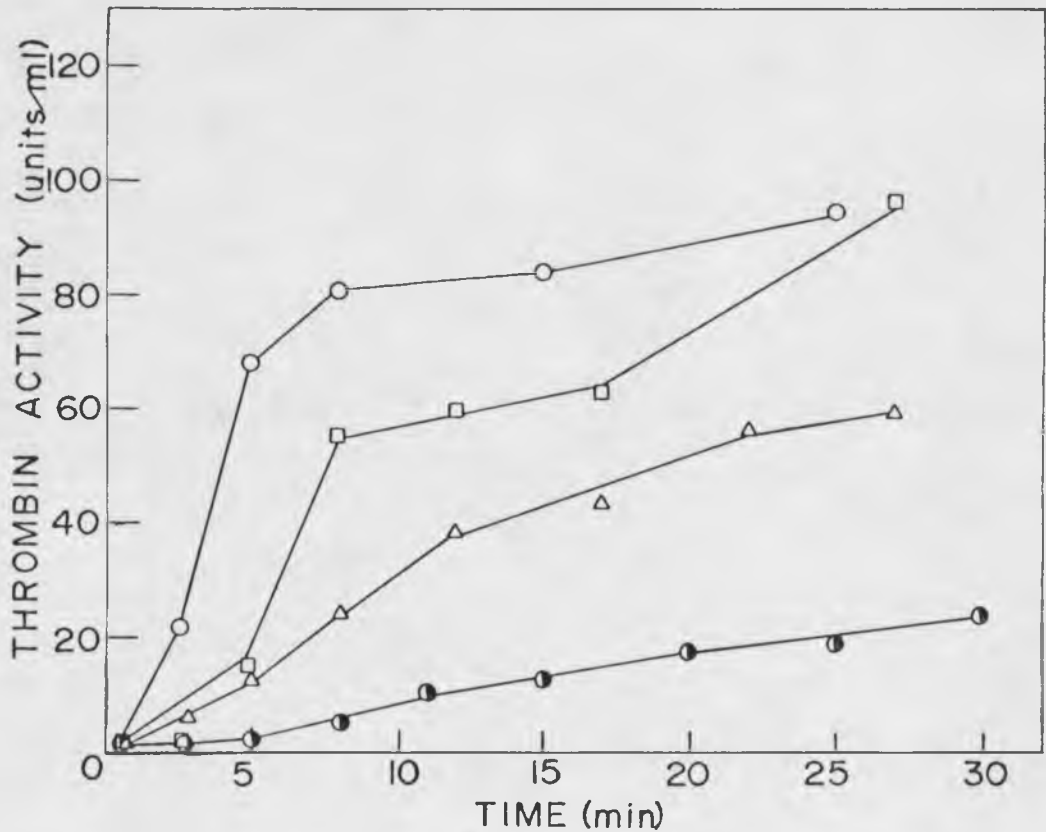


Figure 36. Time course of thrombin generation by Factor Xa, calcium, Factor Va, and varying amounts of PS:PC -- 123 µg prothrombin were incubated with 1.84 µg Factor Xa, 10 mM CaCl₂, 0.25 µg Factor Va (thrombin) and 200 µg (○—○), 100 µg (△—△), 50 µg (□—□), or 16.5 µg (●—●) PS:PC (1:1, w/w). Further details were exactly as described in Figure 31.

fashion. When the calcium concentration was varied, at constant amounts of all the other prothrombinase components, a maximum yield of thrombin was measured in the presence of 10 mM CaCl_2 . Considerable inhibition of both the rate and extent of thrombin formation was observed in the presence of 20 and 40 mM CaCl_2 . In the presence of 5 mM CaCl_2 the reaction proceeded at the same initial rate, but resulted in only about 50% of that yield of thrombin generated in the presence of 10 mM CaCl_2 , after 30 minutes incubation time. If the reaction had been monitored for a longer time the thrombin activity may have approached the same level in both cases.

Finally, when the concentration of the substrate prothrombin was increased at a constant amount of Factor Xa, calcium, phospholipid, and Factor Va^{thrombin}, the initial rate of thrombin formation was the same in all cases, yet a corresponding increase in the amount of thrombin activity generated after 30 minutes incubation time was observed, as illustrated in Figure 37. Prothrombin behaved as a typical substrate in an enzyme catalyzed reaction.

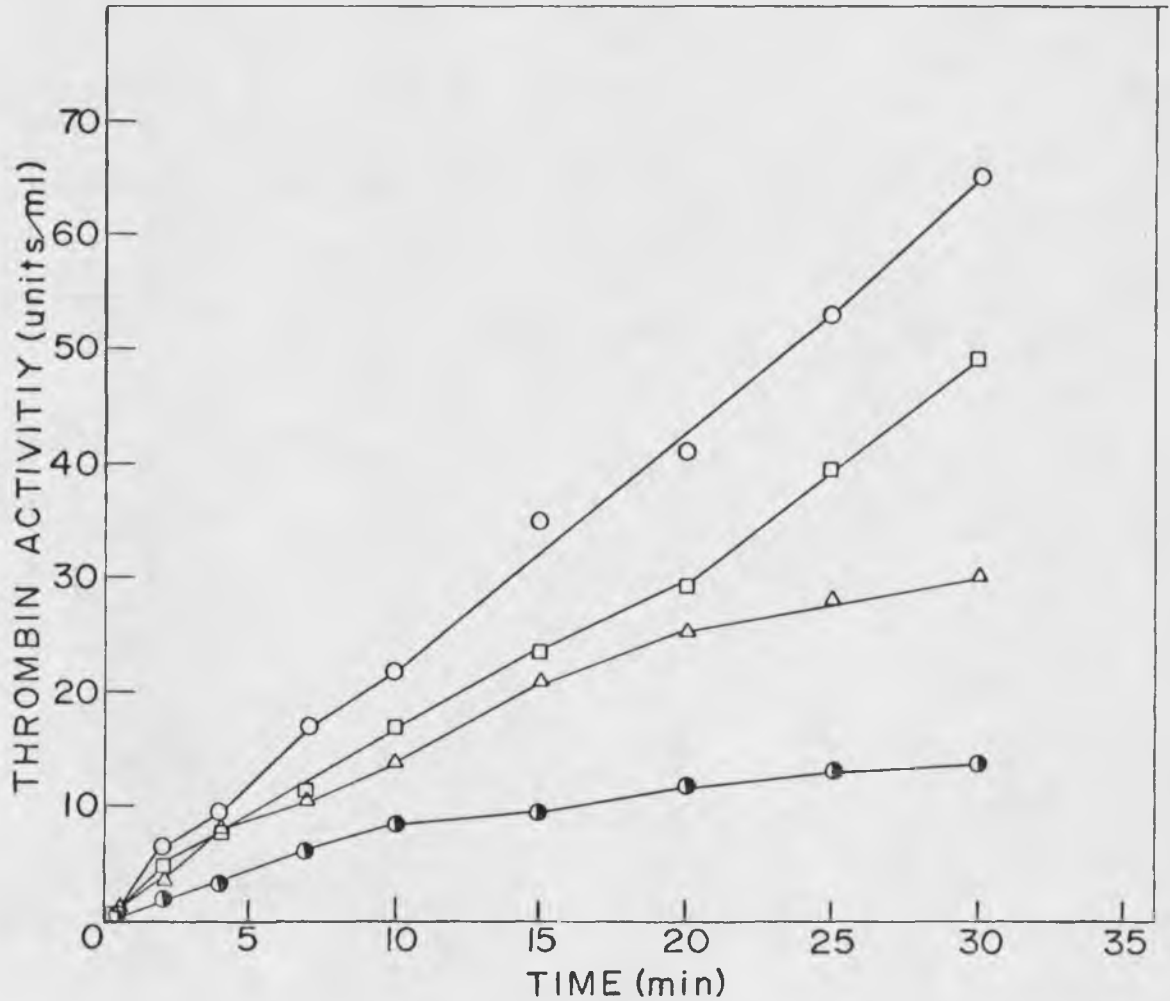


Figure 37. Time course of thrombin generation from varying amounts of prothrombin, by Factor Xa, calcium, PS:PC, and Factor Va -- 1.84 µg of Factor Xa, 10 mM CaCl₂, 0.05 mg PS:PC, and 0.25 µg Factor Va (thrombin) were incubated with 246 µg (○—○), 123 µg (□—□), 61.5 µg (△—△), or 31 µg of prothrombin (●—●). Further details are as described in Figure 31.

