Dissociation and Association of the Oligomeric Forms of Factor V*

George Philip, Julieta Moran, and Robert W. Colman

ABSTRACT: A 5000-fold purification of factor V from bovine plasma was heterogeneous by polyacrylamide disc gel electrophoresis and gel filtration. With gel filtration on Sephadex G-200 and Sepharose 4B, three forms, L, A, and C, were identified. Form L, excluded from Sephadex G-200 and Sepharose 4B columns, as well as 7% polyacrylamide gels, is probably a phospholipid–factor V complex. This conclusion is drawn from the detection of phospholipids when form L was isolated by gel filtration, and the identical properties of factor V bound to phospholipid. Form C was a homogeneous species on analysis by polyacrylamide gel electrophoresis and by sedimentation equilibrium studies. From the molecular weight of 38,000 and the Stokes radius of 5.2 mμ, a frictional ratio of 2.32 was calculated for form C. Form A, probably an oligomer of form C, had a Stokes radius of 8.5 mμ. The dissociation of form A to form C could be induced by the anions $SCN^- > ClO_4^- > NO_3^- > Cl^-$. In contrast, form C could be converted into form A by high concentrations of glycerol. The results may explain conflicting reports on the molecular weights and stability of factor V.

Factor V[1] is a plasma protein which accelerates the conversion of prothrombin into thrombin by activated factor X in the presence of phospholipid and calcium. Ware *et al.* (1947) postulated the existence of different forms of factor V. Although subsequent reports identified only one form, a disparity existed as to the molecular weight of factor V. The different molecular weights reported have been 99,000 (Aoki *et al.*, 1963), 180,000 (Hussain and Newcomb, 1963), 290,000 (Esnouf and Jobin, 1967), and 400,000 (Papahadjopoulos *et al.*, 1964). Barton and Hanahan (1967) observed two active forms of factor V; their estimated molecular weights were 350,000 and 70,000.

The stability of factor V can be altered by experimental conditions. Complete loss of factor V activity resulted from freezing and thawing or on dialysis against 0.15 M Tris buffer containing 0.5 M NaCl (Esnouf and Jobin, 1967). Glycerol, in contrast, preserved activity.

The present study was undertaken to reinvestigate the molecular properties of factor V and the effect of experimental conditions upon the molecular weight of factor V. This paper presents evidence of the existence of different oligomeric forms of factor V and the interconversions among these forms.

Experimental Procedures

Materials. Factor V was prepared from bovine plasma as described by Colman (1969). The starting material for gel filtration was the cellulose phosphate eluate obtained with 0.4 M phosphate (specific activity 50 units/mg).[2] The assay for factor V (Colman, 1969) measured the ability of test solutions to correct the defective thrombin formation using plasma artificially depleted of factor V. Similar results were obtained using plasma from a human subject with a congenital isolated deficiency of factor V. Bovine thrombin was purified, characterized and assayed as described previously (Colman, 1969). Sodium heparin USP (150 units/mg) was obtained from Abbott Labs. Inosithin (soybean phospholipids, Associated Concentrates) was homogenized in an appropriate buffer to make a 2% stock solution and stored at −20°. Sephadex G-200, Sepharose 4B, and Dextran Blue 2000 were obtained from Pharmacia Fine Chemicals, hydroxylapatite gel was purchased from Bio-Rad, and lactic dehydrogenase, bovine albumin, and cytochrome C from Sigma Chemicals Co. DFP obtained from Aldrich was diluted to a 1 M stock solution in 2-propanol. Other reagents used were of analytical grade.

Gel Filtration. Sephadex G-200 gel filtration was carried out at 8° using upward flow columns at a pressure of no more than 15 cm of H_2O. The total column volume (V_t) was calculated from the geometry of the column and checked by the elution volume of alanylglycine (171 ml). The void volume (V_0) obtained from the elution volume of Dextran Blue 2000 was 52 ml. The included volume (V_i) was calculated from the equation $V_i = V_t - V_0 - V_g$. Since the water regain of Sephadex G-200 is 20 g of H_2O/g of dry Sephadex, the gel volume (V_g) can be neglected. This assumption introduces an error of less than 2%. The elution volume (V_e) was the volume at the peak of the activity curve. The effective pore radius of the gel beads was calculated at 17.9 mμ using lactate dehydrogenase as a standard. This is in accord with Ackers (1964), whose reported value was 18.7 mμ. The distribution coefficient $K_D = (V_e - V_0)/V_i$, and Stokes radii were calculated as described by Ackers (1964). The columns were further calibrated with bovine albumin and cytochrome C for the estimation of

