II. MECHANISM OF PRODUCTION FROM PROTHROMBIN*

The specific activity of the protein when assayed by a modified two-stage procedure is 1,100 to 1,300 NIH thrombin units per mg.

The molecular weight of the proenzyme determined by gel filtration in both 6M guanidinium chloride, and electrophoresis in sodium dodecyl sulfate is 72,000 ± 5,000. The activation of prothrombin in 25% sodium citrate-defibrinated plasma has been studied by monitoring molecular weight changes and the production of enzymatic activity toward fibrinogen and Nα-tosyl-L-arginine methyl ester.

The results of this study are consistent with a mechanism in which prothrombin is first cleaved to an intermediate of 65,000 daltons. This intermediate is subsequently cleaved to produce two single chain molecules of 39,000 daltons and 24,000 daltons. The 39,000-dalton chain is thrombogenic, giving rise to an active thrombin composed of two disulfide-linked chains (33,000 and 6,000 daltons). Further cleavages in this molecule result in two 28,000-dalton thrombins; one composed of two disulfide-linked chains (18,000 and 10,000 daltons) and the other composed of three disulfide-linked chains (14,000, 4,000, and 10,000 daltons).

The thrombins produced in the activation system are chromatographically and electrophoretically indistinguishable from those in Parke-Davis topical thrombin.

SUMMARY

Bovine prothrombin has been purified by modifications of existing procedures. The purified protein is effectively homogeneous in four analytical gel electrophoresis systems, and is stable during storage in 50% glycerol-H2O at −20°C. The specific activity of the protein when assayed by a modified two-stage procedure is 1,100 to 1,300 NIH thrombin units per mg.

The molecular weight of the proenzyme determined by gel filtration in both 6M guanidinium chloride, and electrophoresis in sodium dodecyl sulfate is 72,000 ± 5,000. The activation of prothrombin in 25% sodium citrate-defibrinated plasma has been studied by monitoring molecular weight changes and the production of enzymatic activity toward fibrinogen and Nα-tosyl-L-arginine methyl ester.

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The thrombins produced in the activation system are chromatographically and electrophoretically indistinguishable from those in Parke-Davis topical thrombin.

At one time, the activation process was thought to be autocatalytic in nature; however, later evidence failed to support this position, and it is almost universally held that contamination of prothrombin with protein and lipid factors is responsible for the observation of an apparent autocatalytic event. The best available data at present suggests that the physiological activation of prothrombin is catalyzed by a "prothrombinase" complex composed of the protein factors V and Xa, calcium, and phospholipid.

The activation of the proenzyme has been studied in numerous laboratories with varying degrees of success. Prothrombin preparations of varying states of purity have been activated by means of crude or semipurified intrinsic or extrinsic clotting factors, and product formation has been assessed by means of chromatographic or electrophoretic techniques, and the generation of clotting and esterase activity. Seegers (1), Lanchantin, Friedmann, and Hart (4, 5), and Aronson and Menache (6) have provided some of the more conspicuous efforts aimed at deciphering the chemical mechanism of prothrombin activation. These studies have suffered from the technical difficulties attending the resolution and characterization of the complex mixture of reactants, intermediates, and products present in the activation mixture. An additional complexity inherent to the system is the surface-active, aggregate-forming nature of prothrombin (7, 8), thrombin (or thrombins) (9, 10), and some of the intermediates (11) of the process.

Many of the difficulties inherent to studies of prothrombin activation are also encountered in studies of thrombin. The application of new techniques for the simultaneous resolution and molecular weight evaluation of polypeptide chain molecular weights in denaturing solvents (12, 13) toward studies of thrombins (14, 15) appear to be adequately suited to studies of prothrombin activation.

