The Activation of Factor V by Factor Xa or α-Chymotrypsin and Comparison with Thrombin and RVV-V Action. An Improved Factor V Isolation Procedure†

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ABSTRACT: Bovine plasma factor V has been isolated by a preparative procedure involving barium sulfate adsorption, QAEC extraction, poly(ethylene glycol) precipitation, and finally chromatography on a desulfated Sepharose 6B column. Factor V was recovered as a single peak in yields of 35–40% with a specific activity of 50–70 representing a purification of 1000–2000-fold relative to the starting plasma. The apparent molecular weight of the purified factor V was 439 000 ± 5000. On sodium dodecyl sulfate gel and analytical gel electrophoresis, this factor V preparation showed multiple bands, but results are inconclusive with regard to a possible subunit structure for this factor. The purified factor V was stable for at least 1–2 weeks when stored at 4 °C in 0.2 M Tris-acetate, 50 mM $CaCl_2$, 10% glycerol, pH 7.5. When stored at −20 °C in 50% glycerol, this preparation was stable for several months. Treatment of the purified factor V with bovine factor Xa, RVV-V, thrombin, or chymotrypsin (but not trypsin) led to a seven- to ten-fold increase in clotting activity and a concomitant decrease in apparent molecular weight. The latter was comparable for each activation system yielding the following average molecular weight values: factor Va^{Xa}, 246 000; factor $Va^{RVV\text{-}V}$, 251 500; Factor Va^{thr}, 239 000; α-chymotrypsin, but not trypsin, can activate plasma factor V yielding a product similar to that observed with the above activators. The molar quantities of each of the activators required varied considerably with thrombin having the highest specific activity and factor Xa the lowest. Activation by factor Xa was greatly facilitated by the addition of phospholipid. In the presence of a mixture of phosphatidylcholine/phosphatidylserine (1:1, w/w), the activation of factor V by factor Xa plus Ca^{2+} required one-third the amount of factor Xa protein as that required in the absence of phospholipid. Even though each of these activators appears to act in an enzymatic manner, the chemical nature of the conversion is unknown at this time.

Factor V represents a circulating plasma protein intimately involved in the intrinsic and extrinsic pathways of blood coagulation in mammals (Nemerson and Pitlick, 1972). In an in vitro system, this factor participates by accelerating the rate of attack of factor Xa,[1] a protease, on prothrombin (in the presence of Ca^{2+}) to yield thrombin. So far as can be ascertained, factor V has no enzymatic activity. Current thinking would place factor V in the role of a large molecular weight co-factor. While there are many unanswered questions with regard to our understanding of the mode of action of factor V, there is general agreement that factor V is an important component of prothrombinase. The latter enzyme, a complex consisting of factor Xa, phospholipid, and Ca^{2+} plus factor Va, is regarded as the most active form of factor Xa yet identified (Barton et al., 1967; Papahadjopoulos and Hanahan, 1964; Esmon et al., 1974).

Even though considerable insight into the mechanism of factor Xa attack on prothrombin has been gained in the past few years (Stenn and Blout, 1972; Esmon et al., 1974; Kisiel and Hanahan, 1974), elucidation of the role of factor V in prothrombinase activity, for example, has simply not maintained an equal pace. In part, this can be attributed to difficulties associated with the isolation and purification of factor V (Day and Barton, 1972). Further complications include lack of any identifiable enzymatic activity in factor V preparations and the probable requirement of an "active" form, i.e., factor Va, in the prothrombinase complex. Consequently, as part of a continuing study on the biochemical nature of the prothrombinase complex and its mode of attachment to prothrombin, an investigation was undertaken with the expressed aim of developing a convenient, reproducible isolation procedure yielding a stable factor V and of exploring the conversion of factor V to a more active form, factor Va, by such reagents as factor Xa, RVV-V, thrombin, and α-chymotrypsin.

The results of this current investigation show that a high purity, stable factor V can be isolated by a relatively simple procedure and that factor Va formed by action of factor Xa, RVV-V, thrombin, or α-chymotrypsin on factor V appears comparable in molecular characteristics.

Experimental Section

Materials

Barium sulfate was purchased from Matheson Coleman and Bell. QAE-cellulose (0.92 mequiv/g) was a product of Schleicher and Scheull, Inc. Sephadex G-150, Sephadex G-200, SP-Sephadex C-50, Sepharose 6B, and Dextran Blue 2000 were obtained from Pharmacia Fine Chemicals. Russell's viper venom (no. VR1EF) was purchased from Miami Serpentarium. Sodium lauryl sulfate, Coomassie brilliant blue R,

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[1] Abbreviations used: PC, phosphatidylcholine; PS, phosphatidylserine; RVV-V, factor V activator of Russell's viper venom; factor V, factor V (plasma); factor Va, activated factor V; factor Xa, activated factor X; factor Va^{Xa}, factor V activated by factor Xa; factor $Va^{RVV\text{-}V}$, factor V activated by RVV-V; factor Va^{thr}, factor V activated by thrombin; PEG, poly(ethylene glycol); QAE, diethyl(2-hydroxypropyl)aminoethyl; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; DFP, diisopropyl fluorophosphate.

bovine serum albumin, aldolase, γ-globulin, bovine carbonic anhydrase, myoglobin, soybean trypsin inhibitor (type II-S), heparin (grade I), and 2-mercaptoethanol were products of Sigma Chemical Co. DNP-glycine was obtained from Mann Research Laboratories, Inc. Benzamidine hydrochloride was an Aldrich Chemical Co. product. Apoferritin was purchased from Calbiochem. Pyronin Y, acrylamide, and *N,N'*-methylenebisacrylamide were recrystallized from chloroform and acetone, respectively, according to Loening (1967). Poly(ethylene 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 was treated according to McPhie (1971). Bovine Topical thrombin was a Parke, Davis and Co. product. Phosphatidylserine and phosphatidylcholine were prepared from bovine brain and hen's eggs, respectively, by Dr. M. Gamo in this laboratory. 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). In the latter technique, oxalated human plasma was aged at 37 °C until the clotting time in the factor V assay exceeded 60 s. Human brain thromboplastin was prepared according to Biggs (1972). Factor VII and factor X deficient plasmas were purchased from Sigma Chemical Co. (St. Louis). α-Chymotrypsin (47 units/mg), CDI, was purchased from Worthington Biochemical Corp. Trypsin (type III) and TPCK were Sigma Chemical Co. products.

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–Ca^{2+} buffer was prepared from Michaelis buffer with addition of $CaCl_2 \cdot 2H_2O$ to 0.025 M. The pH of each of the above solutions was adjusted to 7.35 by the addition of 1 N HCl.

Methods

A. Protein Measurement. All protein determinations were made by measuring the absorbance at 280 nm, assuming an $E_{280}^{1\%}$ of 10 and correcting for Rayleigh light scattering at 320 nm according to Shapiro and Waugh (1966).