CHAPTER 4

DISCUSSION

Isolation of Bovine Plasma Factor V

Bovine plasma Factor V was isolated in good yields and in a stable form by a scheme which consisted of: barium sulfate adsorption of oxalated bovine plasma, QAE cellulose adsorption and elution of plasma Factor V, polyethylene glycol precipitation of the QAEC extract and chromatography of PEG Factor V on a column of desulphated Sepharose 6B. In order to extract Factor V from bovine plasma with an anion exchange adsorbent, blood must be collected in either sodium or potassium oxalate. It is not extracted to a great extent when the blood is collected in sodium citrate, a more commonly used anticoagulant in the collection of blood. Since the solubility of calcium citrate, 0.85 g/100 ml, is considerably greater than that of calcium oxalate, 0.0067 g/100 ml, the former anticoagulant may remove calcium from the Factor V molecule as well as that in solution in the blood, to a greater extent than the latter. Consequently, more negative charges may be exposed which would facilitate the adsorption of Factor V to an anion exchange adsorbent such as QAE cellulose.

Soon after the plasma is separated from the cellular components of the blood, it is important to treat it with barium sulfate immediately, to adsorb out Factors II, VII, IX, and X. Factors II and X are the precursors of two proteases, thrombin and Factor Xa, each of which is capable of activating Factor V, so their early removal from the preparation will lessen the chances for proteolytic conversion of plasma Factor V to its activated form.

Extraction of Factor V from the barium sulfate adsorbed plasma with QAE cellulose is a procedure originally reported by Dombrose et al. (1972). QAE cellulose has a greater capacity for adsorbing Factor V than TEAE cellulose, used originally by Esnouf and Jobin (1967), and separately by Colman (1969a), to prepare bovine Factor V. After Factor V was adsorbed to the QAE cellulose, greater purification was subsequently realized when the calcium acetate buffers of Dombrose et al. (1972) were used to wash the cellulose and elute Factor V than the sodium phosphate buffers described by Esnouf and Jobin (1967). Furthermore, the inclusion of calcium in this isolation step stabilized the Factor V activity and undoubtedly accounted for the greater recovery of QAEC Factor V prepared according to the scheme developed in this study compared to TEAEC Factor V prepared according to Esnouf and Jobin (1967). QAEC Factor V was recovered in a yield of 70% while TEAEC Factor V was recovered in a yield of 50%.

Dombrose et al. (1972) recalculated their barium citrate adsorbed plasma with non-ionic cellulose, at pH 5.9, in order to stabilize Factor V prior to the QAEC adsorption step. However, in the course of developing the present procedure, it was found that recalcification resulted in an increase in the Factor V activity of the adsorbed plasma. Factor V subsequently extracted from the recalculated plasma with QAEC gave multiple peaks of activity when chromatographed on a column of Sephadex G-200. The activity pooled from the column could be activated only 2-4-fold by RVV-V, whereas the QAEC Factor V prepared according to the present procedure could be activated 10-13-fold under the same conditions. So, by omitting the recalcification step and extracting Factor V at neutral pH, a very stable preparation of Factor V was obtained, which consisted of a single molecular weight form when the preparation was chromatographed on Sephadex G-200.

During subsequent steps in the isolation of Factor V, the properties of activatability and molecular weight were monitored along with recoveries of total Factor V activity and protein, in order to evaluate the extent to which purification of Factor V in its native or precursor form was successful. These criteria were satisfied when QAEC Factor V was precipitated with polyethylene glycol and chromatographed on a column of desulphated Sepharose 6B. Polyethylene glycol precipitation was used successfully by

Schmer et al. (1972) to purify bovine plasma Factor VIII, a large protein similar in function to Factor V but in the activation of Factor X to Factor Xa in the coagulation scheme. Day (1975) claimed that PEG precipitation of TEAEC Factor V resulted in non-enzymatic dissociation to a species of molecular weight 276,000 daltons. However, at no point did he carry out his isolation scheme in the presence of protease inhibitors to prevent any enzymatic proteolysis of Factor V which could easily occur in concentrates of partially purified Factor V such as the PEG precipitate. However, in this study, QAEC Factor V did not appear to dissociate to lower molecular weight species upon PEG precipitation since PEG Factor V could be activated 10-fold by bovine thrombin and it eluted from a column of Sephadex G-200 in a single peak of activity close to the column void volume.

The PEG precipitation step was employed primarily to rapidly concentrate Factor V for application to a preparative column of desulphated Sepharose 6B. When QAEC Factor V was concentrated by ultrafiltration there was frequently and sporadically an increase in Factor V activity which was greater than could be accounted for by the degree of concentration alone. This phenomenon was noted also by Day (1975) when he concentrated his Sephadex G-200 Factor V preparation by ultrafiltration prior to its activation by RVV-V. Since ultrafiltration was also very time consuming,

this method of concentrating large volumes of QAEC Factor V was unsatisfactory.

PEG Factor V was purified an additional 4-5-fold by chromatography on a column of Sepharose 6B which had been treated chemically to remove sulfate ester groups attached to the carbohydrate backbone, as described by Porath et al. (1971). Chromatography on Sepharose 6B without prior chemical treatment to remove sulfate groups, did not achieve much additional purification of PEG Factor V. Factor V eluted from this column in multiple peaks of activity which spread over such a large volume, that it was not well resolved from the bulk of the eluting protein, as shown in Figure 38. However, when the same Sepharose was first treated to remove ionizable sulfate groups, subsequent chromatography of PEG Factor V saw a fairly symmetrical elution profile of Factor V activity, in a reasonable volume, and with considerable separation from contaminating proteins so that additional purification of Factor V was realized, as shown in Figure 39. In addition Factor V was recovered from desulphated Sepharose 6B in a yield which was 20% greater than that obtained from the untreated agarose.