We wish to report data dealing with bovine prothrombin purified by a modification of existing purification techniques, which consistently produces a highly purified, stable prothrombin. The activation of this material in 25% sodium citrate and dilute defibrinated plasma has been studied by means of enzymatic activity production and molecular weight analysis in denaturing solvents. The data obtained from this study provide a means of ordering of intermediates of the activation process in terms of their molecular weights and rate of appearance. Finally it will be demonstrated that the same thrombin species observed in Parke-Davis topical thrombin (15) are produced

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upon citrate activation of prothrombin of near electrophoretic purity.

**MATERIALS**

Bovine blood was obtained from the G. Bartusch Packing Company, St. Paul, Minnesota. Thromboplastin and Ac globulin were from Difco. Other clotting reagents were as described previously (15). Disopropyl fluorophosphate was obtained from Sigma and used as a 1 m solution in isopropanol. DEAE-cellulose was obtained from Schleicher and Schuell, Inc., and purified by the method of Sophianopolous and Vestling (16). Acrylamide and N,N'-methylenebisacrylamide were from Eastman and recrystallized from chloroform and 1-propanol, respectively. Sodium dodecylsulfate was from Mann. Urea was from Sigma, and deionized before use. Guanidinium chloride (ultrapure) was obtained from the Heico Chemical Company, Delaware Water Gap, Pennsylvania. Sulfoethyl Sephadex and 6% Sepharose were from Pharmacia.

All other reagents were of the best available commercial grade.

**METHODS**

**Blood**—Bovine blood (8 volumes) was collected from stunned slaughterhouse animals in plastic buckets containing 2.85% sodium citrate (1 volume). Approximately 50 liters of blood in anticoagulant were collected in each trip to the slaughterhouse. Plasma and cells were separated by means of a continuous flow separator (De Laval No. 518), and the plasma stored in batches of 2 liters each at -20 °C.

**Prothrombin Purification**—The extension of the procedure of Moore et al. (17) reported by Ingwall and Scheraga (8) was further modified to prepare prothrombin from frozen plasma. The plasma was quickly defrosted under warm tap water and centrifuged to remove insoluble material. The procedure of Moore et al. was then applied to the plasma. This purification procedure, which involves adsorption on barium citrate, elution with EDTA-sodium citrate, ammonium sulfate fractionation, and isoelectric precipitation was followed verbatim, with the exception that the prothrombin-containing (NH₄)₂SO₄ pellet was redissolved in 10⁻⁴ m disopropyl fluorophosphate prior to the dialysis step. This precaution, suggested by the work of Cox and Hanahan (7) and Jackson, Johnson, and Hanahan (18) results in consistent preparations, and good yields in later purification steps. The protein from the isoelectric precipitation step was then subjected to chromatography as reported by Ingwall and Scheraga (8), except that DEAE-cellulose rather than DEAE-Sephadex was used. The DEAE-cellulose elution profile differed from that of Ingwall and Scheraga in that only a single peak of protein containing effluent was eluted by the gradient.

The protein in the prothrombin containing column effluent was precipitated by addition of solid (NH₄)₂SO₄ (80% of saturation), centrifuged and the pellet taken up in 50% glycerol-H₂O. The prothrombin solution was then stored at -20 °C. Under these storage conditions, the protein precipitates; however, solution is readily achieved by warming the slurry to 25 °C. The final product is stable for at least 4 months under these storage conditions.

During the early stages of purification, prothrombin activity was determined using the Ware and Seegers (19) method. In accord with the observations of other workers (7, 30), the activation times became progressively longer as the purity of the protein increased. A modified assay procedure was employed in later stages of purification (after isoelectric precipitation), in which the concentrated proenzyme was diluted with Ac globulin and defibrinated plasma prior to activation with thromboplastin. In effect, the plasma factors removed during purification were added back to the assay system. The relative concentration of plasma prothrombin added in the assay system was such that its contribution to the total observed thrombin activity was insignificant. In a typical assay, 30 NIH thrombin units were derived from the material assayed, and about 0.005 NIH thrombin units from the added plasma. The generation of thrombin activity was followed by the removal of from 1 to 10 μl of the activation assay system, and assaying for NIH clotting activity as described previously (15). Prothrombin concentration was estimated spectrophotometrically using the ε₅₆₅ of 14.1 reported by Cox and Hanahan (7).