B. Preparation of Desulfated Sepharose 6B. Sepharose 6B was chemically treated to reduce the number of sulfate ester groups according to the procedure outlined by Porath et al. (1971).

C. Phospholipid Dispersions. Phosphatidylcholine and phosphatidylserine were mixed in equimolar amounts of diethyl ether, and this mixture was evaporated to dryness under a stream of nitrogen. The residue was dissolved in Michaelis buffer, pH 7.35. This suspension was then sonicated at 5-min intervals at the number 5 setting of a Bronson sonifier for a total time of 25 min. The resulting dispersion was centrifuged at 100 000*g* for 1 h and the clear, colorless supernatant used in experiments requiring phospholipid.

D. Gel Electrophoresis. 1. Analytical polyacrylamide gel disc electrophoresis was performed according to Davis (1964) in a Buchler Poly-Analyst apparatus. A photopolymerized 2.5% gel was cast over a chemically polymerized 5% resolving gel. Samples of 20–100 μl, containing 50–100 μg of protein, were electrophorized for 2–3 h at room temperature and subsequently stained and destained according to Fairbanks et al. (1971).

2. Sodium Dodecyl Sulfate. Electrophoresis in the presence of sodium dodecyl sulfate was carried out according to the procedure of Swank and Munkres (1971), in 7.5% acrylamide gels (1:30, bisacrylamide ratio) containing 0.1% sodium dodecyl sulfate 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% sodium dodecyl sulfate, pH 6.8, for 12–15 h, then treated with 5% (v/v) 2-mercaptoethanol and/or made 8 M in urea, and incubated for 1 h at 37 °C. Each sample was made 20% (v/v) in sucrose and pyronin Y was added as a tracking dye. Then, 10–15-μl aliquots of each sample were electrophorized at 25 °C at a constant current of 1.5 mA/gel for 13–15 h. The electrophoresis buffer was 0.1 M Tris-phosphate, 0.1% sodium dodecyl sulfate, pH 6.8. Subsequent to electrophoresis the gels were stained and then destained according to Fairbanks et al. (1971). The gels were individually scanned at 600 nm in a Gilford Model 240 spectrophotometer equipped with a Model 2410 linear transport accessory.

E. Molecular Weight Determination. 1. Sodium Dodecyl Sulfate Gel Electrophoresis. The mobility of each protein species on electrophoresis (as described above) was determined from the densitometry tracing and the apparent molecular weight determined from a linear semilog plot of molecular weight vs. mobility for reduced, standard proteins. Bovine serum albumin (68 000), ovalbumin (45 000), bovine carbonic anhydrase (39 000), and myoglobin (17 200) were used as molecular weight standards.

2. Gel Filtration. The apparent molecular weights of bovine plasma factor V and of the activity resulting from thrombin, factor Xa, RVV-V, or chymotrypsin treatment were determined by gel filtration on a calibrated column of Sephadex G-200, at 4 °C using Michaelis–0.025 M $CaCl_2$, pH 7.35 buffer. A 1.6 × 55 cm column was calibrated with standard proteins: apoferritin (443 000 daltons), γ-globulin (205 000 daltons), aldolase (160 000), and bovine serum albumin (68 000). The void volume was determined with Dextran Blue 2000 and the internal volume was measured with DNP-glycine. Elution volumes of standards and samples were determined by weighing the eluates 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 vs. the K_D value where $K_D = (V_e - V_0)/V_i$, and $V_i = V_e(\text{DNP-glycine}) - V_0$. The apparent molecular weight of the samples applied was determined from the calibration curve.

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 of human brain thromboplastin were preincubated for 30 s at 37 °C, followed by the addition of 0.1 ml of test sample and finally 0.1 ml of 0.025 M $CaCl_2$. Samples already containing calcium were assayed by adding 0.1 ml of deionized water to the factor V deficient plasma–thromboplastin mixture, and the assay was initiated by the addition of 0.1 ml of the factor V sample diluted in Michaelis–0.025 M $CaCl_2$, pH 7.35 buffer. The assay was standardized with normal citrated pooled human plasma and test solutions were diluted so that clotting times were in the 20–30 s 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 corrected for light scattering at 320 nm.

Factor Xa was assayed by the procedure of Bachmann et al. (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.

Thrombin activity was assayed at 37 °C according to a modification of the procedure of Shapiro and Waugh (1966),

Table I: Purification Scheme for Bovine Plasma Factor V.

Fraction	Volume (ml)	Units/ml	Total Act./Units	Spec. Act.	% Recovery	Purification (Fold)
Plasma	11 205	1.82	20 393	0.0284	100	1.0
$BaSO_4$ 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
Polyethylene glycol precipitate	42	270	11 340	13.4	56	472
Desulfated Sepharose 6B	432	18.2	7 862	66.7	39	2349

using a 0.4% solution of bovine fibrinogen which was the I-2 fraction obtained according to the procedure of Blomback and Blomback (1956). Thrombin activity which was determined from a standard log-log plot of clotting time vs. thrombin concentration constructed with an NIH standard thrombin preparation (Lot B-3, 21.7 NIH units/mg).

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 min at 25 °C. A control, containing buffer in place of the RVV-V sample, was run under similar conditions. Each sample was assayed for factor V activity and the RVV-V activity calculated as the factor V 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.

Preparation of Bovine Plasma Factor V. In a typical run, 21 l. of bovine blood were collected at a local meat packing house in polyethylene buckets containing 0.1 M potassium oxalate, and a mixture of benzamidine hydrochloride, heparin, and soybean trypsin inhibitor (each at 10 mg/l. of blood). The contents were mixed well and within 1 h were centrifuged at 2300*g* at 4 °C. The plasma was recovered and centrifuged a second time for 15 min at 2300*g* at 4 °C and then adsorbed at room temperature with $BaSO_4$, 100 g/l., for 20 min with constant stirring. This was followed by centrifugation at 2300*g* for 10 min to remove the $BaSO_4$. The supernatant solution, or adsorbed plasma, was then diluted 1:1 with deionized water and the pH adjusted to 7.0 with 1 N HCl. This diluted plasma was then treated batch-wise with QAE-cellulose (9 g per l. of original plasma) for 20 min at room temperature with constant stirring. The mixture was allowed to stand out for 30 min and the cellulose subsequently collected by centrifugation at 2300*g* for 10 min at 4 °C. The QAE-cellulose pellet was washed with 0.055 M calcium acetate, pH 7.25, until the adsorbance of the wash solution at 280 nm read 0.10 or less. Bovine plasma factor V was then desorbed from the cellulose pellet with 0.11 M calcium acetate, pH 7.25, in a volume $^1/_{10}$ that of the starting plasma. The final extract was treated with 10^{-3} M benzamidine hydrochloride and 50 mg/l. of soybean trypsin inhibitor (storage at 4 °C). This (QAEC) factor V preparation was further purified by poly(ethylene glycol) precipitation and subsequent chromatography on desulfated Sepharose 6B as follows: The QAEC factor V sample was titrated to pH 6.0 with 1 N acetic acid and treated with 50% poly(ethylene glycol) (PEG) 6000 to a final (PEG) concentration of 12% at 4 °C. The precipitate was collected by centrifugation at 4000*g* for 15 min at 4 °C. The pellet was dissolved in 0.20 M Tris-acetate, 50 mM $CaCl_2$, 10% glycerol, pH 7.5, and then dialyzed against the same buffer for 10–12 h before being applied to a column of desulfated Sepharose 6B, 5 × 90 cm, equilibrated in the same buffer. The column was developed in the same buffer and the factor V active fractions were collected and stored at 4 °C until needed.