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

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

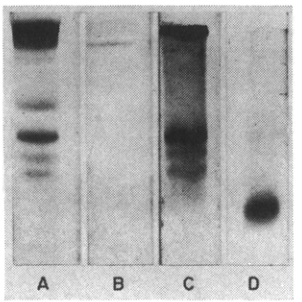

FIGURE 1: Polyacrylamide disc gel electrophoresis of the cellulose phosphate eluate and the different forms of factor V: (A) cellulose phosphate eluate; (B) form L; (C) form A; and (D) form C. Forms L, A, and C were obtained from Sepharose 4B column (see text).

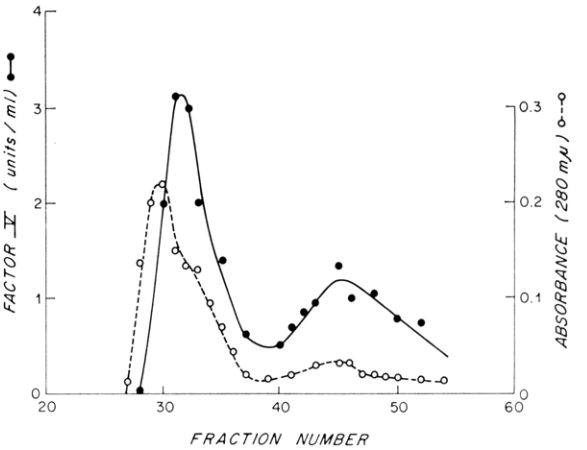

FIGURE 2: Gel filtration of cellulose phosphate eluate on Sephadex G-200 column. Concentrated cellulose eluate (2 ml, 30 units/ml) was injected on a 2.5 × 3.5 cm Sephadex G-200 column equilibrated with 0.04 M potassium phosphate buffer, pH 7.0, containing 10% glycerol. The same buffer was used for elution. Fraction volume was 1.8 ml.

molecular weight (Andrews, 1964). Sepharose 4B gel filtration was carried out in similar fashion to Sephadex G-200 except that a downward flow column was used. The total volume of the column was 156 ml and the void volume (V_0) was 52 ml. Unless otherwise specified, 0.04 M potassium phosphate buffer (pH 7.0 containing 10% glycerol by volume) was used for all the gel filtration experiments with factor V. The water regain of Sepharose 4B is 25 g of H_2O/g and V_g was neglected.

Disc Electrophoresis. Separation was performed in 7.5% polyacrylamide gel (Tris-glycine buffer, pH 9.3, at 8°; Davis, 1964; Ornstein, 1964). The sample gel was sometimes omitted, and the sample layered on the running gel in 25% glycerol. Protein concentration was determined in a Beckman DU spectrophotometer from the absorbency at 280 mμ. It was assumed that $\epsilon_{280}^{1\%}$ was 10. Protein solutions were concentrated by the use of an Amicon ultrafiltration apparatus (Blatt *et al.*, 1965) with a UM-10 or XM-50 membrane.

Ultracentrifugation. Sedimentation equilibrium studies were performed in a Spinco Model E analytical ultracentrifuge by the meniscus depletion method of Yphantis (1964). Raleigh interference optics were used for concentration measurements. A double-sector cell with Sapphire windows contained the 0.1-ml samples. Equilibrium was considered achieved if pictures taken at 18 and 24 hr were identical. The fringes were measured using a Nikon 6 microcomparator. Partial specific volume was taken as 0.73 (Esnouf and Jobin, 1967). The average molecular weight was obtained from the slope calculated by least-squares analysis of ln c *vs.* r^2.