**Electrophoresis**—Electrophoresis in SDS¹ was conducted according to the method of Weber and Osborn (13) with the exception of the modifications previously reported (15). Urea-atic acid electrophoresis was performed according to the method of Panyim and Chalkley (21). Disc gel electrophoresis was performed according to the method of Davis (22) at pH 8.8. Isoelectric focusing was according to the method of Wrigley (23) in pH 3 to 10 gels. With the exception of the SDS gels, which were stained with Coomassie blue, all acrylamide gels were stained with Amido black. Excess stain was removed electrophoretically. Absorbance scans of stained gels were performed with a Gilford model 2228 spectrophotometer equipped with a Gilford model 850 gel scanner. Integration of gel scans was accomplished by means of a planimeter.

The effect of disulide cross-links on protein mobilities in SDS may result in low values for the apparent molecular weight (Mₐpp) of nonreduced proteins (15, 24). For all of our experiments, Mₐpp values were determined for unknowns by comparison with the mobilities of reduced standard proteins. The difference in SDS apparent molecular weights (Mₐpp values) of the reduced or nonreduced states is most often within the limits of error of the technique (±10%). In cases in which other molecular weight data determined either by sedimentation equilibrium or gel filtration in 6 M guanidinium chloride are available, those values will be used in discussing the results.

**Other Methods**—N-{α-tosyl-L-arginine methyl ester hydrolysis was determined using the spectrophotometric method of Hummel (25). Thrombin purified from Parke-Davis topical thrombin was prepared by the method of Lundblad (26). Clotting assays were as described previously (15). Sodium citrate concentrations are expressed as weight per volume solutions of the dihydrate.

**Gel filtration in 6 M guanidinium chloride was done according to the method of Fish, Reynolds, and Tanford (12).**

**RESULTS**

The prothrombin purified by our modifications of existing procedures is stable, and has a specific activity of from 1100 to 1300 NIH thrombin units per mg. Fig. 1 presents a photograph of acrylamide gel electrophoretograms prepared with the purified protein. Using the Davis (22) and urea-acetic acid (21)

¹ The abbreviations used are: SDS, sodium dodecyl sulfate; TAME, N-{α-tosyl-L-arginine methyl ester.
² The term Mₐpp has been used to designate molecular weights obtained using comparative techniques.
FIG. 1. Photograph of acrylamide electrophoresis gels prepared with purified prothrombin. 1, Davis Method (22) (pH 8.6); 2, urea, acetic acid (pH 3.2); 3, SDS (sample unreduced); 4, SDS (sample reduced).

electrophoretic techniques, the material is effectively monodisperse. On SDS gels, about 5% of an impurity can be detected. The material is also effectively homogeneous in isoelectric focusing experiments in acrylamide gels.

The generation of thrombin activity from prothrombin on incubation at 37°C in 25% sodium citrate has been used as a means of detecting Factor X contamination in purified prothrombin samples (7, 8, 27). Our preparations do not generate thrombin when exposed to 25% sodium citrate for at least 50 hours. For a typical preparation, a 1 mg per ml solution of prothrombin will give a clotting time of about 1000 sec after 50 hours of incubation. In one preparation, no thrombin activity was observed after 300 hours of incubation in this medium. The impurity responsible for the thrombin generation in 25% sodium citrate (presumably Factor X) is apparently present in small but varying quantities; but at any rate, it does not affect the stability of our preparations under the storage conditions employed.

Gel filtration of reduced, carboxymethylated prothrombin in 6 M guanidinium chloride gave a molecular weight of 70,000 ± 5,000. This value is in good agreement with the values of 68,000 to 74,000 daltons previously reported (7, 8) for prothrombin purified on DEAE-cellulose or Sephadex.