RVV-V Purification. RVV-V was prepared by a modification of the procedure of Esmon et al. (1973). Crude RVV-V was chromatographed on Sephadex G-150 equilibrated 0.05 M sodium acetate, 0.5 M NaCl, pH 5.0. The RVV-V active portion of the eluate was pooled, concentrated by ultrafiltration, and dialyzed against 0.05 M sodium acetate, 0.5 M NaCl, pH 5.0, and applied to a column of SP-Sephadex C-50 previously equilibrated in 0.05 M sodium acetate, 0.5 M NaCl, pH 5.0. A linear gradient of 0.5 M NaCl to 1 M NaCl in 0.05 M sodium acetate, pH 5.0, was applied and the RVV-V activity was removed at approximately 0.7 M NaCl. The latter eluate was dialyzed against 0.1 M Tris-HCl, pH 7.5, concentrated by ultrafiltration and stored at −20 °C in 0.5-ml aliquots in polyethylene tubes. On sodium dodecyl sulfate electrophoresis the RVV-V appeared to contain only one major band of 26 000–28 000 dalton molecular weight.

Bovine Thrombin. Bovine thrombin was prepared according to a modification of the procedure of Lundblad (1971). Two vials of Parke-Davis thrombin containing 10 000 NIH units/vial were reconstituted in 0.1 M sodium phosphate, 10^{-3} M ϵ-aminocaproic acid, pH 6.5, and dialyzed 12–15 h against the same buffer. The sample was charged onto a column of SP-Sephadex C-50 previously equilibrated in 0.1 M sodium phosphate, pH 6.5; this was followed by equilibrating buffer until all of the A_{280} adsorbing material was eluted. The buffer was then changed to 0.25 M sodium phosphate, pH 6.5, after which the thrombin was eluted as a sharp peak. The thrombin was pooled, concentrated by ultrafiltration, and stored at −20 °C in polyethylene tubes.

The specific activity of this purified thrombin was 2400 NIH units/mg protein. On sodium dodecyl sulfate gel electrophoresis, this preparation appeared to contain both α and β forms, with α the predominant species.

Bovine Factor Xa. This factor was isolated by the preparative gel electrophoresis method described by Kisiel and Hanahan (1973), subsequent to the activation of purified plasma factor X by RVV-X or immobilized trypsin. The factor Xa showed predominately a single band on analytical gel electrophoresis and the specific activity of repeated preparations ranged from 600 to 1000 units per mg protein.

Activation of Bovine Plasma Factor V by: (a) RVV-V. A total of 1–5 units of factor V in 0.20 M Tris-acetate, 50 mM $CaCl_2$, 10% glycerol, pH 7.5, was treated with RVV-V at 25 °C in a polycarbonate tube to a final concentration of 5 mM $CaCl_2$ and from 2.5 to 10 μg of RVV-V/ml, in 0.1 M Tris-HCl. After addition of RVV-V at zero time, aliquots of the activation mixture were withdrawn at 5-min intervals to assay for factor V activity.

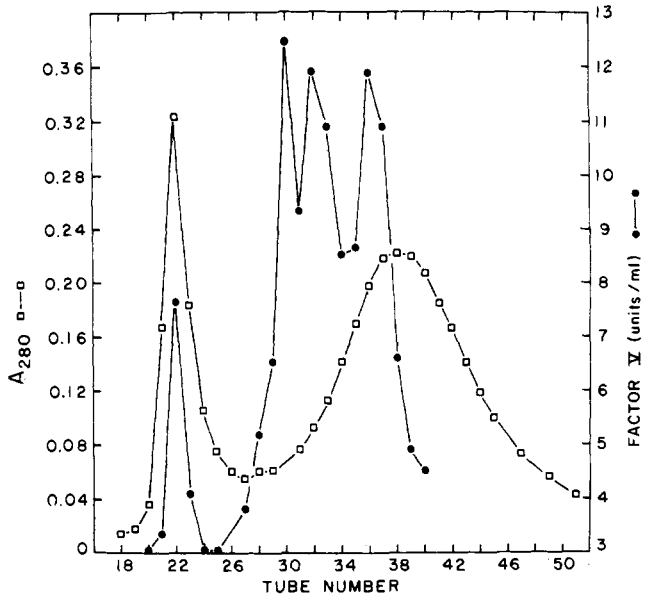

FIGURE 1: Elution profile of a PEG precipitate of factor V on Sepharose 6B. Thirty milliliters of QAEC-factor V (see Table I) was precipitated with PEG 6000 and redissolved in 1 ml of 0.20 M Tris-acetate, 50 mM $CaCl_2$, 10% glycerol, pH 7.5, and applied to a 1.6 × 55 cm column of Sepharose 6B. The column was eluted with the same buffer at a flow rate of 10 ml per h and 2.0-ml fractions were collected and analyzed. Further details are provided in the text.

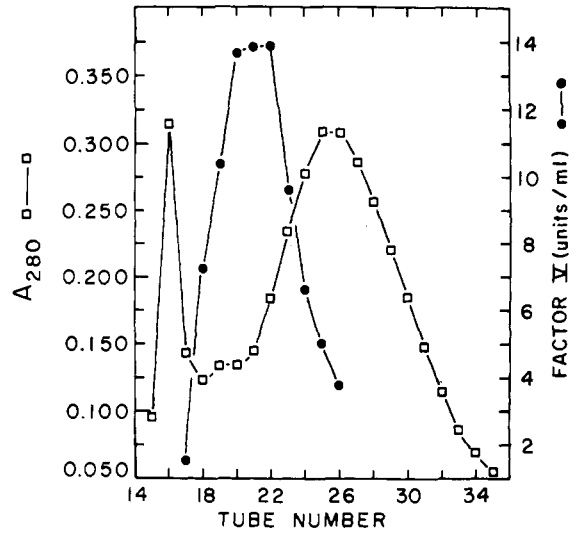

FIGURE 2: Elution profile of PEG precipitated factor V on desulfated Sepharose 6B. Sample and conditions were exactly the same as in Figure 1, except that desulfated Sepharose 6B was used in place of Sepharose 6B.

