The Conversion of Human Prothrombin to Thrombin by Sodium Citrate

ANALYSIS OF THE ACTIVATION MIXTURE*

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Since the discovery that bovine prothrombin when dissolved in 25% sodium citrate is autocatalytically converted to thrombin (2), several reviews have appeared which summarize the many chemical and biological changes occurring during this transformation (3-5). Recently the findings that poly-L-lysine, as well as a number of polyamino acids, initiates prothrombin activation (6) have suggested the importance of considering nonenzymic activators in the blood coagulation mechanism.

Apart from some ultracentrifugal observations of bovine prothrombin during citrate activation mentioned briefly in review articles (4, 7), the protein changes which occur during this activation process, to our knowledge, have not been examined in detail. Also, the conversion of human prothrombin to thrombin by citrate has not been studied as extensively as the corresponding conversion in the bovine system, except for the work of Alexander (8). His studies have dealt mainly with the mechanism whereby citrate and other anions activate certain clotting factor impurities found in most prothrombin preparations; his point of view is not shared by Seegers and Marciniak (9) (see discussion following and Reference 8).

In the present work with human preparations we have not attempted to study the mechanism of prothrombin activation, but have focused our attention on the changes in molecular size and thrombin activity which occur when prothrombin is exposed to concentrated citrate solutions. The data obtained indicate certain similarities and dissimilarities to the phenomena reported for the bovine species. In addition, smaller molecules obtained after activation of the zymogen have been identified and partially characterized. Similar experimental approaches might be helpful in studies of the activation mechanism itself and eventually for an understanding of the role of other clotting factors in this transformation.

EXPERIMENTAL PROCEDURE

Materials

Human prothrombin was isolated in 30 to 40% yield from acid-citrate-dextrose blood by a method previously described (10). A total of 10 prothrombin preparations, each of approximately 25 mg of protein isolated from 800 ml of plasma, was employed in this study. They had specific activities ranging from 1500 to 2400 NIH units per mg of protein. Additional analytical measurements are presented elsewhere (10). Further purification of the preparations to a specific activity of from 2800 to 3200 NIH units per mg can be achieved by exclusion chromatography on Sephadex G-200 at pH 6.0 (11). Unfortunately, this technique, although useful as an analytical tool, yields too little material for the type of study to be described. Therefore, as will be seen subsequently, the preparations employed are only partially purified and contain small amounts of other clotting factors (10).

In certain experiments, other proteins were used for comparison purposes. These included bovine fibrinogen Fraction I and crystalline bovine albumin (Armour), and 7S γ-globulin, ovalbumin, trypsin, pepsin, chymotrypsin, ribonuclease, and cytochrome c (Mann Research Laboratories). Sephadex was obtained from Pharmacia. Unless otherwise stated, all other reagents were of analytical reagent grade.

Methods

Assay of Prothrombin and Thrombin Activity—Prothrombin was assayed by the clotting time of a standardized bovine fibrinogen solution after conversion to thrombin, with the two-stage method of Ware and Seegers (12). Thrombin was measured in a similar system (5), omitting the first-stage conversion of prothrombin to thrombin. Both procedures were standardized with varying concentrations of Lot 3B NIH thrombin (specific activity, 21.7 units per mg) dissolved in 0.154 M NaCl. A graphical plot of the logarithm of the fibrinogen clotting time (range, 13 to 35 sec) versus the logarithm of the NIH thrombin activity (range, 0.4 to 2 NIH units per ml) gave a straight line with a slope of 0.76. An identical relationship was obtained with the human thrombin preparations to be described subsequently. It has been reported (3) that the Iowa unit, for which there is no suitable standard but with which a great deal of information has been obtained in the past, is 1.25 times the NIH unit.