Effect of Anions on Thrombin Activation[3] *of Factor V.* Form A of factor V (6–8 units/ml in 0.01 M phosphate buffer, pH 7.0) was incubated at 25° with an equal volume of the same buffer containing the anion at various concentrations. At specified intervals, 0.05-ml aliquots were transferred to 0.95 ml of Veronal buffer (0.02 M Veronal in 0.15 ml of NaCl and 0.1 M sodium oxalate, pH 7.4). The resulting solution was used for thrombin activation experiments as described by Colman (1969). Initial activity was determined by a parallel run in which buffer was substituted for thrombin. The stability of factor V and changes in thrombin activity were monitored in control experiments, indicating activity and susceptibility to thrombin were constant throughout the course of the individual experiments. The concentration of glycerol in the incubation mixture was less than 1%.

Analysis of Lipids. Lipids in form L of factor V were established by extraction by minor variations of the procedure of Folch *et al.* (1957). Each extract was further divided into phospholipids and neutral lipids by thin-layer chromatography in two solvent systems (Skipski *et al.*, 1964; Gottfried, 1967).

Results

Disc Electrophoresis of Cellulose Phosphate Eluate. Bovine factor V separated by polyacrylamide gel electrophoresis is composed of at least 7 bands (Figure 1A). The specific activity of the cellulose phosphate eluate (50 units/mg) was comparable with that reported by Esnouf and Jobin (1967) and Barton and Hanahan (1967).

Gel Filtration of Cellulose Phosphate Eluate on Sephadex G-200. Sephadex G-200 gel filtration of factor V reveals the presence of several proteins (Figure 2). The activity eluted with a peak at fraction 45, henceforth referred to as form C, usually had specific activity greater than that of form A (peak fraction 32) and corresponded to the protein peak.

Gel Filtration of Cellulose Phosphate Elutate on Sepharose 4B. Since form A of factor V elutes close to the void volume of the Sephadex column, Sepharose 4B gel filtration, which separates larger molecules, was used instead. Figure 3 illustrates the results obtained by gel filtration of the cellulose phosphate eluate on a Sepharose 4B column and shows three distinct activity peaks. The forms at these peaks were separately pooled, concentrated, and rechromatographed on Sephadex G-200 column. The forms eluted from Sepharose 4B column with peaks at fractions 32 and 43 corresponded, respectively, to form A and form C from Sephadex G-200.

Characterization of Form L of Factor V. The additional activity peak noted at tube 15, corresponding to the void volume of the Sepharose 4B column (Figure 3), represents a form of factor V (form L) with a molecular weight greater than two million. Because of form L's large molecular size, it did

[3] The term "activation" as used in this paper represents only an increase in measured activity and does not connote a change from a precursor to an active entity.

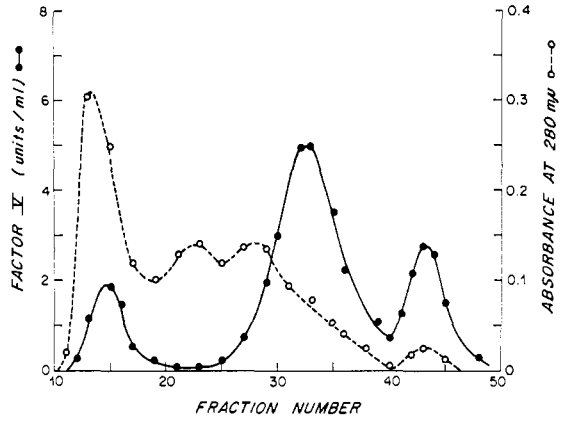

FIGURE 3: Gel filtration of cellulose phosphate eluate on Sepharose 4B column. Concentrated cellulose phosphate eluate (3 ml, 60 units/ml) was fed on a Sepharose 4B column 2.5 × 33 cm equilibrated with 0.04 M potassium phosphate buffer, pH 7.0, containing 10% glycerol. The same buffer was used for elution. Fraction volume was 3.7 ml.

