Bovine Thrombin

PURIFICATION AND CERTAIN PROPERTIES*

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SUMMARY

An examination is made of the fractionation of a crude thrombin preparation obtained by bioactivation, namely, Parke Davis thrombin, topical. Columns of carboxylic resin, cellulose phosphate, diethylaminoethyl cellulose, and Sephadex G-100 were studied. The starting material and all products obtained by using only carboxylic resin or cellulose phosphate are shown to be impure and to contain a nonenzymatic inhibitor. A simple procedure is given for isolating a stable thrombin of high, constant specific activity. The procedure involves removal from solution, first, of inhibitor and some nonthrombin protein by DEAE-cellulose at pH 7, 1/2 = 0.1 (phosphate), and 23°. A cellulose phosphate column is next used under the same conditions to adsorb thrombin and pass some nonthrombin protein. After additional inactive protein is washed off at pH 7 and 1/2 = 0.15, thrombin is eluted at 1/2 = 1.0 to give stock thrombin. The procedure at this point gives a minimum yield of 91.1 ± 8.4% of the original activity. Gel filtration of stock thrombin on Sephadex G-100 separates an average additional 24% of the absorbance of stock thrombin as an inactive protein and gives a single thrombin peak. By comparison with known proteins the peak position corresponds to a molecular weight of 36,000 ± 1,600 (95% confidence limits). The last 0.6 of the peak has constant specific activity which corresponds to 1,160 ± 130 NIH units per absorbance unit or 2,260 ± 250 NIH units per mg. At ionic strength 1.0, pH 7, and 4°, stock thrombin preparations lose activity to the maximum extent of 0.1% per week. At 1/2 = 0.1, pH 7, and 23°, diluted stock thrombin (20-sec clotting time) loses 1.6% of its activity per day. This is shown not to be due to an inhibitor, an extraneous enzyme, or the presence of 5 × 10⁻⁴ M KCN, which had to be added to prevent bacterial growth. The mechanism appears to be a zero order denaturation. Attempts to concentrate stock thrombin by ammonium sulfate precipitation or osmotic solvent removal have frequently led to the production of inactive aggregates. Acetone precipitation from 0.15 M calcium chloride at pH 7 has been successful in giving concentrations up to 10 absorbance units per ml.

Solutions containing thrombin (EC 3.4.4.13) are obtained by activating prothrombin preparations (1, 2), most frequently by bioactivation, which requires the addition of crude fractions of plasma, serum, or tissue. Bioactivation thus introduces a variety of impurities in addition to those which may be present in the prothrombin. The difficulties of preparing and activating prothrombin have been circumvented in several laboratories by using commercial thrombins, such as that of Parke Davis and Company (3). This material has been used as such, for example, by Kazal, Grannis, and Tocantins (4) and by Silver and Murray (5). As will be shown, Parke Davis thrombin is excessively unstable for many purposes.

A significant improvement in the specific activity of commercial thrombin with high yield was obtained by Rasmussen (6), who used a carboxylate resin column. This is the purification procedure most frequently used, for example, by Seegers, Levine, and Shepard (7) and Magnusson (8) with bioactivated bovine prothrombin, and by Miller and Copeland (9) to obtain human thrombin from a commercial prothrombin which they bioactivated. Our results show that PD-T purified by the Rasmussen procedure does not have significantly improved stability. Instability is evident for one of the above preparations (7) but, generally, stability has not been adequately studied. This is attributed to the problems encountered in the quantitative assay of thrombin, particularly when assay data obtained on different days are to be compared. Studies of stability are obviously important to any consideration of physical and chemical properties.

We have utilized PD-T on the basis that a successful fractionation procedure could be used for mixtures purer than those

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1 The abbreviations used are: PD-T, Parke Davis thrombin; stock-T, stock thrombin; DEAE-T, diethylaminoethyl cellulose thrombin; CP-T, cellulose phosphate thrombin; resin-T, resin thrombin.
of commercial thrombins, and, further, that commercial thrombins could then be used as sources in laboratories not equipped to carry out the process starting with plasma. Fractionation was investigated with respect to the extent to which stability and purity could be improved while maintaining a yield near 90%, thus avoiding the possibility of selectively discarding thrombin components. A simple procedure is described. This procedure is examined with respect to the impurities removed, particularly thrombin inhibitor. The thrombin product is characterized with respect to storage and physio-logical conditions and with respect to molecular weight and purity. Of the several techniques available for examining purity, we have chosen the combination of Sephadex filtration and a comparison of specific activities of the individual effluent fractions. The latter has required the development of tech-niques which permit the statistical errors of the assay to be revealed and to be made small.