Massachusetts, November 22 and 23, 1963, and has appeared in abstract form (1).
Citrate Activation of Human Prothrombin—Lyophilized preparations of human prothrombin were activated by either dissolving them directly in 25% (w/v) 0.07 M trisodium citrate (calculated on the basis of the anhydrous salt) or first dissolving them in distilled water and then adding an equal volume of 50% (w/v) sodium citrate. In all instances, activation was carried out in sealed plastic test tubes at 23 ± 1° with prothrombin concentrations of 5 to 15 mg of protein per ml.

Dialysis of Activation Mixture—At appropriate intervals after solution of prothrombin in sodium citrate, an aliquot was taken for activity and protein analysis, and the remainder, usually 1 to 2 ml, was placed in a Visking casing and dialyzed at 23° against 9 volumes of distilled water in a plastic container with a magnetic stirring bar. The casing, which had previously been boiled in EDTA (13), was tied off at one end and the other end was wrapped and tied around a length of hollow glass tubing (1 × 6 cm). This served to expose the solution to atmospheric pressure, since in early experiments it was found that osmotic pressure gradients in a closed dialysis casing increased its permeability to protein, which was thus lost. The absorbance of the dialysate was recorded at 10-min intervals at 250 μM (Beckman DU spectrophotometer) on 3-ml samples, which were returned to the dialysate immediately after measurement.

Conductivity measurements of the dialysate were performed on 0.1-ml aliquots diluted with water with the use of an Industrial Instruments conductivity bridge. When equilibrium had been reached, usually in 45 to 60 min as measured by conductivity, the sample inside the bag was recovered; its volume, conductivity, activity, and protein concentration were measured; and it was then subjected to both sedimentation velocity analysis and exclusion chromatography.

Sedimentation Analyses—Sedimentation velocity experiments were performed at 20° with a Spinco model E ultracentrifuge as previously described (10), except that 12 mm, 4° single sector cells were used. For analyses in which the salt concentration was sufficiently high to skew the pattern, i.e. 2.5% sodium citrate (0.097 M, Γ/2 = 0.5), a base-line was run concurrently with solvent alone. The maximum refractive index gradient was then estimated by superimposing the base-line on the protein pattern and measuring the vertical distance between the two. Final corrections for the sedimentation coefficient were made in the customary manner (14), to a solvent with the density and viscosity of water at 20°. Density measurements were performed by standard pyrenometer techniques, and viscosity measurements were made in a No. 50 Cannon-Ubbelohde semimicro dilution viscometer.

Exclusion Chromatography—This procedure was carried out on Sephadex G-100 columns in a manner similar to that of Whitaker (18), except that columns (1 × 60 cm) equilibrated with 0.097 M sodium citrate (Γ/2 = 0.5, pH 7.8) were used. Volumes of sample (1 ml) in 0.097 M sodium citrate were placed on the column and 1-ml fractions of effluent were collected in polyethylene tubes with a Gilson Medical Electronics constant volume fraction collector. Protein was analyzed chemically and by absorbance measurements at 250 μM (16, 17). Void volumes (V₀) were determined on each column experiment with 5 mg of 7S γ-globulin per ml, with which the elution volumes (Vₑ) of a number of commercially available proteins, listed above, were compared. Components of dialyzed citrate activation mixtures and nonactivated prothrombin were treated similarly and, in addition, measurements of prothrombin and thrombin activity were determined on the effluents. Both V₀ and Vₑ were estimated graphically to the nearest 0.2 ml by triangulation (18). Flow rates were controlled at about 0.2 ml min⁻¹ cm⁻², by vertical adjustment of the buffer reservoir above the column. In other experiments, desalting on Sephadex G-25 and exclusion chromatography in phosphate buffers of lower ionic strength were carried out by procedures previously described (10, 11).