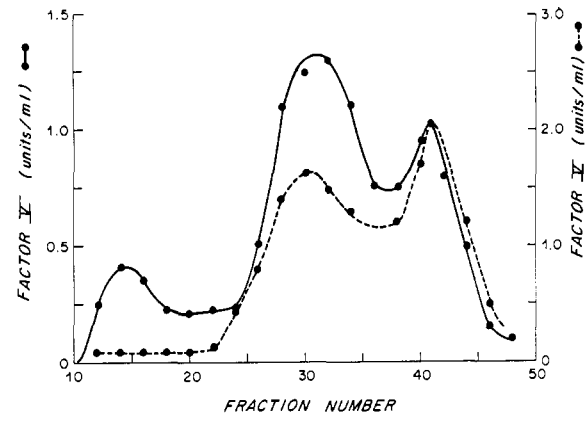

FIGURE 4: Gel filtration of forms A and C of factor V and a mixture of factor V and inositol on Sepharose 4B column. The protein concentrations are omitted for the sake of clarity. The curve illustrated by dashed lines represents the activity profile of Sepharose 4B gel filtration of the cellulose phosphate eluate. Fractions 27–45 were pooled and concentrated, and 3 ml of the concentrate (28 units/ml) was mixed with 1 ml of a 2% suspension of inositol. After stirring for about 30 min at room temperature, the mixture was gel filtered through the same column. The solid lines represent the factor V activity profile. Fraction volume was 3.8 ml. Other details were as in Figure 3.

not penetrate the 7% polyacrylamide gel (Figure 1B) but remained at the origin. Factor V activity was successfully eluted from this excluded band. However, when the pore size of the polyacrylamide gel was enlarged (4% instead of 7%), form L entered the gel as a single band. Form L was always visibly turbid when isolated in 0.04 M phosphate buffer. The turbidity was greatest when the ionic strength was lowered by dialysis against distilled water or dilute phosphate buffer (0.005 M, pH 7.0). Turbid solutions could be solubilized by dialysis against 0.3 M KCl. When the turbid concentrate (containing 10% glycerol) was centrifuged at 20,000*g* for 1 hr; the precipitate contained 90–95% of the activity while the supernatant had little or none. Similar results were obtained by the removal of glycerol by dialysis against phosphate buffer (0.005 M phosphate, pH 7.0) and subsequent centrifugation at 5000*g*. After extraction of the lipid from form L, the total lipids were identified as 60% phosphatides and 40% neutral lipids. The phosphatide fraction was tentatively analyzed to contain primarily phosphatidylcholine and sphingomyelins and trace amounts of phosphatidylethanolamine and combined phosphatidylinositol-phosphatidylserine. The neutral lipid fraction consisted primarily of triglycerides and cholesterol ester and trace amounts of monoglycerides and an unidentified slow-moving spot.

During the formation of prothrombinase activity, factor V is bound to a phospholipid with factor X and calcium. In the present study the gel filtration technique was used to examine the binding of factor V to inositol. Fractions which corresponded to forms A and C from the Sepharose 4B column were pooled and concentrated at room temperature. After incubation of the concentrate and inositol for 30 min, the concentrate was again subjected to gel filtration on the Sepharose 4B column. For clarity and ease of comparison, only the activity curves are shown in Figure 4. Some of the factor V activity now appeared in the void volume of the column where the inositol was eluted, suggesting that form L, obtained from Sepharose 4B columns, is probably factor V bound to phospholipids. The nature of the phospholipids and the mode of binding are currently under investigation.

Further Purification of Form A. The elution pattern of form A was not characteristic of a homogeneous species. The activity peak did not correspond to the protein peak, and several protein bands were detected on polyacrylamide gel electrophoresis (Figure 1C). The specific activity of form A was approximately half that of the starting material. Form A was purified about threefold over the pooled Sepharose fraction with a yield of 36% (Table I). Polyacrylamide electrophoresis of this purified fraction now revealed only two bands rather than four. A similar purification was achieved by chromatography of form A in potassium phosphate buffer (0.04 M) on an hydroxylapatite column, washing with 0.1 M phosphate, and elution with 0.3 M phosphate (pH 7.0). In 10% glycerol at −50°, form A retained all its activity after 3 months.

Characterization of Form C. Form C obtained from Sepharose 4B column appeared as a single band after electrophoresis in polyacrylamide gel (Figure 1D). The specific activity (112 units/mg of protein) was more than twice as high as cellulose phosphate eluate. This form was unstable even in the presence of 10% glycerol, losing up to 50% activity in 12 hr at 6°. In the absence of glycerol, activity was almost totally lost at 6° in 12 hr or in frozen solution at −50°. Form C could be stored indefinitely and would retain its potency when the fractions were concentrated (100 units/ml or more) and stored in 50% glycerol at −50°. As noted below, however, this procedure results in a change in molecular size.