Desulphated Sepharose 6B Factor V was similar to PEG and QAEC Factor V in that it consisted of a single molecular weight species when chromatographed on Sephadex G-200, as well as rechromatographed on desulphated Sepharose 6B. Also, upon isolation it could be readily

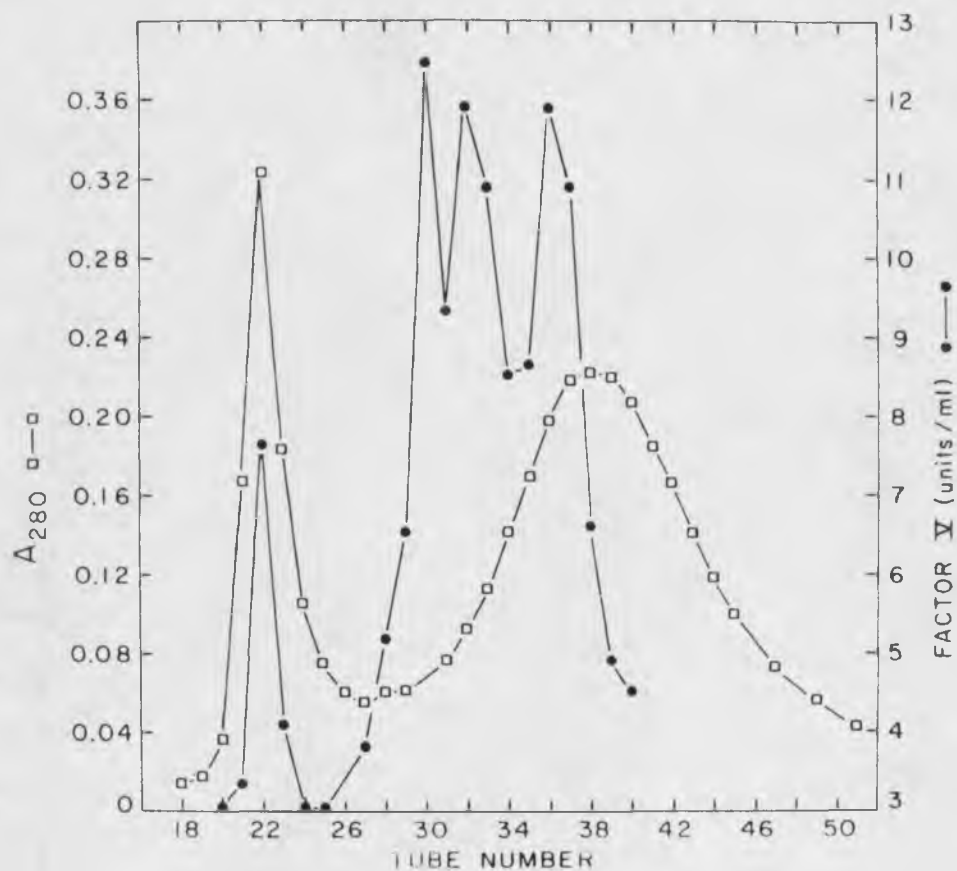


Figure 38. Elution profile of PEG-Factor V on Sepharose 6B -- Thirty ml of QAEC Factor V were precipitated with PEG 6000, dissolved in 1 ml of 0.20 M Tris acetate, 50 mM CaCl₂, 10% glycerol, pH 7.5, and applied to a 1.6 x 55 cm column of desulphated Sepharose 6B at a flow rate of 0.17 ml/min. Two ml fractions were collected and analyzed for Factor V activity (●—●) and for protein (□—□).

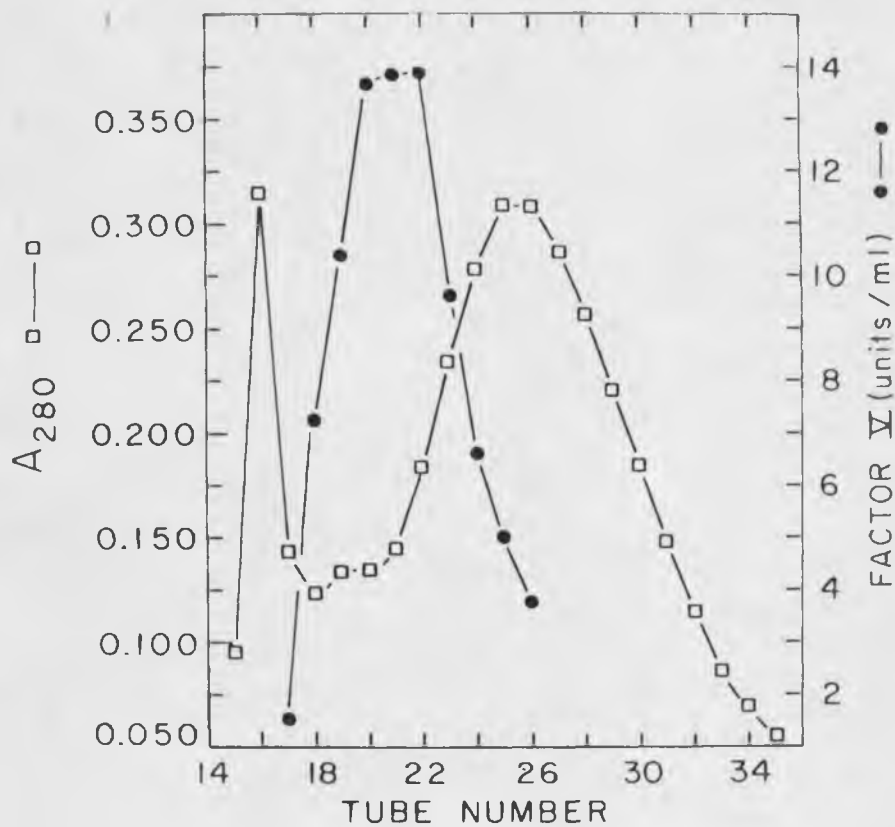


Figure 39. Elution profile of PEG-Factor V on desulphated Sepharose 6B -- Sample and conditions were exactly as in Figure 38 except that desulphated Sepharose 6B was used in place of Sepharose 6B.

activated 8-10-fold by thrombin, Factor Xa, RVV-V, or chymotrypsin and when stored in the isolation buffer, 0.20 M Tris acetate, 50 mM CaCl₂, 10% glycerol, pH 7.5 at 4°C at a protein concentration of 0.3 to 0.5 mg/ml for 3-4 weeks only 10-15% of the original activity was lost and Factor V could still be activated 8-10-fold by RVV-V. Because of its stability and the fact that Factor V was recovered in good yields (39%) by this procedure, the preparation lends itself to further purification of Factor V when newer or more unique techniques of protein isolation become available.

The procedure for isolating bovine Factor V developed in this study could accommodate varying amounts of plasma for work-up. Generally 15-18 liters of bovine plasma were processed through the QAEC step in one day, followed by PEG precipitation and desulphated Sepharose 6B chromatography on the next. Within three days the isolation could be completed. Work-up of large amounts of plasma was hampered only by the time required for centrifugation of whole blood to obtain the plasma, and by the amount of material applicable to the desulphated Sepharose 6B column as one sample.

Bovine Factor V prepared in this manner was free of other clotting activities but was not homogeneous with respect to protein as evidenced by analytical disc and SDS acrylamide gel electrophoresis as well as sedimentation velocity ultracentrifugation, and rechromatography on

desulphated Sepharose 6B. Efforts to further purify Factor V using conventional techniques of protein isolation such as ion-exchange chromatography, preparative disc gel electrophoresis and isoelectric focusing were unsuccessful. When QAEC Factor V was subjected to ion-exchange column chromatography on DEAE or CM cellulose, DEAE-Biogel, or QAE-Sephadex, further purification was not realized due to large losses of Factor V activity and the anomolous manner in which Factor V eluted from any one of these ion-exchange columns. When the ionic strength of the eluting buffer was increased either stepwise, or gradually by applying a gradient, the Factor V activity desorbed in a broad peak which tailed throughout the column effluent. In addition, the bulk of the sample protein co-eluted with the activity so that Factor V was not well resolved from contaminating proteins. The activity recovered could not be activated by any of the enzymes observed to activate native Factor V, and when applied to a calibrated column of Sephadex G-200 any of these Factor V preparations eluted in a volume corresponding to an apparent molecular weight of approximately 200,000 daltons, a value characteristic of activated rather than plasma Factor V. Identical problems were encountered with the other purification techniques tried, such as preparative polyacrylamide gel electrophoresis, isoelectric focusing, or adsorption to and elution of Factor V from phospholipid aggregates.