A sample of prothrombin was activated by incubation in 25% sodium citrate, and dilute, defibrinated plasma at 37°C. The stored prothrombin sample (about 5 mg per ml) in glycerol-H2O was diluted in half with cold 50% sodium citrate and dialyzed versus 25% sodium citrate at 4°C for 12 hours. At this point, an aliquot of defibrinated plasma in 25% sodium citrate was added to the prothrombin sample such that the final dilution of plasma was 200-fold, and the activation mixture incubated at 37°C.

Aliquots of the prothrombin for electrophoretic analysis taken at the moment of citrate addition, after dialysis, and at the time of plasma addition (zero time).

At given intervals following the addition of the defibrinated plasma, samples were taken for the determination of clotting and L-TAME esterase activity using the NIH (15) and Hummel (25) procedures, respectively. All clotting assays were performed in quintuplicate. Fig. 2 presents the time course of the production of clotting and L-TAME esterase activity during activation. Both curves were normalized on the basis of 100% being the maximal activity generated. In this experiment, 55% of the thrombin clotting activity observed on two-stage analysis of the original sample was generated.

In agreement with the observation of Lanchantin et al. (4), there is a coincident rise in the L-TAME esterase and clotting activity. The initial observation of the activity was at about 30 hours after the addition of plasma. Following maximal thrombin generation, there is a plateau in the activity activation profile followed by a slight decrease in L-TAME esterase activity, and a precipitous drop in clotting activity. Again these observations are in agreement with those reported by Lanchantin et al. In our accompanying communication (15) we have established that the differential declines of clotting and L-TAME esterase activity in Parke-Davis topical thrombin can be correlated with the
conversion of a 39,000 dalton thrombin to at least two 28,000-dalton thrombins of reduced clotting activity.

At numerous intervals during the activation process, aliquots of the activation mixture were removed and the activation stopped by 1:1 dilution of the aliquot into glacial acetic acid. The acidified protein samples were desalted by dialysis versus 0.2 M acetic acid, and lyophilized. The dry desalted protein samples were taken up in 1% SDS, 0.025 M sodium phosphate buffer pH 7.1, heated in a 100° H2O bath for 30 min, and subjected to acrylamide gel electrophoresis in SDS. The electrophoretograms prepared in this fashion are depicted in Fig. 3. The starting material after dilution with sodium citrate is on the left (−2) and thrombins purified from Parke-Davis topical thrombin by the Lundblad procedure are on the right (11a). The gel just prior to the zero time gel is the sample taken immediately before plasma addition and the gel labeled zero represents the sample obtained at our defined zero time (addition of plasma). Inspection of the gels presented in Fig. 3 leads to two important observations. The first is that a significant alteration of the prothrombin has occurred during the dialysis into 25% sodium citrate. Approximately 30% of the prothrombin has been converted to an intermediate with an SDS Mapp of 60,000 with disulfide bonds intact. The second observation of importance is that the presence of the electrophoretic bands characteristic of the large (Mapp unreduced 40,000) and small (Mapp unreduced 25,000) thrombins in the SDS gels suggests substantial quantities of these materials long before thrombin activity is noted in the assay system.

The gels depicted in Fig. 3 were scanned, the scans integrated, and the relative percentage of fractions of each component determined at each time of sampling. Fig. 4 presents a graphical representation of the relative percentage of each protein component present during the time course of the activation process.

In the article accompanying this one (15), it has been established that two weight classes of thrombin with molecular weights of 39,000 and 28,000 are present in purified Parke-Davis topical thrombin. On the SDS system, with disulfide bonds intact, the 39,000-dalton material has an Mapp unreduced of 40,000, while the two 28,000-dalton components have Mapp values unreduced of 25,000 and 23,000. The larger thrombin is the most active with respect to clotting activity. In Fig. 4, it can be seen that the relative concentration of a component with an Mapp unreduced of 40,000 is the protein in maximum concentration at about 24 hours activation time. However, the onset of clotting activity did not occur until about 30 hours, and maximum activity was not realized until about 60 hours of activation. A second apparent anomaly occurs in the observation of a biphasic curve for the appearance of material whose Mapp unreduced is 25,000.