RESULTS

Sedimentation Velocity and Exclusion Chromatography Analysis of Prothrombin before Activation—Before activation of prothrombin to presumably smaller molecular weight units, it was important to know whether the zymogen dissociated in dilute solution under the experimental conditions and also if the preparations contained appreciable quantities of smaller molecular weight contaminants. An example of the concentration dependence of the sedimentation coefficient of a prothrombin preparation is shown in Fig. 1 along with a similar experiment with bovine serum albumin for purposes of comparison. These analyses were limited to protein solutions exceeding 2 mg per ml because the skewness of the boundary produced by the high salt concentration did not permit accurate estimates of the sedimentation rates over a sufficient period of time at lower concentrations. Extrapolation of the values to zero concentration, noted by the dashed lines in Fig. 1, gives Sₑₚ, w values similar to those obtained for these preparations in more dilute salt solvents (1) The form of the curve does not suggest a dissociating system.

Because of the limitation of the sedimentation studies to protein solutions exceeding 2 mg per ml, experiments were carried out to determine whether there was any evidence of dissociation of prothrombin by Sephadex G-100 exclusion chromatography. Fig. 2 shows that from 1.3 to 9.0 mg per ml the protein and activity of these preparations have identical elution volumes (Vₑ) of 20. Lack of homogeneity of the preparation by this method is indicated by the failure of the specific activity to be constant with elution volume.

In experiments duplicating the one presented in Fig. 2, three pools of effluent, corresponding to the dashed horizontal lines in

![Fig. 1. Concentration dependence of the sedimentation coefficient of human prothrombin and bovine serum albumin at 20° in 2.5% sodium citrate (0.097 M), pH 7.8, Γ/2 = 0.5. Dashed lines represent extrapolation to zero concentration. See the text for explanation.](http://www.jbc.org/fig1.png)
the figure, were desalted on Sephadex G-25, lyophilized, and reconstituted in 2.5% sodium citrate. At a protein concentration of 3 mg per ml Pool 2 showed $V_s = 20$ for the protein and activity on Sephadex G-100 and $s = 4.3$ on sedimentation analysis. Pool 3 was identical with Pool 2, although the specific activity of the peak at $V_s = 20$ was about 20% less. Pool 1 could not be analyzed because of insufficient material recovered. However, it showed two peaks upon ultracentrifugation, the major one sedimenting at approximately 98, and a smaller sedimenting component of about 4.5S.

While the data presented above gave no evidence for dissociation of prothrombin on the gel columns, the activity was distributed over a rather wide effluent volume of 15 to 30 ml. To determine whether or not this distribution was characteristic of another protein with a molecular size similar to prothrombin, BSA was used since its sedimentation and diffusion coefficients are about the same as those of the zymogen (1). In all experiments, 5 mg of BSA per ml were chromatographed on Columns (1 x 60 cm) and found to have an identical effluent volume range. This range is also about the same as had been found by Whitaker (15) for proteins of this size.

Assay of Prothrombin-Thrombin Activity and Protein during Prothrombin Activation by Sodium Citrate—Fig. 3 presents one of five experiments in which activity measurements were performed at intervals after activation of a prothrombin preparation with 25% sodium citrate. Upon activation, prothrombin activity falls markedly, as measured by the two-stage system, and virtually disappears in 3 hours. During this time, relatively little thrombin forms, suggesting, perhaps, the evolution of an intermediate component in the system. The yield of thrombin is maximal between 10 to 24 hours after activation, after which it falls off gradually. In five experiments, with both single and pooled lots of human prothrombin, the maximal yield of thrombin produced averaged 75% of the total prothrombin activity available, with a range of 55 to 90%. This variation in maximum activation could not be ascribed to the specific activity of the preparations used. "Seeding" the system with preformed thrombin in a ratio up to 1 unit of thrombin to 20 units of prothrombin markedly increased the rate of thrombin formation, but had no appreciable effect on the total yield. In one experiment, 25% ph-trisodium isocitrate gave a yield of thrombin in 24 hours of 16% of the theoretical maximum based on prothrombin activity. In two experiments, 5 mM NaCl had no activating effect.