Dissociation of Factor V during these isolation procedures may be a consequence of the high ionic strength required to desorb it from ion-exchange columns, or the alkaline pH to which Factor V was exposed for long periods of time (15-20 hours) during preparative polyacrylamid electrophoresis. Even when calcium was included in the column or electrophoresis buffers in these procedures, great losses of Factor V activity were still incurred and the elution behavior of both the Factor V activity and accompanying proteins remained anomolous.

Consequently, in order to prepare Factor V in its native or precursor form, so that a study could be made of its activation by various proteolytic enzymes, absolute purification had to be sacrificed except for the exclusion of other clotting factors from the final preparation.

Properties of Bovine Factor V

Bovine Factor V was isolated with a specific activity of 60 U/mg in a yield of 39% representing a 2400-fold purification relative to the starting plasma. The specific activity of this preparation was slightly greater than that of Esnouf and Jobin (1967) or Colman (1969a) who reported a value of 50 U/mg and 49 U/mg with an overall purification of 4000 and 2800, respectively. However, their preparations probably represent a lesser degree of purification since they both isolated a species of Factor V with a

molecular weight of 300,000 which is a dissociative form of plasma Factor V having a higher specific activity than plasma Factor V, according to Day and Barton (1972).

Inasmuch as nearly all of the published preparations contain multiple forms of Factor V, of varying intrinsic specific activities, it is difficult to compare the degree of purification claimed in these preparations with the preparation of Factor V reported in this study.

The molecular weight of Factor V, determined by Sephadex G-200 gel chromatography was $439,000 \pm 4000$ daltons. No other molecular weight forms of Factor V were detected in this preparation. This value agrees well with those reported for partially purified bovine Factor V by Papahadjopoulos et al. (1964) of approximately 400,000; Dombrose and Seegers (1973) of $380,000 \pm 7000$; Kahn and Hemker (1972) of 400,000; and Hanahan et al. (1972) of 400,000.

Philip et al. (1970) found that bovine plasma, collected in the presence of DFP, resolved into two peaks of Factor V activity when chromatographed on Sepharose 4B. These corresponded to forms A and C, with a molecular weight of 300,000 and 38,000, respectively, of the preparation of Colman (1969a). However, it was found in this study that chromatography of unfractionated bovine plasma on a calibrated column of Sephadex G-200 gave only one peak of Factor V activity which eluted close to the void volume to

give an apparent molecular weight of 400,000 daltons. Consequently the isolation procedure for bovine Factor V reported in this study was developed to preserve this form as representative of native or plasma Factor V.

The molecular weight of Factor V estimated by chromatography on a calibrated column of desulphated Sepharose 6B was 538,000 daltons and that of Factor Va^{RVV-V} was 298,000 daltons. These values were reproducible and are significantly higher than those determined on Sephadex G-200. Factor V is almost excluded from the dextran matrix of Sephadex G-200, but can easily penetrate that of Sepharose 6B. Although Sepharose gels have a lower charge density than Sephadex gels, the fact that Factor V is able to penetrate the Sepharose matrix provides an opportunity for greater interaction with it. Moreover, Sepharose contains ionizable sulfate and carboxyl groups which tend to repel a negatively charged protein applied to such a column. Although the Sepharose used in the experiments reported here was altered to remove the sulfate groups, no steps were taken to mask the carboxyl groups. Furthermore, it is likely that Factor V and Factor Va were negatively charged under the chromatographic conditions at which their molecular weights were determined since human Factor V was reported by Rosenberg et al. (1975) to have a pI of 5.1 to 5.4. Consequently both Factor V and Factor Va may have been excluded early from the Sepharose column due to ionic

repulsion between the protein and negatively charged groups on the column even though the ionic strength of the column buffer was made to 0.35 to minimize any such interactions. Although the density of sulfate groups on Sepharose 6B was not quantitated either before or after they were chemically removed, this modification appeared to represent a sufficient change in the matrix to significantly sharpen the elution profile of Factor V from this column. However, it may not be an adequate gel filtration medium for estimation of the molecular weights of these proteins. Crone (1974) observed that two acidic proteins were excluded early from a column of Sepharose 4B at low ionic strength. He attributed this to repulsion between the anionic proteins and similarly charged groups on the gel. However when the ionic strength of the elution buffer was increased the volumes in which these same proteins eluted increased, corresponding to lower molecular weights.

One other explanation for the high molecular weights of Factor V and Factor Va^{RVV-V} on desulphated Sepharose 6B, is that these proteins were aggregating during gel chromatography with the result that they were excluded early from the column. However, this seems unlikely since in any one experiment no more than 0.5 mg/ml protein eluted from the column.

Several techniques used to analyze this Factor V preparation indicated that it was heterogeneous.

Sedimentation velocity analyses of Factor V revealed a rapidly sedimenting and polydisperse system. A sedimentation coefficient of 80, determined for Factor V, is quite high and thereby indicates that protein aggregation may have occurred during the ultracentrifuge run.

Analytical disc gel electrophoresis indicated that there were at least six major components in the Factor V preparation, and the Factor V activity was localized in a single, moderately staining band near the top of the resolving gel. Due to non-specific staining of the Coomassie Blue dye throughout the gel, it was difficult to make a densitometric scan to determine the relative amount of Factor V protein in this preparation. However, a visual inspection of the relative staining intensities of the bands indicated that Factor V was not the major protein constituent of this preparation. This protein pattern was not altered when the preparation was subjected to conditions of ionic strength varying from 0.01 to 0.50. Assuming that the structure of the proteins comprising the Factor V preparation was not altered during analytical disc gel electrophoresis, regardless of the prior conditions of ionic strength, the molecular integrity of these proteins was apparently not affected by the concentration of positive or negative charges in their molecular environment.

In contrast, vanMourik et al. (1974) observed that Factor VIII dissociated at low ionic strength and was stable

in high ionic buffers at neutral pH activity. Analytical acrylamide gel electrophoresis of Factor VIII at an ionic strength of 0.01 M revealed multiple bands of increasing mobility relative to a sample prepared at an ionic strength of 0.14 M, which contained 1-2 bands of low mobility. In addition, reducing the ionic strength of a Factor VIII sample saw a significant loss of activity. These workers concluded that Factor VIII dissociated at low ionic strength due to electrostatic repulsion between its subunits and that the existence of Factor VIII in an aggregated state was promoted by a medium of high ionic strength to neutralize these intramolecular repulsive forces.

Electrophoresis of Factor V in the presence of SDS and urea under nonreducing conditions revealed 5-6 major protein bands while under reducing conditions approximately 13-14 bands were observed. Since this preparation of Factor V was heterogeneous it is difficult to make any conclusions on a possible subunit structure for Factor V.

An important feature of this Factor V preparation was its activatability. The clotting activity of Factor V could be readily enhanced 8-10-fold by several different enzymes, and this was a consistent feature of the preparation. This characteristic and the molecular weight determined for Factor V on Sephadex G-200 were two properties which were preserved in the procedure by which Factor V was isolated from plasma, and were considered characteristic of

the molecular integrity of Factor V in its precursor or plasma form.

In spite of the physical heterogeneity of this preparation of Factor V, it did not contain any other clotting activities, except for trace amounts of Factor VIII. This feature facilitated a study of the direct effects of several proteolytic enzymes, some extrinsic and some intrinsic to the blood clotting scheme, on the activity and molecular size of Factor V, as well as the effect of Factor V, per se, on the conversion of prothrombin to thrombin.