These apparent anomalies are explained by the data presented in Fig. 5. Here the same protein samples whose electrophoretograms appear in Fig. 3, were reduced in 1% mercaptoethanol, and the polypeptide chain molecular weights and compositions were calibrated versus reduced standards. The numbers on the gel tubes correspond to activation times. The two gels at the extreme right labeled IIa correspond to purified samples of Parke-Davis topical thrombin from different lots.
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5. SDS gel electrophoretograms of reduced samples taken during the timed activation study described in the text. A representative sample of the total number of gels is depicted. The vertical column on the left of the gels corresponds to the SDS $M_{\text{app}}$ reduced of the major stained protein bands, when gels were calibrated versus reduced standards. The numbers on the gel tubes correspond to the time of sampling during activation. The two gels at the extreme right labeled IIa correspond to reduced samples of thrombin purified from two different lots of Parke-Davis topical thrombin.

$M_{\text{app}}$ values in the nonreduced state in SDS are 25,000 and 23,000.

If one examines the gels depicted in Fig. 5, one can see the formation of a trace of a component with a molecular weight corresponding to 33,000 beginning to appear at 34 hours. As time of activation progresses, there is a gradual increase in the stained band corresponding to 33,000 daltons at the expense of the band at 40,000 daltons. At 60 hours of activation, the band at 33,000 daltons becomes the major component.

In a similar fashion, the $M_{\text{app}}$ of the 25,000 component present during the early stages of activation remains unchanged on reduction of disulfide bonds. As activation time progresses, this band almost entirely disappears before the onset of thrombin activity. In the late stages of activation, reduced polypeptide chain components are observed with $M_{\text{app}}$ values consistent with those derived on reduction of the smaller thrombins.

The molecular weights of components whose mobilities in SDS were unaffected by reduction (40,000 daltons and 25,000 daltons) were confirmed by gel filtration of these reduced carboxymethylated proteins in 6 M guanidinium chloride. In this system, these materials behave as linear random coiled polypeptide chains of $M_{\text{app}}$ values of 39,000 and 24,000, respectively.

In Fig. 6 are presented the relative fraction of each reduced polypeptide chain $M_{\text{app}}$ class as a function of time. These data were obtained by integration of absorbance scans of the gel data of Fig. 5. Here the coexistence of reduced polypeptide chains of $M_{\text{app}}$ values 40,000 and 33,000 daltons is most obvious. Further, comparison of Figs. 6 and 2 reveals that the NIH clotting and L-TAME esterase activity curves paralleled the increase of the 33,000-dalton reduced polypeptide chain. The diminution of clotting activity, with relatively constant L-TAME esterase activity, a condition consistent with the degradation of the 39,000 dalton thrombin, is coincident with the increased quantity of reduced polypeptide chains which originated from the smaller thrombins (about 18,000 ± 2,000, 14,000 ± 1,000, and 10,000 ± 1,000 daltons) (15).

The upper curve of Fig. 6 demonstrates the rapid production and decline of the reduced polypeptide chain of $M_{\text{app}}$ 25,000.
The disappearance of the 25,000-dalton component is coincident with the rapid rise of a component of $M_{app}$ 20,000, which apparently breaks down rapidly to a 17,000-dalton component and this to a 14,000-dalton component.