In duplicate experiments, aliquots of the citrate activation mixture were taken at the same intervals depicted in Fig. 3, and placed directly into either 1.5 M NaOH, 5% Na$_2$CO$_3$, or acetate buffer. They were then correspondingly analyzed for protein by the biuret methods of Henry, Sobel, and Berkman (16) and of Lowry et al. (17), and for $\alpha$-amino nitrogen (18). No significant change in protein concentration with time of activation was detected. Absorbance measurements for ninhydrin-reactive $\alpha$-amino nitrogen were also constant with time. The sodium citrate itself had no effect on these colorimetric methods.

Sedimentation Studies and Exclusion Chromatography Analysis of Prothrombin after Activation by Sodium Citrate—After activation of the prothrombin preparations by 25% sodium citrate for varying periods of time, the mixture was dialyzed as described under "Methods." This procedure had several distinct advantages. It permitted a rapid equilibration and 10-fold dilution of the salt concentration with only a 2-fold dilution of the protein. In addition, the prothrombin-thrombin conversion was virtually stopped by this procedure and both zymogen and enzyme activity remained stable for at least 8 hours at room temperature.

After dialysis, the protein and activity in all experiments were quantitatively recovered from the dialysis casing, and no material with an absorbance at 280 mg was found to have passed

The abbreviation used is: BSA, bovine serum albumin.
tern. Photographs were taken at the times indicated after the rotor reached full speed. Phase plate angles were at 60° except for Experiment F, which was 45°. Sedimentation is from left to right.

FIG. 4. Tracings of schlieren sedimentation patterns of human prothrombin before and at varying periods after 25% sodium citrate activation. Dialysed activation mixture (1 ml) was applied to columns (1 x 60 cm) equilibrated and eluted with 2.5% (w/v) sodium citrate, pH 7.8, \( \Gamma/2 = 0.5 \). Volumes (1 ml) were collected and analyzed for protein, prothrombin, and thrombin activity as indicated. Elution volumes \( (V_e) \) were uncorrected for the slight differences in void volumes of the three different columns used in these experiments. Recoveries in the experiments presented averaged 99% (range, 86 to 110%) for protein and 102% (range, 83 to 114%) for activity. Experimental samples correspond to those in Fig. 4. Experiments A, B, and F were run with the same prothrombin preparation; Experiments C, D, and E, with different preparations. Additional details given in the text.

Fig. 5. Sephadex G-100 elution pattern of human prothrombin before and at varying periods after 25% sodium citrate activation. Dialysed activation mixture (1 ml) was applied to columns (1 x 60 cm) equilibrated and eluted with 2.5% (w/v) sodium citrate, pH 7.8, \( \Gamma/2 = 0.5 \). Volumes (1 ml) were collected and analyzed for protein, prothrombin, and thrombin activity as indicated. Elution volumes \( (V_e) \) were uncorrected for the slight differences in void volumes of the three different columns used in these experiments. Recoveries in the experiments presented averaged 99% (range, 86 to 110%) for protein and 102% (range, 83 to 114%) for activity. Experimental samples correspond to those in Fig. 4. Experiments A, B, and F were run with the same prothrombin preparation; Experiments C, D, and E, with different preparations. Additional details given in the text.

SCHLIEREN PATTERN AT:

EXPT. TIME ACTIVATED:

PROTEIN CONC.:

32 min. 80 min.

A-1 UNACTIVATED 10 mg/ml.

A-2 UNACTIVATED 40 mg/ml.

B 6 MIN. 3.0 mg/ml.

C 1 HOUR 3.0 mg/ml.

D 2 HOURS 5.2 mg/ml.

E 6 HOURS 5.4 mg/ml.

F 16 HOURS 4.4 mg/ml.