Activation of Bovine Plasma Factor V

Several purified proteolytic enzymes were observed to affect the activity, and in some cases the molecular size of Factor V. Bovine and human thrombin, Factor Xa, RVV-V, and α -chymotrypsin all served to enhance the clotting activity of Factor V 8-10-fold at pH 7.5, and 25°C over a period of 10-20 minutes. Among these activators, bovine thrombin had the greatest specific activity toward Factor V and Factor Xa had the least, when this latter activator was supplemented only by calcium. Activation of Factor V by human thrombin required approximately 10-fold more protein than required for activation by bovine thrombin. This difference probably reflects the different species sources of thrombin.

The specific activity of the Factor V formed by the action of these proteases was the same in each case. Although activation gave a 10-fold increase in the clotting activity of plasma Factor V, when activated Factor V, in each case, was isolated by chromatography of the activation mixture on Sephadex G-200, it had a specific activity 20 times greater than that of its precursor. As can be seen in Figure 27, less than 50% of the sample protein co-eluted with the Factor Va activity peak from the column. The bulk of the protein remained at the column void volume. This material is probably composed of protein contaminants in the Factor V preparation but it could also be an inactive precursor form of plasma Factor V which is not susceptible to activation by any of the above enzymes.

Trypsin and pronase either did not alter the activity of Factor V or produced large losses of activity. Table 3 summarizes the effects of the various proteolytic enzymes tested, on the activity of Factor V, prepared according to the procedure described in this study,

Activation by any of these proteolytic enzymes was generally carried out in the presence of calcium because of the greater stability of Factor V under these conditions. However, activation of Factor V by thrombin or by RVV-V did not require either calcium or phospholipid, as did that by Factor Xa. Removal, or at least reduction of the level of calcium in a Factor V sample by dialysis saw a 50% loss of

Table 3. The effect of various enzymes on bovine Factor V.

	Final Conc. (per ml) ^a	Activate ^b	Inactivate ^c
Bovine Thrombin	0.36 µg	X	X
Human Thrombin	6.0 µg	X	X
Factor Xa	(+ PL) 4 µg 12 µg	X	
RVV-V	2.5 µg	X	
α-chymotrypsin	3.1 µg	X	X
Trypsin	0.1-1.0 µg		X
Pronase	0.1-10 µg		X

^aThree to five units/ml of Factor V were treated with each enzyme, at that concentration indicated in the table, in the presence of 5 mM CaCl₂, at pH 7.35 and 25°C.

^bActivate: A 7-10-fold enhancement of the initial level of activity of Factor V over a period of 10-20 minutes under the conditions described.

^cInactivate: A decline in the initial level of activity of Factor V immediately after mixing with a given reagent or after a 7-10-fold increase in the same activity had been observed, under the conditions described.

activity but the remaining Factor V was apparently intact since it was still a viable substrate for subsequent activation by thrombin or by RVV-V.

The addition of PS:PC (1:1, w/w) to a Factor V activation mixture containing thrombin or RVV-V did not affect the rate or the extent of activation. Factor V can bind to phospholipid even in the absence of calcium (Papahadjopoulos and Hanahan, 1964; Jobin and Esnouf, 1967), but evidently this association did not render it more susceptible to activation by thrombin. And, since thrombin cannot bind to phospholipid with or without calcium (Barton and Hanahan, 1969), the presence or absence of calcium and phospholipid would not be expected to affect the reactivity of this enzyme, per se, toward Factor V.

Activation of Factor V by α -chymotrypsin was not investigated in enough detail to determine the effect of calcium or phospholipid on this interaction.

Activation by Factor Xa did require calcium and was significantly enhanced by the further addition of phospholipid. The contribution of these cofactors to the activation of Factor V mimics those conditions under which prothrombin is converted to thrombin by Factor Xa. However, some activation of Factor V probably does occur even in the absence of calcium, as has been observed for the activation of prothrombin by Factor Xa, but at such a slow rate that the activity of Factor V would probably have to be monitored

for one hour or longer in order to detect a significant change. In the presence of calcium the extent to which Factor V was activated reached a maximum within fifteen to twenty minutes at 25°C, and when the reaction was carried out in the presence of added phospholipid, the initial concentration of Factor Xa could be reduced at least 3-fold to achieve a similar degree of activation within the same time period. The concentration of the enzyme, Factor Xa, contributed to this reaction in a stoichiometric manner. Increasing amounts of Factor Xa, added to a constant amount of Factor V, calcium and phospholipid, gave a corresponding increase in the rate and the extent of activation of Factor V. These observations are inconsistent with the behavior expected for a typical enzyme. In a simple enzyme catalyzed reaction, increasing the initial enzyme concentration affects only the rate of the reaction while the extent of the reaction, or the amount of product formed, is the same in each case. Yet, the extent to which Factor V was activated by Factor Xa was dependent on the initial concentration of the enzyme. Factor Xa then may have a regulatory role as well as a catalytic one in this reaction.

Activated Factor V produced by Factor Xa action was relatively stable especially when the reaction was carried out in the presence of both calcium and phospholipid. Addition of excess Factor Xa in the presence of calcium alone or with calcium and phospholipid did not result in a

large loss of Factor V activity indicating that Factor Xa probably is capable only of limited proteolysis of Factor V.

Activation by thrombin, RVV-V or α -chymotrypsin was generally followed by a loss of Factor V activity. Losses were least in the presence of RVV-V, the Factor V activity declining only 20% after maximum activation was observed. Addition of an excess of RVV-V to Factor V did not accelerate the rate or extent of inactivation so these activity losses were most likely due to an inherent instability of Factor Va compared to its plasma form rather than to further proteolysis by the enzyme.

However, inactivation by thrombin is probably due to unrestricted proteolysis of the Factor V molecule since the addition of excess thrombin to Factor V enhanced both the rate and extent of the activity loss. It was noted by Colman (1969a) that the loss of Factor V activity after thrombin activation could not be prevented by the addition of hirudin, suggesting that Factor Va^{thrombin} is inherently unstable just as Factor Va^{RVV-V} was observed to be.

In view of this dual effect of thrombin on Factor V, it is tempting to speculate that the action of thrombin on Factor V represents a control mechanism for the generation and destruction of this coagulant activity in the intrinsic coagulation pathway.

Thrombin was reported by Legaz et al. (1973) also to activate and destroy the activity of Factor VIII and these

workers speculated that this may be a mechanism for regulating the coagulant activity of Factor VIII.

Inactivation of Factor V was most pronounced in the presence of α -chymotrypsin. The interaction of α -chymotrypsin and Factor V was not studied in great detail so it is difficult to conclude why inactivation as well as activation occurs in the presence of this enzyme, other than to advance those reasons cited above, namely the instability of Factor Va relative to plasma Factor V, and extensive proteolysis of Factor V by the enzyme.

Activation of Factor V by each of the four enzymes listed above also resulted in a considerable reduction in the molecular size of plasma Factor V, as estimated by gel filtration on Sephadex G-200. Activation of plasma Factor V, with an apparent molecular weight of 439,000 daltons, produced a new, more active species of Factor V with an apparent molecular weight of 240-250,000 daltons. Table 4 lists the molecular weight of Factor Va as produced by the action of thrombin, Factor Xa, RVV-V, and α -chymotrypsin.

The molecular weight of Factor Va^{thrombin} determined in this study agrees well with that reported by Papahadjopoulos et al. (1964) for bovine Factor V and the values reported by Kahn and Hemker (1972), who found similar changes in both bovine and human Factor V upon activation by thrombin.

Table 4. Molecular weights of Factor Va formed from plasma Factor V by various proteolytic enzymes, as determined by Sephadex G-200 gel chromatography.