The relative percentage of the chains of SDS $M_{app}$ 17,000 $\pm$ 2,000, 13,000 $\pm$ 2,000, and 11,000 $\pm$ 1,000 in the upper portion of Fig. 6 declines and then increases in quantity. This increase in these components is concomitant with the fall in clotting activity. The decrease in clotting activity while L-TAMe esterase activity remains constant has previously been interpreted as a conversion of the most active 39,000-dalton thrombin to two 28,000-dalton thrombin molecules of decreased clotting ability. The polypeptide chain components derived from these low molecular weight thrombins are 18,000 $\pm$ 2,000, 14,000 $\pm$ 1,000, and 10,000 $\pm$ 1,000 daltons (15). The coincident increase in the percentage of polypeptide chains, whose $M_{app}$ values in SDS are essentially identical with those derived from the 28,000-dalton thrombins with the decrease of clotting activity lead us to suggest that these chains are derived from the 28,000-dalton thrombins. The increase of the relative fraction of these components during the decline of clotting activity is again reproduced in the lower curve of Fig. 6.

Further evidence for the existence of the smaller thrombins was derived from sulfoethyl Sephadex C-50 chromatography of the thrombin-active products present in the system after 128 hours of activation. Gradient elution of thrombins from such a column, using the procedures described in the accompanying article (16), produced an elution profile similar to that reported for Parke-Davis topical thrombin. L-TAMe esterase-specific activity was constant for all of the enzyme eluted, while NIH clotting activity was clearly biphasic. The thrombins eluted from the column were indistinguishable from those prepared from Parke-Davis topical thrombin in comparative SDS electrophoretic studies of the proteins in the reduced and unreduced states.

While SDS electrophoresis of reduced polypeptide chains has been demonstrated to be accurate within about $\pm$10% (13), analysis of unreduced polypeptide chains can result in consistently low estimates of molecular weight for some proteins. By reference to the composition versus time data of Figs. 4 and 6, several identities between reduced and unreduced intermediates become obvious. Thus, prothrombin with disulfides reduced has an $M_{app}$ of 75,000 and an $M_{app}$ of 72,000 with disulfides intact. The first intermediate produced has an $M_{app}$ reduced of 65,000 and 60,000 unreduced. The second thrombogenic single chain intermediate formed has an $M_{app}$ of 40,000 in both the reducing and nonreducing systems. Similarly, the $M_{app}$ of the 25,000-dalton nonthrombogenic component produced concurrently with the 40,000-dalton component is unaffected by reducing conditions. These suggested identities among single chain components are presented in Table I. Also presented in Table I are the molecular weight data accrued for the two molecular weight classes of thrombin purified from Parke-Davis topical thrombin. The proposed identities between SDS reduced and unreduced data for thrombin are more difficult to comprehend since none of the active thrombins are single chain molecules, and hence show dramatic alteration upon reduction. For most of the intermediates, prothrombin and thrombin, additional molecular weight data are available and these data, together with best molecular weight estimates considering all procedures used are also presented in Table I.

The time course of the activation process as analyzed in Figs. 2, 4, and 6, together with the identities proposed in Table I, permit the construction of a hypothetical mechanism for prothrombin activation in the system used. Fig. 7 presents the proposed mechanism in diagrammatic form.

According to the proposed mechanism, prothrombin is degraded sequentially to a single chain molecule of 65,000 daltons (Intermediate 1). This component is cleaved to form two single chain molecules with molecular weights of 39,000 (Intermediate 2) and 24,000 (Intermediate 3). Intermediate 3 is nonthrombogenic, and possesses no L-TAMe esterase or clotting activity. Intermediate 2 is the single chain precursor of the first, most active thrombin, IIa-A.