**EXPERIMENTAL PROCEDURE**

**Materials**

**Chemicals**—All chemicals were reagent grade and were used without further purification.

**Distilled Water**—The general supply of distilled water was passed through a mixed bed, ion exchange resin (hydrogen and hydroxyl forms), then through a Millipore filter, HA grade, and was stored in a polyethylene container.

**Proteins**—Thrombin, topical was obtained from Parke Davis Company, bovine Fraction I from Armour and Company. bovine albumin from Sigma, and egg albumin, five times crystallized B grade, and α-chymotrypsin, crystallized salt-free A grade, from Calbiochem.

**Column Materials**—Diethylaminoethyl cellulose, Cellex-D, obtained from Bio Rad Laboratories, Richmond, California, had an exchange capacity of 0.86 meq per g; cellulose phosphate, Cellex-P, obtained from Bio-Rad Laboratories, had an exchange capacity of either 0.76 or 0.90 meq per g. No differences due to capacity were observed. Carboxylate resin, Amberlite CG-50, type I, was obtained from Rohm and Haas Company, Philadelphia; Sephadex, Sephadex G-100, 140 to 400 mesh, was obtained from Pharmacia International, New Market, N. J.

**Containers**—All containers used for thrombin were made of polyethylene, and all columns were constructed of Lucite.

**Absorbance**—By definition, 1 absorbance unit of a material is that amount per ml which for a 1-cm path length has a unit corrected absorbance at pH 7.0 and λ = 280 nm. All absorbances were corrected for scattering by subtracting 1.7 times the apparent absorption at 320 nm, and all measurements were carried out with a Beckman model DU spectrophotometer.

**Fluorometric Recording**—Column effluents were frequently monitored by a Turner model 111 fluorometer which filters peaking at 280 and 320 nm were used to illuminate and monitor, respectively. Readings were recorded by a Texas Instrument Servo-riter II. The fluorometer sample cell, of 0.6-ml volume, was connected to the exit of the column by a total of 1 cm of Servo-riter II. The fluorometer sample cell, of 0.6-ml volume, was connected to the exit of the column by a total of 11 cm of polyethylene, and all columns were constructed of Lucite.

**Buffers**—All buffers, except those used in preparing fibrinogen, contained 5 × 10^{-4} M potassium cyanide to suppress microorganisms. Phosphate was the only anion for buffers at pH 7.0 except for stock fibrinogen-diluting buffer, in which ionic strength was due one half to sodium phosphate and one half to sodium chloride.

**Preparations of Thrombins**

**Stock T and DEAE-T**—Cellex-D and Cellex-P first had fine particles removed. Cellex-D was then packed tightly in a column 5 cm in diameter and 7 cm long. Cellex-P was similarly handled to form a column 2.5 cm in diameter and 7 cm long. When they were connected in series, the flow rate through the columns was approximately 50 ml per min under a 60-cm head of water pressure.

Columns were individually washed with acid and base, equilibrated with phosphate buffer at pH 7.0 and Γ/2 = 0.1, and connected in series. PD-T, 800 mg, was dissolved in 320 ml of the equilibrating buffer, applied to the Cellex-D column, and followed by 400 ml of the same buffer. The Cellex-P column was disconnected and washed with 250 ml of phosphate buffer at pH 7 and Γ/2 = 0.15. Thrombin was eluted with buffer at Γ/2 = 1.0, one half due to NaCl, at pH 6.75. The first 50 ml were discarded and the next 50 ml were collected. The eluted thrombin, which will be referred to as stock thrombin, was stored at 4°C. This procedure can be carried out completely in 8 hours. When PD-T was passed through the Cellex-D column alone, a thrombin solution was obtained which will be referred to as DEAE-T.