Form of Factor Va	Molecular weight (daltons)
Factor Va ^{thrombin}	239,000 \pm 4000
Factor Va ^{RVV-V}	250,000 \pm 4000
Factor Va ^{Xa}	246,000 \pm 4000
Factor Va ^{α-chymotrypsin}	238,000 \pm 4000

The molecular weight of Factor Va^{RVV-V} reported in this study is very similar to values reported by Kahn and Hemker (1972) for both human and bovine Factor Va^{RVV-V}, and by Hanahan et al. (1972) for bovine Factor Va^{RVV-V}.

A report by Day (1975) indicated that Factor Xa could alter the molecular weight of plasma Factor V. He prepared Factor Va by activation of a TEAEC extract of bovine plasma Factor V with Factor Xa, and as a result, he observed a change in the molecular weight of plasma Factor V by Sephadex G-200 gel chromatography, essentially identical to that observed in the current study.

The activation of Factor V by α -chymotrypsin is unique to this study also and no comparative values are available on the molecular weight of Factor Va generated from its plasma precursor by this enzyme.

Activation of Factor V by thrombin, RVV-V, Factor Xa, or α -chymotrypsin, probably occurs by proteolysis of the native protein, but the lack of a homogeneous preparation of Factor V made it difficult to correlate activation with specific structural changes in the Factor V molecule. SDS acrylamide gel electrophoresis of plasma Factor V revealed 13-14 protein bands ranging in molecular weight from 105,000 to 30,000 daltons while the electrophoretic pattern of Sephadex G-200 purified Factor Va^{RVV-V} revealed a similar distribution of protein bands, but changes were apparent in the intensity of a few discrete bands. A band of 105,000 daltons appeared to decrease in intensity while bands of 85,000 and 30,000 daltons appeared to increase in intensity. But, because of the uncertainty as to which bands comprise the Factor V molecule and which are merely contaminants, it is difficult to conclude that the relative changes in the electrophoretic pattern of Factor Va^{RVV-V} actually reflect alterations in the subunit structure of plasma Factor V as a result of activation. Because RVV-V appears to be specific for Factor V, among the coagulation proteins, it is possible that these changes are indicative of proteolysis of Factor V upon activation by RVV-V. However, conclusions on the subunit structure of Factor V or on those molecular events which occur when plasma Factor V is converted to activated Factor V must await the availability of a homogeneous preparation of plasma Factor V.

In addition to altering the specific activity of Factor V, as well as its molecular weight, thrombin, Factor Xa, RVV-V and α -chymotrypsin appeared to exert their action on Factor V in an enzymatic manner. A number of kinetic and chemical data on the activation of Factor V by each of these enzymes support this conclusion and these are discussed in the following section.

Mode of Activation of Factor V

Each of the enzymes shown to activate Factor V in this study were chemically treated with inhibitors which have been demonstrated to inactivate serine proteases. When either thrombin or Factor Xa was treated with DFP both of their individual clotting activities were inhibited as was their ability to activate Factor V. Thrombin was inhibited relatively easily while Factor Xa was inhibited only after prolonged exposure to high concentrations of DFP. Since DFP has been shown to specifically label a serine residue in the active site of these enzymes without causing any gross structural alterations, these experiments unequivocally demonstrate that the activation of Factor V by thrombin or by Factor Xa is an enzymatic process.

In addition both of these enzymes were inactivated by conditions of extreme heat. This treatment results in a physical denaturation of the enzymes so it was not

unexpected that they were subsequently unable to activate Factor V. In contrast RVV-V was not inhibited by either DFP, or heat treatment in its ability to activate Factor V. This is an extremely stable enzyme as was noted by Hanahan et al. (1972), who reported that partially purified RVV-V was stable to heating at 95°C over a pH range of 1.0 to 7.0. However the resistance of RVV-V to inactivation by DFP is in contrast to the reports of Esmon and Jackson (1973) and Esmon, Owen, et al. (1973) who reported that RVV-V activity in the crude venom and in highly purified preparations was inhibitable by DFP. It is unfortunate that RVV-V was not inhibited by DFP since such a phenomenon would serve to classify RVV-V as a serine protease together with the other enzymes shown to activate Factor V in this study, and would directly demonstrate that the action of RVV-V on Factor V was enzymatic.

Thrombin was also inhibited by TLCK, which has been demonstrated to modify a histidine residue in the active site of this enzyme (Glover and Shaw, 1971). Both the clotting activity of thrombin and its ability to activate Factor V were abolished by TLCK. In contrast the activity of Factor Xa was not affected by TLCK or by TPCK, in agreement with the previous observations of Fujikawa et al. (1972). As a result of these observations the specificity of thrombin and Factor Xa are to some degree differentiated.

The ability of α -chymotrypsin to activate Factor V was inhibited by TPCK which has been demonstrated to modify a histidine residue specifically in the active site (Schoellmann and Shaw, 1963). Activation of Factor V with α -chymotrypsin was very reproducible yet a similar serine protease, trypsin, only inactivated Factor V presumably as the result of random proteolyses. These observations contrast with those of McKee, Andersen, and Switzer (1975) who found that the activity of human Factor VIII was not altered by low concentrations, or was only diminished by high concentrations of trypsin. It is likely that Factor VIII and Factor V are structurally related since they have similar functions in the coagulation scheme. It is likely then that they would be activated by a common mechanism but the reportedly opposing effects of α -chymotrypsin and trypsin on these two proteins may indicate somewhat different requirements for their activation.

Further evidence supporting an enzymatic mode of action of these several proteases in activating Factor V comes from a number of kinetic data obtained for each of these interactions.

Activation of a second aliquot of Factor V added to a mixture of Factor V and either thrombin, RVV-V, or Factor Xa after activation of the initial Factor V was complete, saw a second enhancement of Factor V activity which proceeded to the same extent as the first in each case.

except for that with thrombin. Here degradation of Factor V may have obscured the second activation. These observations would indicate that the enzyme in each case was not inactivated but was still viable after activation of the first amount of Factor V, to subsequently activate the second.

In the case of thrombin activation, the addition of increasing amounts of thrombin to a constant amount of Factor V resulted in a corresponding increase in the apparent rate of activation but the extent of activation of Factor V was the same regardless of the initial concentration of thrombin. And in the case of activation by RVV-V, the addition of a constant amount of RVV-V to varying amounts of Factor V saw the same apparent rate of activation in each case yet the extent of activation increased in proportion to the initial amount of Factor V added. These separate observations indicate that both thrombin and RVV-V are each behaving as a true enzyme in activating Factor V.

When Factor V was treated with Factor Xa or with RVV-V in the presence of calcium and the activated Factor V formed was subsequently isolated from the activation mixture by chromatography on Sephadex G-200, the activator in each case was well separated from Factor Va.

Factor Xa not only eluted in a volume separate from Factor Va^{Xa} but also in one which was essentially the same as that in which a sample of Factor Xa alone eluted from the

same column. Moreover nearly 80% of the Factor Xa activity applied on the column was recovered and when it was pooled, concentrated and subsequently mixed with a fresh aliquot of Factor V, at least a five-fold activation ensued.

Similarly ^{125}I -RVV-V was quantitatively recovered in a single peak well separated from both Factor V and Factor Va^{RVV-V} on a similar column of Sephadex G-200. Furthermore the ^{125}I -RVV-V eluted in a volume near the column inclusion volume which was comparable to that in which unlabeled RVV-V eluted on the same column. And when the ^{125}I -RVV-V was pooled and added to a fresh aliquot of Factor V significant activation was observed. From each of these experiments it is obvious that neither Factor Xa nor RVV-V form a complex with Factor V as a result of activating it. Furthermore neither enzyme is consumed in this reaction since they can be quantitatively recovered from their individual reaction mixtures. And, the enzymatic function of each was not appreciably altered since both were fully capable of additional activation of fresh substrate.