(b) Bovine Thrombin. A total of 1-5 units of factor V in 0.20 M Tris-acetate, 50 mM $CaCl_2$, 10% glycerol, pH 7.5, was incubated with thrombin in a polycarbonate tube at 25 °C to a final concentration of 5 mM $CaCl_2$ and 0.36-1.4 μg or 0.88-3.5 NIH units of thrombin/ml. After the addition of thrombin aliquots of the mixture were removed periodically to assay for factor V activity.

(c) Factor Xa. Factor V in 0.20 M Tris-acetate, 50 mM $CaCl_2$, 10% glycerol, pH 7.5, was mixed either with PC-PS (1:1, w/w), and bovine factor Xa, or with factor Xa alone, in a polycarbonate tube at 25 °C. A total of 1-5 units of factor V was mixed to a final concentration of 3-4 μg/ml or 2.4 Ortho units of factor Xa, 5 mM $CaCl_2$, and 0.05 mg of PC-PS/ml (1:1, w/w). In a separate experiment, 1-5 units of factor V was mixed to a final concentration of 12 μg/ml or 7.2 Ortho units/ml factor Xa and 5 mM $CaCl_2$. The addition of factor Xa was taken as zero time; thereafter aliquots were withdrawn at intervals to assay for factor V activity.

(d) α-Chymotrypsin and Trypsin. A sample containing Factor V, in amounts roughly from 1 to 5 units in 0.20 M Tris-acetate, 50 mM $CaCl_2$, 10% glycerol, at pH 7.5, was diluted to a final concentration of 5 mM $CaCl_2$. To this mixture α-chymotrypsin was added to make a final enzyme level of 3-6.2 μg/ml and incubation conducted at 25 °C. The addition of enzyme was taken as zero time and thereafter aliquots of the activation mixture were removed at 5-min intervals to assay for factor V activity.

In a similar manner, factor V was treated with trypsin, varying in levels from 0.1 to 1.0 μg/ml, at pH 7.35 and 25 °C. Aliquots of the reaction mixture were removed at appropriate time intervals and assayed for factor V activity.

Results

I. Factor V. Purification and Characteristics. Quantitative data for a typical purification scheme of bovine plasma Factor V are given in Table I. Preparation of factor V through the extraction step with QAE-cellulose is a modification of the procedure of Dombrose et al. (1972). Recalcification of bovine plasma according to Dombrose et al. resulted in the recovery of more than one molecular weight species of factor V which were of lower molecular weight and higher specific activity than plasma factor V. Consequently the final preparation could not be activated more than two- to four-fold with RVV-V. Elimination of this step in the isolation procedure and extraction of the diluted plasma with QAE-cellulose at pH 7.0 resulted in a highly stable factor V preparation. Plasma factor V was recovered in good yield at this step. On Sephadex G-200 column chromatography, QAE-cellulose factor V behaved as a single, molecular weight species of 439 000 ± 5000 daltons. The washings and extraction of the QAE-cellulose with dilute calcium acetate buffers resulted in a preparation of higher specific activity and stability comparable to that described by Esnouf and Jobin (1967), employing TEAE-cellulose and sodium phosphate buffers.

Precipitation of QAE-cellulose factor V with polyethylene glycol 6000 provided a rapid method for concentrating large volumes of QAE-cellulose extract for subsequent gel filtration and allowed a further purification with no apparent alteration in the factor V molecule.

Inasmuch as plasma factor V elutes close to the void volume on Sephadex G-200, chromatography of a PEG precipitate of factor V on Sepharose 6B was undertaken and revealed a separation of the factor V activity from void volume protein. However, in this instance the activity and the total protein spread over such a large volume that little purification of factor V was achieved (Figure 1). Subsequently, chemical modification of Sepharose 6B by the procedure of Porath et al. (1971), for removal of sulfate ester groupings, resulted in a gel filtration medium on which the same factor V preparation eluted in a reasonably symmetrical peak and in a limited eluting volume so that a decided purification was accomplished (Figure 2). Also, the recovery of factor V activity from the desulfated Sepharose was 20% better than that eluted from untreated Sepharose 6B.

Through use of the scheme outlined in Table I, factor V was isolated in a 30-40% yield with a specific activity of 60-70, representing a 1000-2000-fold purification relative to the starting plasma. This isolation has proven to be highly reproducible and resulted in a highly stable factor V preparation

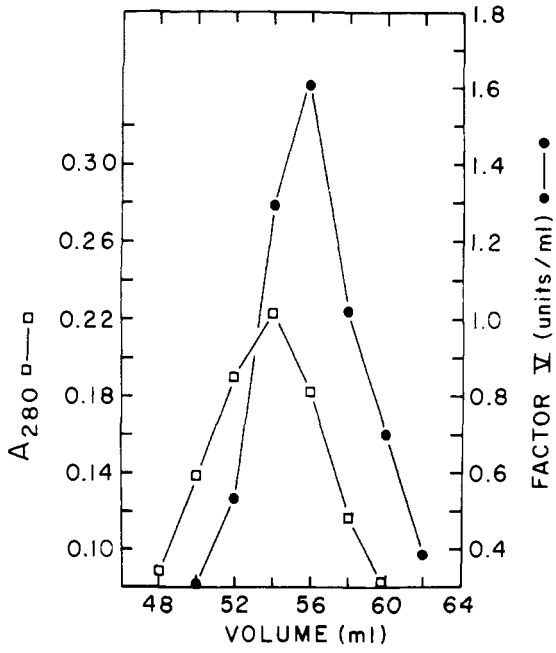

FIGURE 3: Behavior of factor V from desulfated Sepharose 6B on a Sephadex G-200 column. The factor V eluate from Figure 2 was concentrated by ultrafiltration on an Amicon PM-10 membrane to 1.0 ml and then applied to a 1.6 × 55 cm column of Sephadex G-200. The latter column was eluted with Michaelis–0.025 M $CaCl_2$, pH 7.35, at a flow rate of 10 ml/h and 2.0-ml fractions were collected and analyzed. The K_D for the factor V was 0.03.

which retained 80–100% of its original activity upon storage at 4 °C for 1–2 weeks, at a protein concentration of 0.3–0.5 mg/ml. When stored at − 20 °C in the presence of 50% glycerol, the preparation is stable for several months. When this preparation was assayed for the presence of other clotting factors, only trace amounts of factor VIII were detected.