The structures proposed for the next two thrombin-active components (IIa-B, IIa-C) are largely drawn from the data and conclusions of the preceding article (16). The identity drawn between the thrombin produced in the activation system de-

| Component       | SDS $M_{app}$ unreduced | SDS $M_{app}$ reduced | Gel filtration $M_{app}$ reduced, Carboxymethylated | Sedimentation equilibrium not reduced | Best value |
|-----------------|--------------------------|-----------------------|---------------------------------------------------|--------------------------------------|------------|
| Prothrombin     | 72,000                   | 79,000                | 70,000                                            | 68,000-74,000 (7, 8)                  | 72,000     |
| Intermediate 1  | 60,000                   | 65,000                | 30,000                                            | 39,000                                | 65,000     |
| Intermediate 2  | 40,000                   | 40,000                | 24,000                                            | 39,000                                | 39,000     |
| Intermediate 3  | 25,000                   | 25,000                | 33,000, 6,000 (14)                                | 39,000                                | 39,000     |
| Thrombin IIa-A  | 40,000 (15)              | 33,000 (15)           | 33,000                                            | 33 + 6,000 (14)                       | 28,000     |
| Thrombin IIa-B  | 23,000-25,000 (15)       | 17,000, 14,000, 10,000 (15) | 20,000, 14,000, 10,000, 4,000 (14, 15) | 28,000 (14) | 28,000 (18,000 + 10,000) |
| Thrombin IIa-C  |                          |                       | 14,000, 4,000, 10,000                             |                                      |            |

a Gel filtration in 6 M guanidinium chloride.
b The estimate of best value is based upon the relative experimental precision of the techniques used, and consistency between the molecular weights for a given protein with disulfides intact, and those for the polypeptide chains derived from it on reduction.
c In addition to this component, a series of stained protein bands in the 60,000- to 63,000-dalton range are also observed. These have been interpreted as arising from a nonthrombin productive degradative pathway.
d The electrophoretic resolution of these components is quite concentration dependent. These components are listed together, as we have not yet succeeded in resolving them chromatographically.
The mechanism for prothrombin activation suggested (Fig. 7) is consistent with the molecular weight, clotting and L-TAME esterase activity data reported here and in our preceding article (15). The molecular weights we have determined for bovine prothrombin by gel filtration of the reduced, carboxymethylated, randomly-coiled protein in 6M guanidinium chloride and in SDS electrophoresis (70,000 to 75,000) are consistent with the values reported in other laboratories. The molecular weight reported for the 39,000-dalton thrombogenic intermediate suggests that this material is probably the same as the prothrombin activation intermediate reported by Seegers et al. (11). Seegers et al. have reported the isolation of a "prothrombin" with a molecular weight of 38,500. Lanchantin et al. (5) have reported the observation of a nonthrombogenic 25,000-dalton component in their citrate activation studies of human prothrombin.

A good deal of speculation on the mechanism of prothrombin activation has been generated in the past and has lead to the formulation of a number of models for the prothrombin molecule. Among the features of the various prothrombin models proposed are the following. (a) Two thrombins are derived from a single prothrombin molecule (28). (b) Factor Xa activity is derived from the prothrombin portion of the prothrombin (11). (c) The latent thrombin structure in prothrombin comprises portions of the amino-terminal sequence plus other indeterminate portions of the molecules (29). The nonthrombin portion of the prothrombin molecule is a second protease possessing L-TAME esterase activity (29).

The maximal observed specific activity of bovine prothrombin is about 1,800 NIH thrombin units per mg, while that of our most active bovine thrombin is 2,720 NIH thrombin units per mg (15). Our best estimate of the molecular weights of prothrombin, and the most active, largest thrombin are 72,000 daltons and 39,000 daltons, respectively. If we assume quantitative conversion of prothrombin to thrombin, 1 mg of prothrombin would yield 39.72 mg of thrombin. This would yield in the formation of about 1,480 NIH thrombin unit per mg of prothrombin. The observed maximal specific activity of our purified zymogen is about 88% of the expected value. Considering the fact that it is doubtful that one can obtain quantitative conversion of prothrombin to exclusively the most active 39,000-dalton thrombin molecule, without some degradation of that component to the less active 28,000-dalton thrombins, a value of 88% conversion is quite reasonable. Other evidence for the lack of a second thrombin derived from prothrombin is the observation that the only other component derived from prothrombin of significant size during the activation process (24,000 daltons).