Molecular Size of Factor V in Native Plasma. The possibilities were investigated that the various molecular forms of factor V are artifacts induced during collection or purification by partial proteolysis, especially due to thrombin. Beef blood (nine volumes) was collected into one volume of the following anticoagulants: (1) 0.1 M sodium oxalate, (2) 1% sodium heparin, and (3) 0.1 M sodium oxalate containing DFP in a

TABLE I: Purification of Form A.[a]

Procedure	Volume (ml)	Factor V Activity (units/ml)	Sp Act. (units/mg)	Recovery
Starting material	5	50	20	
0.04 M Phosphate eluate (pooled supernatants)	15	1	3	6
0.15 M Phosphate eluate	10	2	6	8
0.3 M Phosphate eluate	10	9	56	36

[a] Fractions corresponding to form A from Sepharose 4B columns were pooled and concentrated. The concentrate which was used as the starting material was made 0.3 M in potassium phosphate by addition of 0.5 M phosphate buffer, pH 7.0. The phosphate was precipitated by adding 1 M $CaCl_2$ and centrifuged at 2500*g* for 20 min at 2° to collect the precipitate of calcium phosphate. The supernatant had no activity. The precipitate was washed three times with 0.04 M phosphate and then once with 0.15 M phosphate, pH 7.0. Activity was eluted with 0.3 M phosphate, pH 7.0. Washings and elution were carried out by suspending the precipitate in the respective buffer, for 10–20 min. with stirring at 2°, followed by centrifugation to collect the supernatant which was assayed for activity.

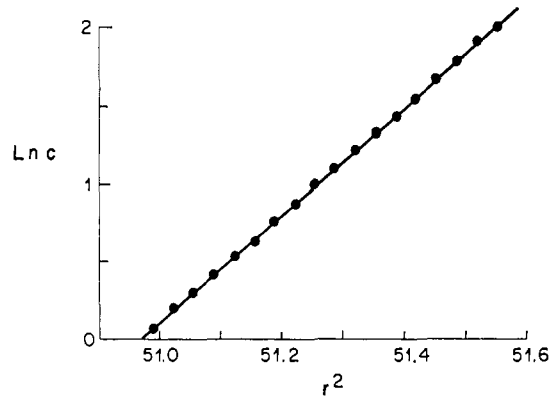

FIGURE 5: Sedimentation equilibrium studies of form C of factor V. Form C from Sepharose 4B column was concentrated and dialyzed exhaustively against 0.01 M potassium phosphate, pH 7.0, containing 0.2 M KCl. The natural logarithm of this relative concentration (ln *c*) was plotted against the square of the distance from the axis of rotation (r^2). These results were obtained after 24 hr at 14.8° and 40,055 rpm. The concentration of form C used was about 0.2 mg/ml.

concentration of 10^{-2} M. Each blood sample was immediately centrifuged and the plasma removed. Within the day, 3 ml was filtered on Sepharose 4B columns previously equilibrated with Tris (0.02 M, pH 7.4) and containing 0.15 M sodium acetate and 10^{-3} M DFP. In each of three experiments the factor V activity peaks corresponded identically with forms A and C of the purified factor V preparations (Figure 3). Form A constituted 75% of factor V activity; form C, 25%.

The effectiveness of the DFP was measured in separate experiments in which DFP (10^{-3} M) inactivated 2 units of thrombin with a half-life of 0.25 min. Even after 12 hr, the time of gel filtration, the DFP in the buffer inactivated thrombin with a half-life of 2 min.

Determination of Molecular Weights of Forms C and A. Electrophoretically homogeneous form C was exhaustively dialyzed against 0.005 M phosphate buffer (pH 7.0) containing 0.2 M KCl. The molecular weight of form C was determined by sedimentation equilibrium (Yphantis, 1964). Complete depletion of meniscus concentration could not be achieved below 30,000 rpm. The linear relationship of ln *c vs.* r^2 (Figure 5) suggests the homogeneity of form C. The molecular weight was found to be 38,000 ± 2,000.