The Sephadex pattern at 2 hours of activation (Fig. 5D) shows that the thrombin now appearing in the system (Fig. 3) is associated with the major protein peak at \( V_e = 25 \). This pattern also suggests the presence of a peak with a \( V_e = 20 \), comparable to that of the original preparation. This was not apparent in the 1-hour experiment (Fig. 5C), but may be explained by the fact that different prothrombin preparations were used in these two experiments. At 6 hours (Figs. 4E and 5E), the sedimentation boundaries remain about the same, but the Sephadex elution pattern suggests the emergence of another activation product at \( V_e = 28 \). At 16 hours (Figs. 4F and 5F), after maximum activation of prothrombin had been obtained, two distinct but diffuse boundaries in the sedimentation pattern and four protein peaks in the Sephadex elution pattern were evident. The latter correspond to \( V_e = 17 \) (the heavy protein impurity eluted at the \( V_0 \) peak, \( V_e = 20 \) (the original prothrombin activity peak), \( V_e = 25 \) to 26 (thrombin activity), and \( V_e = 28 \) (an inactive fragment). In this experiment, the peak enzyme activity corresponded to 4000 NIH units per mg, approximately twice the peak activity of the original zymogen (cf. Fig. 5, A and F). There is also some suggestion of a fifth peak in the 16-hour elution pattern of \( V_e = 36 \). Although this peak is poorly defined and may represent "trailing," its appearance was observed in each of the three elution patterns of 16-hour activation mixtures examined.

In three experiments in which 16-hour activation mixtures were dialyzed and placed on Sephadex, eluents from the columns corresponding to \( V_e = 20 \) to 25 and \( V_e = 27 \) to 30 were pooled, desalted on Sephadex G-25, and lyophilized. The dried frac-
Whitaker (15) has described such a system in which a series of... for proteins of similar partial specific volume and frictional ratio... essentially globular proteins emerges from Sephadex G-100 columns at distinct \( V_e:V_0 \) ratios in a linear relationship to the... of their Stokes radii and diffusion coefficient than molecular weight. His studies have indicated, however, that with tightly cross-linked G-100 gels, the correlation of \( V_e \) with molecular radii... formation of inactive fragments, presumably of lower molecular weight. Under these conditions.

In one experiment, a prothrombin preparation was activated in sodium citrate for 144 hours. The sedimentation analysis showed two major boundaries of \( s = 3.2 \) and \( s = 2.0 \), the two peaks being of nearly equal area. The Sephadex elution pattern was qualitatively similar to that seen at 16 hours, but the thrombin activity was considerably less, and there appeared to be more of the inactive protein at the higher \( V_e \). This suggests that the decline of thrombin activity from a maximum at 16 to 24 hours to less than 50% at 144 hours (Fig. 3) is associated with the formation of inactive fragments, presumably of lower molecular weight.

Ackers (19) has recently presented evidence that the chromatographic separation of proteins on gel columns is more a function of their Stokes radii and diffusion coefficient than molecular weight. His studies have indicated, however, that with tightly cross-linked G-100 gels, the correlation of \( V_e \) with molecular radii for proteins of similar partial specific volume and frictional ratio is essentially the same as its correlation with molecular weight.

Whitaker (15) has described such a system in which a series of essentially globular proteins emerges from Sephadex G-100 columns at distinct \( V_e:V_0 \) ratios in a linear relationship to the logarithm of their molecular weights. As shown in Fig. 6, we have found the same relation to hold for a number of similar proteins. From these data we have estimated the following molecular weight ranges for the protein components of the activation mixtures: (a) heavy protein contaminant, more than 100,000; (b) prothrombin activity, approximately 68,000 to 75,000; (c) thrombin activity, approximately 35,000; (d) inactive fragments, approximately 25,000 and 10,000. These estimates assume that the protein is globular, as is the bovine material (20), and also that the protein components do not interact in such a way as to modify the \( V_e \) values. The data appear to justify the latter assumption. Moreover, in all experiments (see, for instance, Fig. 2, Curves A and B) \( V_e \) was independent of concentration as determined by measurements of either activity or protein.