Generation of Thrombin from Prothrombin in the Presence of Factor Xa, Calcium, Phospholipid, and Either Factor V or Factor Va

Enhancement of the clotting activity of Factor V implies that it is a more reactive participant in the scheme of blood coagulation. However, the significance of the conversion of plasma Factor V to a more active form was

determined by comparing the effect of plasma Factor V versus Factor Va on the rate and extent of conversion of prothrombin to thrombin in an in vitro system containing highly purified clotting factors.

Previous investigators (Jobin and Esnouf, 1967; Barton et al., 1967; Esmon et al., 1974a, 1974b) observed that the conversion of prothrombin to thrombin occurs at the fastest rate and to the greatest extent in the presence of a complete prothrombinase enzyme consisting of Factor Xa, calcium, phospholipid and Factor V. Only one component, namely Factor Xa, can convert prothrombin to thrombin alone; neither calcium, phospholipid, nor Factor V alone, or when in combination, cannot effect the production of thrombin activity from prothrombin.

In this study similar observations were made: The rate of conversion of prothrombin to thrombin by Factor Xa was enhanced 2-fold by the addition of calcium, while the subsequent addition of phospholipid enhanced the reaction about 50-fold. The further addition of Factor V of Factor Va tremendously enhanced both the apparent rate and the extent of thrombin formation approximately 1000-fold, yet neither form of Factor V alone was capable of generating thrombin from prothrombin. If plasma Factor V was added to a mixture of Factor Xa, calcium and phospholipid (PS:PC) followed immediately by the addition of prothrombin, a brief lag period of 30-60 seconds was observed before

thrombin activity was detectable. However when Factor Va, as formed by the action of Factor Xa, thrombin or RVV-V, was added in place of plasma Factor V, thrombin activity was measurable immediately. These observations were similar to those of Prentice et al. (1967) who subsequently concluded that plasma Factor V was inactive and had to be altered by thrombin to participate in the prothrombinase reaction. However, Giddings and Bloom (1975) demonstrated that for a given amount of phospholipid, less protein in the form of Factor Va was required to saturate it than was observed for the same amount of protein in the form of Factor V. If this indicates a greater affinity of Factor Va for phospholipid as compared with plasma Factor V, then the brief lag period observed here and by previous investigators may reflect a time dependent orientation of plasma Factor V on the phospholipid surface which is not required for Factor Va because of its greater intrinsic affinity for phospholipid.

If this lag period reflects the time required for Factor V to be activated by thrombin, then some thrombin must be generated before Factor V can participate in the prothrombinase reaction, and consequently, this step would be a bottleneck in the formation of the prothrombin activator.

Activation of Factor V by thrombin constitutes a feedback mechanism which not only stimulates its own formation by converting plasma Factor V to Factor Va, but

also limits this reaction by subsequent degradation of Factor V. Since thrombin cannot bind to phospholipid in vitro, it must be released into solution from the prothrombinase complex as it is generated from prothrombin. Therefore it must diffuse back to the surface where Factor V is adsorbed in order to activate and/or inactivate it. However it was demonstrated in this study for the first time that Factor Xa can activate Factor V, and this reaction occurs most efficiently in vitro under the same conditions where Factor Xa converts prothrombin to thrombin. Activation of Factor V in the presence of calcium and phospholipid required approximately three times less Factor Xa relative to Factor V than that used in formation of the prothrombin-converting principle. Maximal activation of Factor V under the former conditions required 10-15 minutes at 25°C but if the amount of Factor Xa in this reaction was increased so that the relative proportion of Factor Xa to Factor V was the same as in the thrombin generation experiments, one would expect activation of Factor V to be completed much faster. Consequently it is not unreasonable that the lag period represents that time required for activation of Factor V, not by thrombin, but by Factor Xa. And once it is activated, Factor V may or may not require a time dependent re-orientation on the phospholipid surface. This reaction not only constitutes a new interaction in the blood coagulation scheme but also

provides a feed-forward type of mechanism to accelerate the formation of thrombin, which in turn converts fibrinogen to fibrin. In view of the role of phospholipid, to accelerate the activation of Factor V as well as prothrombin by Factor Xa, the apparent conditions under which Factor V and thrombin would interact in vivo, make for a sluggish reaction by comparison. Consequently the extent to which this reaction would occur in vivo, where Factor V is bound to the surface of the platelets, seems remote.

The interaction of Factor Xa and thrombin with Factor V, in the framework of the prothrombinase reaction is illustrated in Figure 40.

The difference in the rate of thrombin generation when plasma Factor V was initially present compared to that when Factor Va was initially present, is similar to that reaction in which Factor X is converted to Factor Xa in the intrinsic system by Factor IXa, calcium, phospholipid, and either Factor VIII, or Factor VIIIa. It was observed by Rapaport and his associates (1963, 1966) that Factor Xa activity evolved at a faster rate when Factor VIII was pre-treated with thrombin before it was mixed with Factor IXa, calcium and phospholipid, than when Factor VIII was added in its native form. These workers concluded that Factor VIII as such, is essentially inactive in coagulation and thrombin modification is required for its participation in the intrinsic scheme and it may be that Factor IXa, analogous

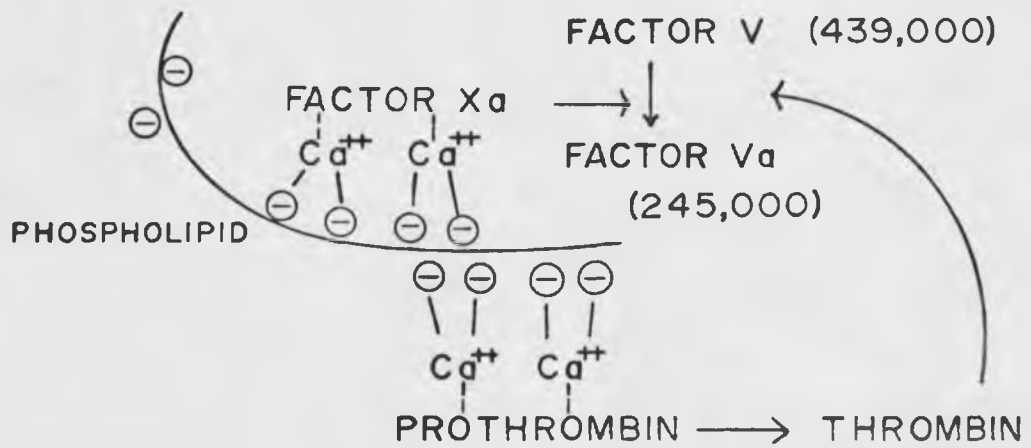


Figure 40. The interaction of Factor Xa and thrombin with Factor V, in the framework of the prothrombinase reaction.

to Factor Xa in the prothrombinase reaction, can convert Factor VIII to Factor VIIIa.

When the contribution of Factor V and Factor Va were compared in the absence of added phospholipid, a more significant difference was observed in the rate and extent of thrombin formation. A lag period occurred in both reactions before thrombin activity was observed but it was only 4-5 minutes when Factor Va was added initially, compared to 21 minutes when plasma Factor V was added initially and once thrombin formation ensued, it occurred at a greater rate and to a greater extent in the presence of Factor Va than in the presence of Factor V. From these observations it seems that each form of Factor V must have some direct effect on either Factor Xa or prothrombin, or both. However, separate attempts to demonstrate complex formation between Factor V, or Factor Va and Factor Xa, or between Factor V, or Factor Va and prothrombin were unsuccessful. As discussed in a previous section, when Factor V and Factor Xa are mixed in the presence of calcium and subsequently chromatographed on a column of Sephadex G-200 in the presence of calcium, the two activities are completely separable. Similarly when Factor V or Factor Va is mixed with prothrombin in the presence of calcium and subsequently chromatographed under the same conditions as the Factor V-Factor Xa mixture, the Factor V and prothrombin activities elute separately from the column.