Direct estimations of molecular weight from a calibration curve (obtained by plotting elution volumes against logarithm of molecular weights (Andrews, 1964)) assumes that the frictional ratios are the same for the calibrating proteins and the unknowns. By this method, the molecular weight of form C is 160,000 and that of form A, 310,000. That the molecular weight (160,000 *vs.* 38,000) for form C by the two methods differs so markedly suggests that the frictional ratio of form C is greater than that of the proteins used for calibration.

The Stokes radius (a_0) of a spherical molecule can be calculated from the weight of the molecule if the frictional ratio is 1.00. The Stokes radius (*a*) was calculated from the K_D on Sephadex G-200 (Ackers, 1964). The frictional ratio is then defined as a/a_0. The distribution coefficients are tabulated in Table II and Stokes radii of the various forms of factor V are computed. Using the molecular weight of 38,000 for form C, the corresponding value for a_0 is 2.2 and the frictional ratio was calculated to be 2.3.

Interconversion of Forms A and C. Several observations suggested that form A was an oligomer of form C. Both forms are present in most preparations. Since the activity of form C was stabilized by 50% glycerol, conditions which might convert form C into the more stable form A were investigated. A preparation of factor V containing 30% A and 70% C (Figure 6) was stored in 50% glycerol for 48 hr. Subsequent gel filtration revealed that the composition had changed to

TABLE II: Stokes Radii and Molecular Weights of Forms A and C.[a]

Forms of Factor V	Number of Determinations	K_D	Stokes Radius (mμ)	Molecular Wt	Frictional Ratio
A	6	0.05	8.5 ± 0.1[b]		
C	8	0.22	5.2 ± 0.2	38,000[c]	2.32

[a] The cellulose phosphate eluate was concentrated and gel filtered through Sephadex G-200 as in Figure 3. Each determination was obtained in a separate experiment with calibrated columns. Stokes radii were calculated as described by Ackers (1964). The mean pore radius of the Sephadex G-200 gel was found to be 17.9 mμ using lactate dehydrogenase as calibrant. [b] Standard deviation of the mean. [c] From sedimentation equilibrium studies (see text).

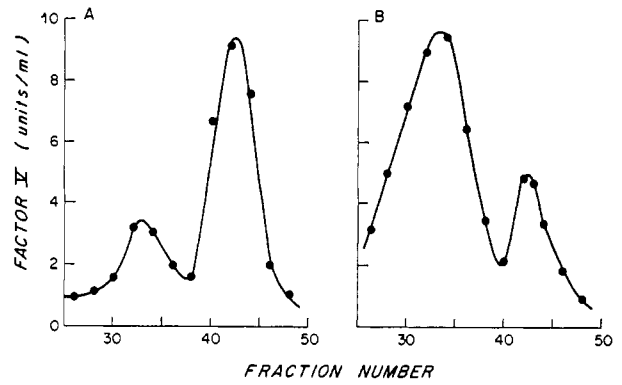

FIGURE 6: Sepharose 4B gel filtration of a factor V preparation before and after incubation with 50% glycerol. The concentrated cellulose phosphate fractions were made 50% in glycerol and one part (3 ml, 175 units/ml) was gel filtered immediately. (A), and the other part (4 ml, 175 units/ml) was gel filtered after keeping at −5° for 48 hr (B).

77% A and 23% C (Figure 6B). To further verify this effect, a preparation of form C (100 units/ml) which was electrophoretically homogeneous was stored in 50% glycerol for 48 hr and analyzed by gel filtration on Sepharose 4B. The results were identical with those shown in Figure 6B, thus, strongly indicating that form A is an oligomer of form C.

Forms A and C react differently upon exposure to purified thrombin. Preparations constituted primarily of form A show a twofold increase of activity on exposure to thrombin; when form C predominates, no activation is observed (R. W. Colman, unpublished results, 1970). Therefore, the increase in activity of factor V on exposure to thrombin may be taken as a measure of the relative concentrations of forms A and C. Using this technique, a time-dependent increase in the extent of thrombin activation was found when form C (100 units/ml) was stored in 50% glycerol. Conversely, form A progressively lost the ability to be activated by thrombin when glycerol was absent and form A gradually changed to form C.