Bovine factor V, as isolated, appears to consist of only one molecular weight species when examined by gel chromatography on Sephadex G-200 (Figure 3) and has an apparent molecular weight of 439 000 daltons. The specific activity of bovine factor V prepared according to the above procedure is approximately 60 units/mg protein (range: 50–70). This value is comparable to the factor V isolated by Esnouf and Jobin (1967), but it is difficult to compare these two preparations since the latter investigators apparently isolated a different molecular weight species of factor V which probably has a higher specific activity independent of the extent of purification. Similarly, the factor V preparation of Dombrose et al. (1972), most likely contained one or more molecular weight species of factor V of higher specific activity than plasma factor V. This would account for their yields of 100% or greater. The preparation described here can be activated seven to ten fold by RVV-V, factor Xa, thrombin, or α-chymotrypsin which is also the same extent of activation observed for QAEC factor V using these same reagents.

Analytical disc electrophoresis of the desulfated Sepharose 6B factor V preparation using a 5% resolving gel, revealed six stainable protein bands. When one gel was sliced in 2–3 mm sections and subsequently examined for factor V activity, it was observed that the activity was located in a moderately staining band near the top of the resolving gel, when compared with a duplicate gel which had been stained for protein with Coomassie blue. When a similar analysis of a preparation of factor $Va^{RVV\text{-}V}$ which had been chromatographed on Sephadex G-200 following activation was made, a nearly identical pattern was observed and the factor V activity was located at the same position in the gel as plasma factor V. This protein banding pattern of the factor V preparations was unchanged when the preparation was subjected to varying conditions of ionic strength prior to analytical electrophoresis. Samples of factor V, after dialysis for 12–15 h against 0.01 M Tris-acetate, pH 7.5, containing NaCl from 0.0 to 0.14 M, gave nearly identical patterns on subsequent analytical electrophoresis of 50 μg of protein in each case. In another series of experiments, the electrophoretic pattern of factor V in 0.2 M Tris-acetate, pH 7.5, was not influenced by the presence or absence of (50 mM) Ca^{2+}.

Sodium dodecyl sulfate electrophoresis of factor V under nonreducing conditions revealed five major protein bands with molecular weights of 120 000, 86 000, 80 000, 57 500, and 50 000 daltons, respectively. And, sodium dodecyl sulfate electrophoresis under reducing conditions revealed a total of 13 peptide bands in the factor V preparation. Five major staining bands with molecular weights of 120 000, 93 500, 80 500, 75 000, and 51 200 daltons were apparent, in this sample. These electrophoretic patterns were a reproducible feature of all factor V preparations.

II. Activation of Bovine Factor V by Factor Xa, Thrombin RVV-V, or α-Chymotrypsin. Activation of bovine factor V occurred in the presence of bovine factor Xa, bovine thrombin, the factor V activator present in Russell's Viper Venom, or α-chymotrypsin. In all cases, this activation took the form of a decided increase (usually over seven fold) in factor V activity, as measured by the clotting assay and of a significant decrease in molecular weight, as determined by Sephadex gel chromatography. Specific features of these studies are presented below.

(a) RVV-V. Activation of bovine plasma factor V in the presence of 5 mM Ca^{2+} with the purified activator from Russell's viper venom proceeded rapidly, reaching a maximum level of factor V activity in 8–10 min followed by a slow decline in activity (Figure 4A). When 1–5 units of factor V was treated with 10 μg of RVV-V in the presence of 5 mM Ca^{2+}, the factor V activity increased eight to ten fold at pH 7.35 and 25 °C. The same amount of factor V activity in the presence of buffer alone did not change appreciably under the same conditions, for upwards of 25 min. Also, the same rate and extent of activation were attained by RVV-V in the absence of calcium.

Activation by RVV-V resulted in a change in the apparent molecular weight of bovine factor V. After treatment with RVV-V, the factor V eluted from the Sephadex G-200 column in a volume corresponding to a molecular weight of 250 000 daltons.

Activation of the same amount of factor V by varying amounts of RVV-V over a four-fold concentration range resulted in a corresponding increase in both the initial rate of activation and the final level of factor V activity. A similar observation was noted by Schiffman et al. (1969), and they concluded that RVV-V was consumed in the activation reaction. However it was further observed in this study that the addition of more factor V to the original activation mixture, after the initial rise in factor V activity had leveled off, resulted in a subsequent increase in activity to the same level or even greater than that measured during the initial activation. This suggests that RVV-V was available in a fully activated mixture to react with the added factor V, and this observation contrasts with the results reported by Schiffman et al. (1969) on a similar experiment.

RVV-V could not be inhibited by DFP in its ability to activate factor V. When treated with DFP at the levels reported to be sufficient to inactivate RVV-V by Esmon and Jackson

(1973) or even with greater amounts of DFP and for up to 24 h, RVV-V could still activate factor V to 78% of the control RVV-V. The latter was carried through the same procedure except that an aliquot of isopropyl alcohol was added in place of DFP.

In confirmation of a previously published report (Hanahan et al., 1972), RVV-V appeared to be stable to heat treatment in its ability to activate factor V. Heat-treated RVV-V was able to activate factor V, after 10 min incubation at 25 °C, to 76% of the level of a control portion, which was maintained at 40 °C, prior to the activation reaction.

Activation of factor V by RVV-V appeared to be temperature dependent in that activation at 37 °C proceeded at a faster rate but to the same extent as that at 25 °C.

(b) Thrombin. Treatment of bovine factor V with bovine thrombin produced a maximum activation in 7–8 min at pH 7.35 and 25 °C. When 1–5 units of factor V in the presence of 5 mM Ca^{2+} was treated with 0.7–1.4 μg of purified thrombin, the factor V activity increased rapidly to eight to ten fold of the basal level (Figure 4B). The same amount of activation was also observed in the absence of calcium. After maximal activation was reached, a gradual decline in factor V activity was observed in the presence of bovine thrombin. The activated Factor V preparation showed an apparent molecular weight upon Sephadex G-200 chromatography similar to that observed with factor $Va^{RVV\text{-}V}$. Thrombin activated factor V eluted in a volume corresponding to an apparent molecular weight of 239 000 ± 5000 daltons.

Activation of factor V by varying concentrations of bovine thrombin resulted in an increase in the initial rate of activation yet the same level of factor V activity was reached with increasing thrombin concentrations over a four-fold concentration range.

In contrast to RVV-V, bovine thrombin did not appear to be heat stable under the same experimental conditions used with RVV-V. Heat-treated thrombin lost all of its clotting activity and was able to activate factor V to only 20% of the control level after 8 min of incubation at 25 °C, at which time the factor V activity had peaked upon treatment with the control thrombin. Bovine thrombin was readily inhibited by both DFP and TLCK. Thrombin, in 0.1 M Tris-HCl, pH 7.5, was treated with a 1000-fold molar excess of DFP in isopropyl alcohol, or with a similar excess of TLCK in 0.1 M Tris-HCl, pH 7.5, for 1 h at room temperature, followed by 12–15-h dialysis against 0.1 M Tris-HCl, pH 7.5, to remove excess inhibitor. Control thrombin samples were treated with aliquots of isopropyl alcohol, or 0.1 M Tris-HCl, pH 7.5, and further processed in a manner identical with the DFP- and TLCK-thrombin samples. In both cases the thrombin clotting activity was abolished as was its ability to activate factor V.