**Resin Thrombin**—This preparative procedure is similar to that described by Rasmussen (9). PD-T, 100 mg in 2 ml, was applied, and the column was washed first with 55 ml of Γ/2 = 0.1 equilibrating buffer, then with 30 ml of Γ/2 = 0.3 phosphate buffer at pH 7. Thrombin was eluted with buffer at pH 7.1 and Γ/2 = 0.5. Yields were near 80% of initial activity.

**Cellulose Phosphate Thrombin**—Columns of cellulose phosphate have been used by Seegers and Landaburu to prepare thrombin (10). A column 1 cm in diameter and 6.75 cm high was packed with refined Cellex-P (dry weight, 1.45 g). This column was washed with acid and base and equilibrated with phosphate buffer at Γ/2 = 0.1 and pH 7.0. The same buffer was used to apply 25 mg of PD-T in 10 ml. The column was then washed with 25 ml of Γ/2 = 0.15 phosphate buffer. Thrombin, eluted with Γ/2 = 1.0 buffer, appeared in the second and third hold up volumes at a yield over 90%.

**Assay of Thrombin Activity**—Clotting times, τ, used to determine thrombin concentration, were determined at pH 7, Γ/2 = 0.1, and 29°C. A 0.15-ml aliquot of thrombin was added to 1.35 ml of fibrinogen containing 0.2 mg of clottable nitrogen per ml. The techniques used in these assays have involved (a) development of a clotting time assay machine in which the only subjective operation is the determination of the end point; (b) establishing the conditions under which purified fibrinogen may be used as a standard reagent; and, (c) establishing by statistical procedures the values of significant variations.

These developments are complicated and will be described fully elsewhere. Certain measurements and conclusions are now cited since they have been used in determining specific activity and in interpreting studies of instability and yield.

An important quantity, the standard deviation of repeated measurements of τ = 20 sec, is 0.12 sec. Thrombin is adsorbed to clean surfaces, even polyethylene and Teflon. Adsorption of polyethylene is variable, between 3 and 7% at τ = 20 sec. The amount of thrombin adsorbed is essentially independent of the
thrombin concentration. The variance due to adsorption can be minimized by saturating the vessel surface with thrombin before use: the surface is thus conditioned. For specific activity measurements the first dilution was discarded and subsequent dilutions were assayed. The storage temperature of stock fibrinogen is important in minimizing the variability between stock fibrinogen tubes. Storage temperatures of $-20^\circ$ and $-90^\circ$ have been examined. Although stock fibrinogen solutions stored at either temperature appear to be stable for at least 8 weeks, $-90^\circ$ represents the better storage temperature, since the standard deviation between a series of daily averages for any single lot of stock fibrinogen is, for $\tau = 20$ sec, 1.0 sec at $-20^\circ$ and 0.5 sec at $-90^\circ$. Fibrinogen stored at $-90^\circ$ was used for all specific activity measurements.

The averages given in the text are accompanied by standard deviations about the average calculated according to the method of Bennett and Franklin (11). Slopes of instability regressions were calculated by minimizing the squared deviations of individual measurements from the line of regression. In certain experiments it was necessary to relate a difference in clotting time to a difference in thrombin concentration. The experimental data so far available suggest that Equation 1 is a useful approximation.

$$T_n / T_0 = \left( \frac{r_n}{r_0} \right)^\gamma$$

Values of $\gamma$ between 0.76 (12) and 1.0 (13) have been reported. In Equation 1 $T_n$ is the test thrombin concentration and $T_0$, that present initially (control).

**Thrombin Yields from Columns**—After a column run, two solutions were compared. The first consisted of an aliquot of the thrombin used for application, diluted by a factor equal to the ratio of the applied thrombin volume to the total volume of all fractions containing thrombin. The second thrombin solution was prepared by combining effluent thrombin aliquots, each of which was a constant proportion of the fraction volume. The fractional yield was then obtained from clotting time assays by Equation 1. For PD-T, preliminary solutions were first made at concentrations of 80 mg per ml. These contained sufficient salt to stabilize them at room temperature until all assays could be performed at the same time.