Influences of Anions on Form A. Thrombin failed to increase the activity of a preparation of factor V after dialysis against 0.3 M KCl for 18–36 hr at 7°. The factor V samples dialyzed against KCl were eluted from Sephadex G-200 as form C. The control (undialyzed) sample was form A. This experiment substantiates further the differences between forms A and C revealed by gel filtration and parallels their susceptibility to thrombin activation.

Attempts to increase the rate of change of form A to form C with higher concentrations of KCl resulted in considerable loss of factor V activity. Therefore, the influence of other anions (0.3 M) was investigated (Figure 7B). Thiocyanate changed form C to form A most rapidly and was followed by perchlorate and nitrate. With ClO_4^- the rate of change increased as the anion concentration was increased (Figure 7A). That these changes were due to the influence of anions was demonstrated by comparing the rate of change with the same anion but with different cations. The rate of change was the same when either 0.4 M $NaNO_3$ or 0.4 M KNO_3 was used.

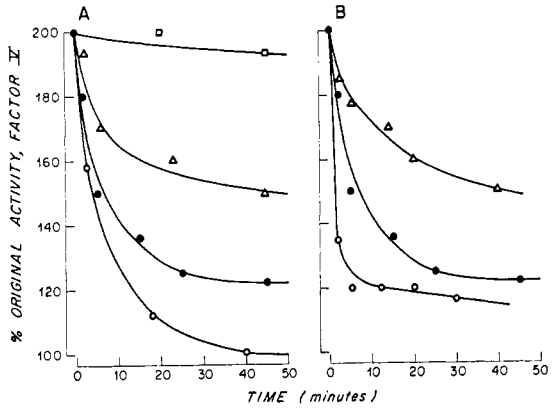

FIGURE 7: Effect of anions on the thrombin activability of form A: (A) effect of different concentrations of ClO_4^- (O—O) 0.33 M, (●—●) 0.3 M, (Δ—Δ) 0.2 M, and (□—□) 0.1 M; (B) effect of different anions (0.3 M) (O—O) SCN^-, (●—●) ClO_4^-, and (Δ—Δ) NO_3^-.

Discussion

The results of this study show that factor V is composed of three forms—form C, form A, and form L. Form C has a molecular weight of 38,000. Form A is apparently an associated form of C with a larger Stokes radius. Form L is a large molecular weight complex of factor V and phospholipids.

The profiles of factor V activity from Sephadex G-200 (Figure 2), Sepharose 4B (Figure 3), and disc gel electrophoresis (Figure 1) indicate that these preparations of factor V are heterogeneous and disagree with earlier reports (Barton and Hanahan, 1967; Esnouf and Jobin, 1967) on the purity of factor V preparations of comparable specific activity. It is interesting that one of these investigations (Esnouf and Jobin, 1967) reported the unexplained finding of a fast-moving component of 32 S; this species was probably eluted in the void volume of the gel filtrations described in the present studies. In addition, these same investigators obtained a 94% recovery of the nitrogen applied to the column, yet only 73% of the weight of factor V could be accounted for by the amino acid composition. This discrepancy could result from the composition of form L of factor V which may be an amalgam of factor V and phospholipids.

Phospholipid is a requisite for optimum accelerating activity of factor V in the conversion of prothrombin into thrombin. Recently, several workers (Papahadjopoulos and Hanahan, 1964; Jobin and Esnouf, 1967) have shown that factor V binds to phospholipid even if no calcium is present. The evidence that form L is a phospholipid–factor V complex is indirect in our studies. Form L contains lipid, most of which is phospholipid, or more specifically, phosphatidylcholine. The latter is effective in the intrinsic coagulation system. Moreover, form L can be produced in a model system by incubating mixed phospholipids with form A and form C. An alternative explanation, although less likely, is that form L may be a higher aggregate of form A. No transformation of form L to form A and form C was effected in any of our experiments. Recently, Ratnoff *et al.* (1969) reported that human factor VIII (anti-hemophilic globulin) emerges in the void volume of a Sepharose 4B column. The dominant components of these fractions were phospholipids. It is interesting to speculate that since both factor V and VIII require phospholipids for their optimal action, phospholipid–protein complexes may represent intermediate reaction products.