The sum of the molecular weight of the intermediates 2 and 3 (63,000) would be consistent with their precursor being any component in the 65,000 to 60,000 molecular size range. We, therefore, cannot exclude the possibility that the observed materials in the *M*_{app}, 60,000 to 65,000 range are actually intermediates in the thrombin productive pathway.
The data we have presented leaves little room for doubt concerning the nature of the intermediate from which thrombin is derived. In our system the results obtained by electrophoresis in SDS of this intermediate with disulfide bonds reduced and gel filtration of the reduced carboxymethylated, randomly coiled, intermediate are consistent with its being a single linear polypeptide chain of 33,000 daltons. The single chain nature of the precursor of IIa-A rules out the possibility that the two chains of IIa A are derived from different ends of the prothrombin polypeptide chain. Rather it appears that this thrombin is derived from a continuous section of the prothrombin molecule.

The question of a second esterase in prothrombin remains unclear. Our data indicate that all of the L-TAME esterase activity generated in the system is associated with components possessing clotting activity (IIa-A, IIa-B, IIa-C). The only other component of significant size, the 24,000-dalton component (obtained by cleavage of the 65,000-dalton intermediate, under the conditions employed for analysis. We cannot, however, rule out the possibility that the 24,000-dalton component (Intermediate 3) may be a protease for which neither fibrinogen nor other component of significant size, the 24,000-dalton component possesses clotting activity (IIa-A, IIa-B, IIa-C). The only activity generated in the system is associated with components in SDS of this intermediate with disulfide bonds intact. Sedimentation equilibrium studies of IIa-A in the unreduced state (15) indicate a molecular weight of 39,000. Both gel filtration of the reduced carboxymethylated protein in 6 m guanidinium chloride and SDS electrophoresis of the reduced protein are consistent with IIa-A being a two chain structure composed of disulfide cross-linked chains of 33,000 and 6,000 daltons. The single chain nature of the intermediate are derived from different ends of the prothrombin polypeptide chain. Rather it appears that this thrombin is derived from a continuous section of the prothrombin molecule.

The prothrombin we have isolated is essentially a single component in three different electrophoretic systems, and in isoelectric focusing experiments. The recent report of Rosenberg and Waugh (30) that activation of crude bovine prothrombin prepared from the plasma of selected purebred animals results in different activation pathways and different thrombins suggests the existence of multiple forms of bovine prothrombin. We have made no attempt at being selective in obtaining our prothrombin, they do not completely rule it out. The size of our blood pool suggests that if prothrombin microheterogeneity exists, it is probable that this would be present in our blood pool. However, since our yields of prothrombin are about 30% based on that present in the original plasma, it is conceivable that our purification procedures are selecting for a single prothrombin species.

The four analytical electrophoretic procedures used for evaluation of our product are quite sensitive for the detection of microheterogeneity. The thrombin generated by plasma-citrate activation of a single electrophoretic species of prothrombin is chromatographically and electrophoretically indistinguishable from that derived from Parke-Davis topical thrombin (15). This observation indicates that the small thrombins which have been demonstrated to be derived from IIa-A in Parke-Davis topical thrombin are not artifacts of that system, but normal prothrombin activation products.

Perhaps the most surprising observation one can make regarding the process of prothrombin activation described here is the transient rather than steady state production of intermediates of the activation process. Each of the intermediates builds up to very high levels before the next process in the activation cycle breaks it down. This circumstance is not due to widely differing rates of the intermediate breakdown processes. Each intermediate appears to be produced at about the same rate as its precursor. The basis for the temporal spacing between intermediates can only be explained by careful kinetic analysis of each partial reaction in the activation scheme.

The data presented here are clearly only the beginning of a careful study of the prothrombin activation mechanism. However, these results do clearly demonstrate the applicability of molecular weight analysis in denaturing solvents to the study of this complex process.

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