The addition of more factor V to the thrombin activation mixture resulted in further increases in factor V activity, as observed with RVV-V. However, due to the apparent rapid inactivation of factor V following its initial activation by thrombin, it is difficult to evaluate the extent of this "second activation".

The activation of factor V by bovine thrombin appeared to be pH dependent in that the maximum rate and extent of activation were achieved at pH 7.5. In addition, this activation was observed to be temperature dependent in that the activation of factor V proceeded faster at 37 °C, but to the same extent as that at 25 °C.

(c) Factor Xa. Bovine plasma factor V was found to be activated seven to ten fold by bovine factor Xa at pH 7.35 and 25 °C in the presence of Ca^{2+} and a mixture of phospholipids,

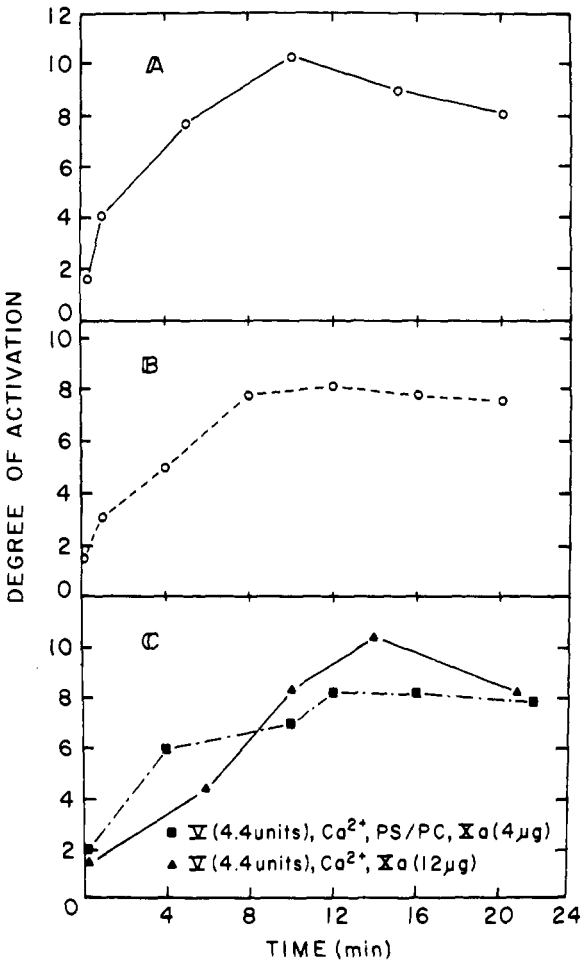

FIGURE 4: Time course of activation of plasma factor V by RVV-V (A), thrombin (B), factor Xa (C). (A) RVV-V: 2.5 units of factor V (fraction obtained from desulfated Sepharose 6B column, see Table I) was incubated at room temperature with 10 μg of RVV-V at pH 7.5. After addition of RVV-V, aliquots of the mixture were withdrawn at the indicated intervals and assayed for factor V activity. Further details are provided in the text. (B) Thrombin; 2.0 units of factor V (same source as in A, above) was incubated at room temperature with 0.4 μg of bovine thrombin at pH 7.5. After addition of thrombin, aliquots of the mixture were withdrawn at indicated intervals and assayed for factor V activity. Further details are provided in the text. (C) Factor Xa: 4.4 units of factor V (same source as in A, above) was incubated at room temperature either with 4 μg of factor Xa in a mixture (total volume, 1.0 ml) containing 0.5 mg of PC–PS (1:1, w/w) and 5 mM $CaCl_2$, pH 7.5, or with 12 μg of factor Xa in a mixture (total volume, 1.0 ml) containing 5 mM $CaCl_2$, pH 7.5. After addition of factor Xa, aliquots were withdrawn at indicated intervals and assayed for factor V activity.

e.g., PC–PS (1:1, w/w) or with Ca^{2+} alone (Figure 4C). In either case, Ca^{2+} was an absolute requirement for the conversion. In the presence of 5 mM Ca^{2+} and 0.05 mg/ml PC–PS (1:1, w/w), 1–5 units of bovine factor V was activated by 4 μg of bovine factor Xa over a period of 8–10 min to seven to ten fold the initial level of factor activity. No change in activity was recorded when plasma factor V was incubated in the presence of Ca^{2+} and phospholipid; an increased level of factor Xa (12 μg) plus 5 mM Ca^{2+} was required to achieve the same increase in factor V activity. It was also found that this increase in factor V activity by virtue of factor Xa action was accompanied by a change in the apparent molecular weight of factor V as determined by gel filtration on Sephadex G-200. Plasma factor V gave an apparent molecular weight of 439 000 ± 5000 daltons while the factor V activity obtained after treatment with factor Xa eluted in a volume corresponding to an apparent molecular weight of 246 000 ± 4000 daltons (Figure 5).

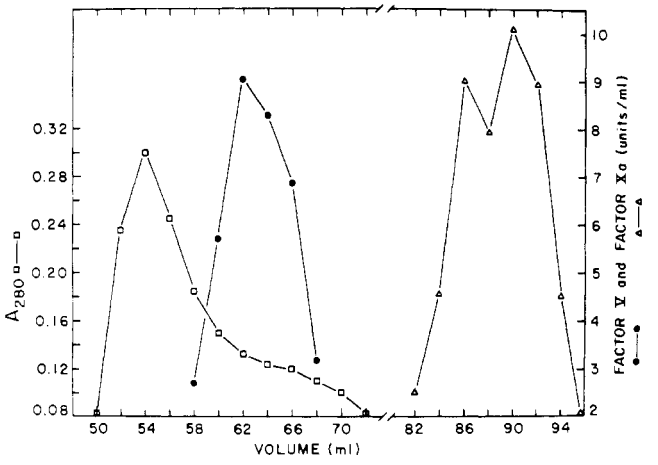

FIGURE 5: Elution pattern of factor V and factor Xa incubation mixture on Sephadex G-200. Factor V, obtained from a desulfated Sepharose 6B column (as described in Methods), was incubated with factor Xa in the presence of Ca^{2+} only as described in the text. This mixture was applied to a Sephadex G-200 column, measuring 1.6 × 55 cm and elution conducted with Michaelis–0.025 M $CaCl_2$, pH 7.35, at a rate of 10 ml/h. Fractions of 2.0 ml were collected and analyzed. The K_D for factor V activity (factor Va^{Xa}) was 0.13.