On agar or cellulose acetate electrophoresis between \( pH \) 7.0 and 9.0, the prothrombin products used in this study migrate as single components with a mobility of an \( \alpha_2 \)-globulin (10). In two experiments, after desalting and lyophilization of a 16-hour activation mixture, two components were evident after agar and cellulose acetate electrophoresis at \( pH \) 8.6. One corresponded to a trace amount of \( \alpha_2 \)-globulin, and the major component had a mobility of a \( \beta_2 \)-globulin.

**DISCUSSION**

Seegers has summarized the biochemical and chemical studies of bovine prothrombin activation (5). Several of these findings are pertinent to the present study. Of particular interest is the fact that, upon citrate activation, bovine prothrombin activity diminishes rapidly before appreciable amounts of thrombin form, suggesting the presence of an inactive intermediate (21). However, the prothrombin activity reappears and then diminishes as more and more thrombin is formed in the activation mixture. In our work with human preparations, we have confirmed the transient formation of an inactive intermediate, but we have no evidence for the reappearance of prothrombin activity. Sampling of the activation mixture of human prothrombin in as close and varying intervals as possible in five different experiments with different preparations did not show any significant deviation from the prothrombin-thrombin relationship depicted in Fig. 3.

It has also been found that, about 60 min after activation, approximately 60% of the nitrogen and tyrosine and 80% of the carbohydrate are cleaved from bovine prothrombin and become soluble in trichloroacetic acid (22). Because of limited quantities of material, we were unable to repeat these experiments. However, we were able to sample the activation system at varying periods of time and could find no evidence for any diminution of protein concentration or increase in \( \alpha \)-amino nitrogen, suggestive of extensive peptide bond cleavage. Conceivably, the methods we used might be insensitive to small changes which might have occurred, but one would imagine that if 60% of the nitrogen became soluble in trichloroacetic acid, presumably indicating evidence for small peptide fragments, this would readily be detected. Evidence for this is further supported by the dialysis and Sephadex column experiments, in which all the protein was recovered from the activation mixtures. Certainly, peptide bonds are broken, as suggested by the fact that human prothrombin has been shown to have an NH₂-terminal alanine, while thrombin has an NH₂-terminal isoleucine (23). The Sephadex experiments at 16 hours (Fig. 5F), however, suggest...
the possibility that fragments of V, = approximately 36 (approximately 10,000 mol. wt.) form during activation.

In two review articles, Waugh et al. (4, 7) have alluded to some preliminary observations on the sedimentation velocity characteristics of Seegers' bovine prothrombin after citrate activation. In these experiments, details of which were not presented, 1 to 2% bovine prothrombin solutions in 25% sodium citrate were diluted 1:10 with water after varying periods of activation and were examined in the ultracentrifuge. The schlieren pattern changed with time from a single component system of a = 4.2 to 4.8s (similar to bovine prothrombin) to a four-component system after 24 hours of activation. These components correspond to (a) a heavy aggregated material containing thrombin activity (approximately 50%); (b) s = approximately 4.1, presumably with thrombin activity (approximately 32%); (c) s = approximately 2.0 (15%); and (d) a small boundary near the meniscus, presumably sodium citrate. In general, our work substantiates the evidence for the latter three components, although we find no evidence for heavy aggregated material with thrombin activity in 2.5% sodium citrate by either sedimentation or exclusion chromatography analysis. Aggregated precipitates occur in 25% citrate solutions, presumably because the protein may be partially insoluble since it is salted out at about 30% citrate.

From all the Sephadex elution patterns at 16 hours of maximum activation, (see, for instance, Fig. 5F) it can be reasonably estimated by planimetry of the patterns that 25 to 30% of the protein present in the original prothrombin preparation was not converted to smaller molecules. Presumably, this material includes 2 to 10% of the large globulin contaminant and 20 to 30% of another protein impurity with a value of V, and s similar to that of prothrombin itself. While it might be argued that this latter fraction may contain chiefly of unconverted prothrombin, we are inclined to believe that it represents an impurity in the original preparation. Its concentration seems to be inversely related to the initial specific activity of the prothrombin product activated. In addition, the biphasic nature of the activation curve (Fig. 3) and the increase in the smaller inactive fragments with time suggest that the thrombin activity may be maximally evolved from the zymogen but is simultaneously destroyed in 25% sodium citrate.