However, when phospholipid is added to the Factor V-Ca²⁺-Factor Xa mixture or to the Factor V-Ca²⁺-prothrombin mixture, prior to chromatography, both proteins in each case elute in the same volume and together with the phospholipid at the column void volume.

These experiments indicate that neither Factor V and Factor Xa, nor Factor V and prothrombin have a strong affinity per se for each other, at least in an in vitro system. Phospholipid then must serve as the cohesive force between them, providing a surface for the local concentration of these proteins in order to react in a most efficient manner. This feature is undoubtedly important when extensive coagulation occurs in vivo and the platelet membrane is altered in such a way so as to expose, or make available a phospholipid surface for adsorption and thereby interaction of the procoagulant proteins.

In spite of what the actual role of Factor V or Va might be in the prothrombinase reaction, Factor Va appears to be a more efficient cofactor than Factor V in this reaction. Whether Factor V must be activated before it can participate in the prothrombinase reaction, is still a matter for speculation. If this is the case, it occurs very rapidly in vitro, within 30-60 seconds as observed in this study; if not, plasma Factor V is less reactive than Factor Va only during this initial time period, since thereafter the rate and extent of thrombin formation are

the same whether plasma Factor V or Va was initially added to the reaction.

However when Factor V and Va were compared on an equal activity basis, that is, when the same number of units of Factor V or Factor Va were added to a prothrombin-forming system, Factor Va was found to be considerably less efficient in enhancing the formation of thrombin than plasma Factor V. As the amount of Factor Va initially added was increased from 1/20th of that amount of Factor V used in the same reaction, to an equivalent amount of Factor V, on a protein basis, both the rate and the extent of thrombin formation progressively increased. Although Factor Va has a specific activity twenty times greater than plasma Factor V, these findings suggest that Factor Va is not twenty times more reactive in the prothrombinase reaction. Instead, the mass of Factor V added to the reaction is important since it has a role in determining both the rate and the extent of thrombin formation, regardless of whether it is initially in the form of Factor V or Factor Va. Factor V then participates in this reaction in a stoichiometric fashion. Likewise, both Factor Xa and phospholipid participate in the formation of thrombin from prothrombin in a stoichiometric fashion. When the initial concentration of Factor Xa or phospholipid was varied, independently of the other prothrombinase components, both the rate and extent of thrombin formation varied accordingly, and,

regardless of the initial amount of prothrombin substrate added to a constant amount of the prothrombinase enzyme complex, the rate of thrombin formation was constant but the amount of thrombin formed varied accordingly.

These observations indicate that the amount of thrombin generated from a given amount of prothrombin by a complete prothrombinase enzyme complex can vary with the individual concentration of Factor Xa, Factor V, and phospholipid, initially added to the reaction mixture. Similar observations were made by Giddings and Bloom (1975), and separately by Rosenberg et al. (1975). The effective concentration of both Factor Xa and Factor V in the reaction is determined by the number of binding sites available for each protein on the phospholipid surface, or on the extent to which each factor can saturate these sites. Phospholipid then enhances the conversion of prothrombin to thrombin not only by providing a surface to effect the local concentration of prothrombin, Factor Xa, and Factor V, but also controls the rate and extent of thrombin formation by limiting the amount of each of these factors which can bind to it and thereby participate in the reaction.

How Factor V or Va exerts such a tremendous effect on the prothrombin conversion reaction remains a provocative question. Factor V may enhance this reaction by increasing the reactivity of Factor Xa toward its substrate, prothrombin. If so, this interaction is mediated only in the

presence of phospholipid since it was observed in the present study that Factor V (Va) and Factor Xa do not form a complex in the presence of calcium only. However, Colman (1970) demonstrated that Factor Xa could attack a synthetic ester at a 3-fold greater rate in the presence of Factor V, calcium and phospholipid, but in the absence of prothrombin, than it could with only added calcium and phospholipid. The degree of this rate enhancement is not very great, so it is not very convincing that Factor V only affects the reactivity of Factor Xa toward prothrombin. Or Factor V (Va) could interact with prothrombin to render it more susceptible to proteolysis by Factor Xa. However, it was observed in the current study that Factor V or Va when mixed with prothrombin in the presence of calcium and the mixture subsequently chromatographed on Sephadex G-200 in the presence of calcium, the two activities eluted on the column in completely separate volumes. In addition, it was observed that when Factor V or Va was mixed with prothrombin in the presence of phospholipid, but no calcium, and the mixture subsequently chromatographed on Sephadex G-200 in the absence of calcium, Factor V or Va eluted in the column void volume with the phospholipid, and prothrombin eluted later in a separate volume. So if Factor V does interact with prothrombin per se, this interaction must likewise be mediated by phospholipid. By this technique then, Factor V or Va and prothrombin do not appear to have

an affinity for each other, in the presence of calcium or phospholipid alone. However, Esmon et al. (1974a) reported that Factor V would bind specifically to prothrombin when it was attached to an insoluble matrix, in the presence of calcium. Furthermore, Esmon et al. (1974a) reported kinetic evidence that Factor V interacts specifically with Intermediate II, a degradative product of prothrombin, formed during its conversion to thrombin (Figure 2). So, although no direct evidence has been reported that Factor V or Va can form a complex with Factor Xa or with Factor II (prothrombin), the several indirect findings that it does affect one or the other, suggest that Factor V (Va) may contribute to the prothrombinase reaction by interacting with both Factor Xa and Factor II, which are concentrated on the phospholipid surface. And by causing a conformational change in Factor Xa to render it more reactive toward prothrombin as well as positioning prothrombin so that those bonds susceptible to proteolysis are situated in the active site of Factor Xa, Factor V would facilitate a most efficient reaction between the two, reflected in a burst of thrombin activity.

For future investigation of the role of Factor V in this reaction it would be of value to determine the composition of the prothrombinase complex, that is, the minimal amount of phospholipid which can bind a known amount of Factors Xa and V to form an active prothrombin-converting

principle. This problem could be approached by using short-chain fatty acid phospholipids, which can exist in monomeric form and can bind to Factors Xa and V, and prothrombin as well. It also would be helpful in this regard to determine the extent to which a molecule of each of these proteins binds to phospholipid. It has been well demonstrated by several investigators that the calcium and phospholipid binding region of prothrombin is localized near the N-terminal portion of the molecule. A similar study could be initiated on the Factor Xa molecule. However to determine either the stoichiometry or the extent of binding of Factor V in the prothrombinase complex, it must be purified to homogeneity. Although this is still a formidable task, the preparation of Factor V developed in this study is a good one with which to start toward this end since it is very stable and can be readily converted to its active form, Factor Va by a number of enzymes. In addition, a homogeneous preparation of Factor V would make possible an investigation of the structural composition of this intriguing protein as well as the molecular mechanism by which it is converted to Factor Va by any one of the enzymes observed in this study to accomplish this. Furthermore the structural determinants for the binding of Factor V to phospholipid could be explored in much the same way that the calcium and phospholipid binding region of prothrombin was elucidated (Benson and Hanahan, 1975).

The elucidation of almost any aspect of the nature of Factor V and its role in the prothrombinase reaction would be a tremendous contribution toward an understanding of the basic mechanisms of blood coagulation,

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