**Sephadex Chromatography**—Columns of Sephadex G-100, 0.95 x 100 cm, were washed for 4 days with deionized column solution (four hold-up volumes) before use. All filtrations were initiated by applying 1.0 ml of protein containing 0.5 to 5 absorbance units. The columns were sealed and flow was controlled at 3 to 4 ml per hour by a Milton Roy chromatographic minipump. Solutions were transferred through Teflon tubing.

For molecular weight determinations, 0.5 to 1.0 absorbance unit of protein was applied and fluorescence was used to monitor the column effluents. Elution volumes are computed from flow rates and times required for chart excursions.

When specific activities were to be measured, a Gilson fraction collector was used to collect aliquots of approximately 0.5 ml, at constant time intervals, into polyethylene tubes ($12 \times 75$ mm). Before a run, 4.5 ml of water were added to each tube expected to receive thrombin activity. For these studies the load was 4 to 5 absorbance units. After filtration was completed, the absorbance of each fraction was measured. Specific activities were then measured by adding 1.0 ml of each thrombin solution to a conditioned vessel containing sufficient buffer to give 0.005 absorbance unit per ml at 280 $\mu\mu$. The clotting time of each dilution was measured, the sequence of dilutions being randomized. The entire procedure minimizes variations, which would systematically introduce longer clotting times for smaller thrombin concentrations.

**RESULTS**

**Thrombin Stability**

Under clotting conditions, i.e., pH 7, $\Gamma/2 = 0.1$, and room temperature ($23^\circ$), the time dependence of the loss of thrombin activity in PD-T solutions is such that experiments should be carried out over a period of days. This requires that bacteriostasis be accomplished, and to this end thrombin solutions were made $5 \times 10^{-4}$ M in KCN.

Stability in dilute solution at $23^\circ$ has been measured for each type of thrombin preparation: PD-T, CP-T, DEAE-T, and diluted stock T at $\Gamma/2 = 0.1$ and resin T at $\Gamma/2 = 0.15$. In each case a thrombin solution was prepared on day zero at a concentration required to give a value for $\tau$ of approximately 20 sec, and one or two clotting times were measured thereafter either daily or every 2 or 3 days with a freshly diluted fibrinogen preparation.

Data were obtained over a 2-year period for 20 preparations of PD-T (six lot numbers), six preparations of Resin-T, four preparations of CP-T, 15 preparations of DEAET, and 14 preparations of diluted stock T. For comparable solutions the clotting time data were handled as follows. For day $n$, the ratios $r_n : r_0$ were calculated. All ratios corresponding to the same day were then averaged. This averaging process was chosen as a result of the magnitude of the day to day standard deviation (5%). If the day to day variations are randomly distributed, the above averaging process permits the relationship between $\tau$ and time for a particular preparation to be evaluated. Fig. 1 gives the average $r_n : r_0$ to time relationships for all types of thrombin except CP-T. The data for CP-T are similar to those for resin-T.

Average clotting time ratios were converted to average thrombin concentration ratios by Equation 1. The resulting $T_n / T_0$ to time relationships are shown in Fig. 2. It should be noted that, although each $T_n / T_0$ ratio is dependent on the choice of $\gamma$ in Equation 1, the shapes of the curves and the conclusions which follow are not.

**Parke Davis Thrombin**—Curve 1 of Fig. 2 reveals that the inactivation of PD-T can be described as the combination of an initial limited rapid decay and a slow residual decay. Thus, PD-T loses an average of 58% of its activity in 6 days, but thereafter the loss is $\sim 2.3\%$ per day. The data for individual PD-T solutions showed a large variability between lot numbers: from consistent losses of 99% to 35% in 7 days.

If the ionic strength of PD-T is increased to 0.3 or 0.5 ($23^\circ$), the $r_n : r_0$ instabilities appear to be linear with respect to time and are 4.3% per day and 3.1% per day, respectively, over a period of 7 days. If the temperature is decreased to $4^\circ$ at $\Gamma/2 = 0.1$, the instability, again appearing linear with respect to time, is decreased to 2.4% per day.