Barton and Hanahan (1967) also detected two activity peaks of a factor V preparation by Sephadex G-200 gel filtration. The molecular weight (Andrews, 1964) for the major component was 350,000. Using this same method in our study, we obtained a molecular weight for form A of 310,000. This suggests that the major component isolated by Barton and Hanahan was also form A. The K_D of 0.05 reported by Papahadjopoulos *et al.* (1964) from gel filtration on Sephadex G-200 is identical with our value for form A. Esnouf and Jobin (1967) reported a molecular weight of 290,000 by sedimentation–diffusion studies. In other studies employing both sedimentation and diffusion techniques, Hussain and Newcomb (1963) reported molecular weight of 182,000. This may support our conclusion that form A represents a higher oligomer of form C. Numerous observations suggest that form C and form A are interconvertible. Freezing and thawing in the absence of glycerol increases the rate of change from form A to C. Storage of homogeneous form C in 50% glycerol-induced formation of form A (Figure 6). Further finding of forms A and C in untreated oxalate plasma suggests that neither of these forms is a laboratory artifact but rather is a natural form of factor V present *in vivo*. As these forms were observed in the presence of DFP or heparin, both of which neutralize thrombin, it is unlikely that partial proteolysis resulted in these two forms during collection or purification (Jackson and Hanahan, 1968; Jackson *et al.*, 1968). Since anions used in the buffer for gel filtration might account for dissociation, acetate, which is lower on the Hofmeister series, was substituted for chloride; no differences were noted. The results with heparin, which inhibits blood clotting, rule out the cation calcium as a reason for the presence of both forms A and C.

The conversion of form A into form C may be explicable in terms of the greater number of polar groups which become exposed during the transformation. This interpretation is consistent with the preservation of form A even in the presence of as little as 10% glycerol as measured by its ability to undergo thrombin activation. The free energy of transfer of charged groups (*e.g.*, amide group) from water to glycol is positive (Tanford, 1964), indicating an unfavorable reaction. Glycerol, which is similar to glycol, can retard (or reverse) the dissociation of proteins if the newly exposed groups are polar (Tanford, 1964). Inorganic salts should have an opposite effect; dissociation should be favored when it occurs concomitant with an increase in polar groups. Since low concentrations of glycerol (10%) prevented loss of thrombin-induced activation of form A, and since higher glycerol concentrations (50%) induced the formation of form A in solution from form C, it is suggested that dissociation of factor V occurs with an increase in exposed polar groups. The increased rate of dissociation with inorganic salt further supports this thesis. A time- and concentration-dependent loss of the ability of factor V to be activated by thrombin following exposure to ClO_4^- was accompanied by the transformation of form A to C.

The effectiveness of the various anions in abolishing the ability of thrombin to activate form A is $SCN^- > ClO_4^- > NO_3^- > Cl^-$. This progression conforms to the Hofmeister (1888) series. The effect of anions on the dissociation of proteins has been studied with many systems. Sealock and Graves (1967) observed partial dissociation of phosphylase *a* in ClO_4^-. The order of effectiveness of anions in depolymerizing glutamic dehydrogenase was $SCN^- > ClO_4^- > Cl^-$ (Wolff, 1964). In decreasing order of effectiveness $NaClO_4$, $NaNO_3$, and KCl dissociated an inhibitor of a bacterial diphosphopyridine nucleotidase (Nagy and Jencks, 1965), and KSCN, $NaClO_4$, and $NaNO_3$ were decreasingly effective in depolymerizing F-actin (Nagy and Jencks, 1965). The mechanism by which anions cause dissociation of form A of factor V may be explained by the more general mechanism of Robinson and Jencks (1965). Acetyltetraglycine ethyl ester was used as a model for proteins and its solubility in the presence of various anions was measured. The effects could be explained by the anionic hydration or "salting in" and electrostatic interactions with the peptide backbone. Ions which increase the protein's solubility are effective in dissociating and denaturing proteins (Robinson and Jencks, 1965).

The stability of factor V has been previously shown to be enhanced by 50% glycerol. On the other hand, in the absence of glycerol, freezing of factor V resulted in a marked loss of activity (Esnouf and Jobin, 1967). Similarly, incubation of factor V with 0.5 M NaCl also results in total inactivation. These observations correlate well with our belief that form C is unstable as compared with form A, and that interconversion between forms A and C depends on glycerol concentration or the presence of inorganic salts.

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