In order to ascertain the nature of this activation, the same amount of factor V in the presence of 5 mM Ca^{2+} was treated with varying amounts of factor Xa over a four-fold concentration range. It was observed that the "initial rate" of the activation reaction increased with increasing factor Xa concentration as did the extent of the reaction, or the final level of factor V activity. However, addition of more factor V to the original activation mixture at the time when the factor V activity had plateaued or began to decline, resulted in a subsequent increase in factor V activity of the total mixture to nearly the same or a greater level of activation than that observed with the initial activation mixture.

Moreover, in a mixture of factor V, Ca^{2+}, and a level of factor Xa sufficient to fully activate the factor V, the factor Xa could be recovered or separated from the factor Va activity by gel filtration of the mixture on Sephadex G-200 (Figure 5). The factor Va and factor Xa activities eluted in separate volumes and the recovered factor Xa not only retained approximately 80% of its original clotting activity but could still activate a fresh portion of the same factor V preparation to 65% of the level observed using a fresh aliquot of factor Xa.

Incubation of factor Xa, in 0.1 M Tris-HCl, pH 7.5, with a 1000-fold molar excess of DFP in isopropyl alcohol for 2 h at 37 °C, followed by dialysis for 12–15 h against 0.1 M Tris-HCl, pH 7.5, resulted in a loss of 80% of the factor Xa clotting activity when compared with a factor Xa control. The latter was an identical sample of factor Xa to which an aliquot of isopropyl alcohol alone was added and processed as with the DFP-Xa. No activation of factor V by DFP-Xa was observed when compared with the activation by the control Xa.

It was also observed here that factor Xa was not stable to heat treatment with regard to its own clotting activity, or its ability to activate factor V. After heating factor Xa in a boiling water bath for 10 min at pH 7.5, it retained only 0.2% of its original clotting activity and was not able to activate factor V to any extent in the presence of Ca^{2+} alone or in the presence of Ca^{2+} and phospholipid, under conditions comparable to those described above.

(d) α-Chymotrypsin and Trypsin. Bovine factor V was activated by α-chymotrypsin at pH 7.35 and 25 °C in the presence of 5 mM $CaCl_2$. When 1–5 units of factor V was treated with 3.1–6.2 μg of enzyme, the factor V activity increased some five to six fold over a time period of 15–20 min. This increase in factor V activity was accompanied by a change in the apparent molecular weight of this factor, similar to that observed with the other activators noted above.

Further it was observed that the activation of factor V by α-chymotrypsin could be inhibited by TPCK, a specific inhibitor for α-chymotrypsin. Treatment of this latter enzyme with a 100-fold excess of TPCK in methanol for 1 h at 37 °C followed by dialysis for 12–15 h to remove excess TPCK resulted in a complete loss in ability of the α-chymotrypsin to activate the factor V. A control preparation of α-chymotrypsin treated with an aliquot of methanol, and further processed as with the TPCK–α-chymotrypsin sample, achieved activation of factor V to the same extent as described above with the untreated enzyme.

Treatment of bovine factor V with bovine trypsin did not result in an activation of this factor and at most levels of trypsin only a loss of factor V activity resulted. When 1–5 units of bovine factor V was treated with 0.1–1.0 μg of trypsin, the factor V activity remained either constant over a period of 15–30 min at 25 °C and pH 7.35 or rapidly decreased over the same time period.

Discussion

Since the structure of plasma factor V can be altered by contact with inorganic salts such as ammonium sulfate and cellulose phosphate (Day and Barton, 1972), the isolation procedure developed in this study was designed to preserve the native form of factor V and to recover this factor from plasma in good yields and with high specific activity. Concomitant with this development, it then became feasible to investigate its conversion to an activated form, factor Va, by four different reagents, factor Xa, RVV-V, thrombin, and α-chymotrypsin. Various facets of these observations are explored below.

A substantial challenge to investigators in the blood coagulation field for a number of years has been to isolate a stable preparation of factor V in good yield. A number of procedures have been reported (Papahadjopoulos and Hanahan, 1964; Esnouf and Jobin, 1967; Philip et al., 1970; Day and Barton, 1972; Dombrose et al., 1972) in which the isolated factor V varied considerably in quality and characteristics. In most instances, these methods utilized bovine plasma as a source material but recently the isolation of factor V from human plasma was reported by Giddings (1974) and by Rosenberg et al. (1975). Giddings outlined a procedure in which the final factor V sample, which was isolated in 11% yield, showed an apparent molecular weight of 300 000. This molecular weight is distinctly lower than the 430 000–440 000 value obtained in our studies on bovine plasma factor V. Though species differences can easily be invoked as a reason for this difference, Kahn and Hemker (1972) did record a molecular weight in the 400 000 range for bovine as well as human plasma factor V. While the isolation procedure outlined by Rosenberg et al. (1975) was provocative, there was a paucity of data on which to base any assessment of the chemical or biochemical characteristics of the purified material.

Perhaps one of the more significant reports in recent years on factor V was that of Day and Barton (1972) who noted that procedures employing highly charged reagents (ionic), such as cellulose phosphate, and ammonium sulfate, led to formation of altered forms of factor V. Later Dombrose et al. (1972) proposed the use of chromatography columns containing quaternary ammonium groups, e.g., QAE-cellulose, for isolation of factor V. On the basis of this information, it became

obvious then that factor V had characteristics suggestive of a hydrophobic protein, and that the isolation procedures should reflect this behavior. This was clearly shown in our experiments in which a factor V preparation passed through a Sepharose 6B column exhibited considerable tailing, and a low recovery of activity. On the other hand, a comparable factor V sample passed through a desulfated Sepharose 6B column exhibited a much sharper elution profile, was recovered in good yields, and has an apparent molecular weight near 439 000 daltons. Another important facet of this latter isolation procedure was the use of poly(ethylene glycol) 6000 to allow concentration of the factor V by precipitation. In our hands, concentration of a factor V preparation by procedures such as ultrafiltration often can lead to an activation of factor V and a concomitant decrease in molecular weight value near 240 000. Consequently by attention to these potential problems, one can obtain a factor V preparation of high purity, in good yields, and of considerable stability.

It has become increasingly evident that factor V, as might have been expected, has characteristics of a glycoprotein. Dombrose and Seegers (1973) reported the presence of 8.7% carbohydrate in their factor V preparation. Recently, Gumprecht and Colman (1975) noted that their factor V sample contained as much as 21% carbohydrate, with mannose, *N*-acetylglucosamine, galactose, and *N*-acetylneuraminic acid as the principal components. In preliminary studies in this laboratory, it was found that factor V contained near 18% total carbohydrate, which consisted of a mixture of galactose, mannose, glucosamine, and sialic acid (Smith, C., and Delaney, S., personal communication).