Because of the heterogeneity of the system studied and the lack of additional biophysical information on the activation products formed, it is difficult to speculate about the molecular weight changes which occur during citrate activation. However, certain relationships emerge from the data which seem helpful in formulating an hypothesis for future experimentation.

From estimates of sedimentation and diffusion data (1, 10), end group analysis (23), and a comparison of the V, /V, ratio with other globular proteins from this study, a molecular weight of 65,000 to 75,000 for human prothrombin would seem reasonable. From the exclusion chromatography experiments, as well as estimates of molecular weights from the sedimentation coefficient in dilute solution in a manner described by Baldwin (24), a value of 35,000 to 40,000 would be estimated for thrombin and about 25,000 for the inactive fragment. An additional activation product was also noted in the Sephadex elution patterns, which when isolated had an s of approximately 1.0 and a molecular weight of about 10,000.

On the basis of specific activity measurements, the peak thrombin values obtainable are approximately 4,000 NIH units per mg of protein. This is almost twice the activity of the prothrombin preparations used and significantly greater than a range of 2,800 to 3,200 NIH units obtainable by further purification of the zymogen (11). Miller and Copeland (13) have reported values as high as 10,000 Iowa units per mg for human thrombin, which if corrected to NIH units would be approximately 8,000 units per mg. Our best preparations of human thrombin, obtained after Amberlite IRC-50 column chromatography of 16- to 24-hour activation mixtures, have ranged from 4,500 to 6,000 NIH units per mg (1). This would seem to suggest that after activation the majority of the protein converts to a fragment of molecular weight of approximately 35,000. An inactive fragment of the parent zymogen or thrombin, which has been altered by this procedure, also is of this approximate molecular weight. Preliminary experiments in this laboratory in which the thrombin fraction has been isolated from the Sephadex columns and further purified by Amberlite IRC-50 chromatography (13) indicate this to be the case.

Last, we would like to emphasize that the preparations used in this study were impure and contained other proteins that are undoubtedly important constituents of the clotting mechanism. To what extent these impurities have obscured the physical events and properties of the activation products noted in this work is not known. Similar studies with prothrombin preparations that are enzymically refractory to citrate activation (8) should be helpful in a more precise interpretation of these data as well as to an understanding of the mechanisms involved.

SUMMARY

The sequence of activity and protein changes following activation of human prothrombin to thrombin in 25% (w/v) sodium citrate solution has been studied by sedimentation velocity analysis and Sephadex G-100 exclusion chromatography. The prothrombin-thrombin transformation appears to follow a change in molecular size with time of activation of approximately 70,000 to 35,000. Thrombin activity is associated with one of the fragments of approximately 35,000 molecular weight, the other being inactive with fibrinogen substrates. No evidence was obtained for substantial breakdown of the activated prothrombin to small peptides or association of thrombin into heavy molecular weight aggregates. The inactivation of prothrombin before appreciable amounts of thrombin have been formed in citrate activation mixtures has been confirmed, although the “reappearance” of prothrombin activity shortly thereafter was not evident. The highest specific activity of human thrombin obtained after 55 to 90% activation of prothrombin is approximately 4000 NIH units per mg of protein. This is about one half the value of highly purified preparations obtained after activation of less pure prothrombin products. The data also suggest the possibility of formation of other fragments of the parent zymogen of approximately 25,000 and 10,000 molecular weight. Although precise interpretation is difficult, these small molecular weight proteins may represent thrombin which has been degraded in size on prolonged exposure to citrate ion.

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