It is evident from the data presented here that bovine plasma factor V can be converted by four different reagents, RVV-V, Factor Xa, thrombin, and chymotrypsin from a high molecular weight form, near 439 000 daltons, to an activated form of lower molecular weight form, near 240 000 daltons. Concomitant with this change there is a significant increase in clotting activity and specific activity associated with this new low molecular weight form. Kahn and Hemker (1972) reported that Russell's viper venom as well as thrombin could activate bovine factor V (and also human factor V). Concomitant with this activation, there was a reduction in molecular size of the bovine factor V from a value of near 400 000 for the parent molecule to one of approximately 195 000 for the activated form. Though specific data supporting a possible mechanism for this conversion are lacking, it appears highly likely that all three activating systems are behaving in an enzymatic manner. For a number of years, the question has been posed as to the form of factor V most active in the prothrombin reaction. Evidence has been steadily accumulating from a number of laboratories supporting the concept that circulating plasma factor V is a less active or inactive form and that this must be "activated" to exert any significant effect on the clotting mechanism. Hence, our data would provide further support for this concept.

It is interesting to note that factors Va and Xa do not appear to form a complex as evidenced by filtration of a factor V–factor Xa activation mixture on Sephadex G-200 (see Figure 5). The Factor Va and Xa activities were completely separable after activation of the factor V had been maximized under the described experimental conditions and in the presence of 25 mM Ca^{2+}. Previous studies (Papahadjopoulos and Hanahan, 1964) had shown that in the presence of added phospholipid, factor V and Xa activities coelute from Sephadex G-200. Consequently phospholipid alone must serve as the adhesive force required for maximum rate of interaction between these two proteins. Further it was shown in our studies that approximately one-third less factor Xa was required to achieve the same rate and extent of activation of factor V in the presence of phospholipid, in this case PS/PC mixtures, plus Ca^{2+} than in the presence of Ca^{2+} only. Thus phospholipid in some manner facilitates the interaction between factor V and factor Xa which otherwise do not appear to have a strong affinity per se in an in vitro system. The importance of the phospholipid may be much greater in an in vivo situation where concentration effects of these coagulation factors may be the limiting condition.

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Evidence That Both Growing DNA Chains at a Replication Fork Are Synthesized Discontinuously†

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ABSTRACT: *Escherichia coli, Bacillus subtilis,* and T7-infected *E. coli* have been labeled with short pulses of [^{3}H]thymidine, and the labeled DNA has been examined by sedimentation in alkaline sucrose. In all three systems, the great majority of the DNA labeled by a short pulse is found in the form of small DNA chains of 10S, the so-called Okazaki pieces. The *B. subtilis* and T7 nascent DNA fragments hybridize with equal efficiency to the separated strands of *B. subtilis* and T7 DNA, respectively. The results suggest that both growing DNA chains at a given replication fork are synthesized discontinuously in the case of *E. coli, B. subtilis,* and T7. We have found that the method used to terminate the pulse affects the size distribution of the labeled DNA; some methods allow joining of nascent DNA fragments after termination of the pulse. Previous reports of discontinuous DNA synthesis on only one growing DNA chain and continuous synthesis on the other DNA chain are probably due to preferential joining of Okazaki pieces on the DNA chain growing in the overall 5′ → 3′ direction.

The well-known work of Okazaki and co-workers has shown that short pulses of [^{3}H]thymidine are incorporated into small DNA chains, the so-called Okazaki pieces, in a variety of bacterial and bacteriophage systems (Okazaki et al., 1968a,b). Pulse-chase experiments have demonstrated that the Okazaki pieces are the precursors of long DNA chains; the latter are indistinguishable in length from the bulk of the DNA. Thus the idea has arisen that one or both of the two growing DNA strands at a replication fork are synthesized discontinuously, and later joined together by DNA ligase.

The question of whether one or both growing DNA chains at a given replication fork are synthesized discontinuously is still not settled. Okazaki et al. (1968a,b) reported that label from a very short pulse appears almost exclusively in small pieces in *E. coli* and T4, implying that both strands are made discontinuously. In three bacteriophage systems, T4, λ, and SPP1, Okazaki pieces hybridize equally to both of the separated DNA strands (Sugimoto et al., 1969; Ginsberg and Hurwitz, 1970; Polsinelli et al., 1969). However, since DNA is now known to replicate bidirectionally in many systems, hybridization data alone cannot be definitive in settling the question.

There have been reports in the case of *E. coli* (Iyer and Lark, 1970; Louarn and Bird, 1974) and *B. subtilis* (Okazaki et al., 1970; Kainuma and Okazaki, 1970) that approximately half the label in a short pulse is found in large DNA chains. DNA hybridization studies as well as other methods have led these workers to conclude that only one DNA strand at a replication fork is synthesized discontinuously in the form of Okazaki pieces, while the other strand is synthesized continuously.

In this paper we report on pulse-labeling studies of *E. coli, B. subtilis,* and T7-infected cells. In all cases, the great majority of the DNA labeled by a short pulse under our conditions is in the form of small DNA fragments, i.e., Okazaki pieces. We have found that the method used to terminate the pulse affects the size distribution of labeled DNA and that some methods allow joining of nascent DNA fragments after termination of the pulse. Previous reports of discontinuous DNA synthesis on only one DNA chain and continuous synthesis on the other DNA chain are probably due to preferential joining of Okazaki pieces on the DNA chain growing in the overall 5′ → 3′ direction.

Materials and Methods

Bacterial Strains. E. coli strains included strain B, 15 TAU *thy*$^-$ *arg*$^-$ *ura*$^-$, and LC 434 F$^-$ *thy*$^-$ *leu*$^-$ B_1^- λ λ*imm 434* (Louarn and Bird, 1974). The *B. subtilis* strain was 168 *thy*$^-$ *trp*$^-$ (Wilson et al., 1966).

Growth, Pulse Labeling, and Lysis of E. coli. Cells were grown in M9 medium supplemented with 0.4% glucose and 0.1% casamino acids. The medium for *E. coli* 15 TAU was supplemented with 2 μg/ml thymine, 20 μg/ml uracil, 50 μg/ml arginine, and 80 μg/ml tryptophan. In some cases [^{14}C]thymine was added to the medium to uniformly label the DNA (0.05 μCi/ml final concentration). The medium for *E. coli* LC 434 was supplemented with 2 μg/ml thymine, 25 μg/ml leucine, and 1 μg/ml thiamine. Cells were grown at 37 °C to 1.5 × 10^8 cells/ml, switched to 25 °C, and grown to 2.5 × 10^8 cells/ml, diluted 1:1 with fresh medium and again grown at 25 °C to 2.5 × 10^8 cells/ml. Five-milliliter aliquots of the culture were pulse labeled at 25 °C by the addition of 50 μl of 1 mCi/ml [^{3}H]thymidine (7 Ci/mmol). Pulses were terminated with 20 ml of −10 °C acetone. The cells were pelleted,

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