**Resin-T**—Curve 2 of Fig. 2 refers to resin-T diluted to $\Gamma/2 = 0.15$ and at $23^\circ$. Here 40% of the initial activity is lost in the first 3 days; the loss is 1.5% per day after 5 days. Many experiments were performed with the objective of obtaining from resin columns thrombin which would be stable at $\Gamma/2 = 0.15$ and $23^\circ$. For example, after thrombin application a column was washed extensively at $\Gamma/2 = 0.35$, an ionic strength just insufficient to
remove thrombin, and a fraction was then collected at an ionic strength just sufficient to elute thrombin. Thrombin activity decreased by ~25% in the first 24 hours. In one experiment, human prothrombin was bioactivated, and thrombin was prepared by the resin column procedure. This purified human thrombin was also found to be unstable.

**CP-T**—Four experiments have been conducted to examine the stability of CP-T at pH 7, $\Gamma/2 = 0.1$, and 23°C. The average initial rapid decay in 3 days was about two-thirds that of PD-T. When elution was carried out at $\Gamma/2 = 0.4$, near the lowest ionic strength which will still result in a yield near 80% in three to four hours on volumes, the rapid decay of test solutions was nearly the same. Stabilities of similar preparations were not reported by Seegers and Landaburu (10). The highest specific activities of CP-T preparations were about half those of stock-T preparations.

**Inhibitor**—A comparison of the stabilities of PD-T (Curve 1 of Fig. 2) and DEAE-T (Curve 3 of Fig. 2) suggests that a material responsible for rapid decay is adsorbed to DEAE-cellulose. This material can be eluted in 1 mM sodium chloride from Cellex-D after thrombin purification. Its presence was revealed as follows. The eluate was diluted to $\Gamma/2 = 0.25$, and an aliquot of this was recombined with an equal portion of the DEAE-T so as to duplicate the protein content of a PD-T solution. The final ionic strength was 0.175. In two experiments the average loss in the first 5 days for the DEAE-T alone was 11% and for the mixture, 48%. In another type of experiment, equal volumes of diluted stock-T and PD-T, both at $\gamma = 15$ sec, were mixed and examined over a period of 7 days. The rapid decay in the mixture was only 0.6 of that in PD-T alone.

It was suspected that the inhibitor could be normal plasma antithrombin. To test this, 1.5 ml of defibrinated plasma, obtained by heating plasma at 56°C for 3 min, were combined with 12 ml of stock-T and 10.5 ml of buffer ($\Gamma/2 = 0.1$). The thrombin-plasma mixture, 18 ml, was purified to give a stock-T preparation. Over a 7-day period this stock-T lost 1.20% of its activity per day. The control (no added plasma) lost 1.86% per day, and the unpurified thrombin and plasma mixture lost ~9.7% per day. These results indicate that antithrombin is removed from purified thrombin and that it could be the inhibitor in PD-T.

**Slow Decay**—Slow decay is shown by all preparations; the mechanism of slow decay has been examined particularly with respect to the DEAE-T and the 14 stock-T preparations. Linearity of the clotting time regression (Curve 3 of Fig 1) was established by determining the number of individual regressions of stock-T which contained a significant quadratic term. Of the 14 stock-T solutions, only two were significantly nonlinear (95% confidence limits). All regressions were then assumed to be linear, with the following results. For stock-T, the average slope is $1.31 \pm 0.25$% per day, and for DEAE-T it is $1.56 \pm 0.45$% per day. For each set, four slopes lay outside the range predicted from the standard deviation of the slope and the sequence of 15 daily measurements. The distribution of slopes thus cannot be assumed to be normal. However, if the distribution is assumed to be normal, the stabilities of DEAE-T and stock-T may be just significantly different at the 95% confidence level, with DEAE-T being the more unstable. If nonrandomness could be taken into account, the slope averages might not be significantly different. The linear relationship between $\tau$ and time of Curve 3 of Fig. 1 has a slope of 1.44% per day. This
The effect of cyanide on slow decay has been examined with concentrations of 0, 5 × 10^{-4}, 1.6 × 10^{-4}, and 4 × 10^{-3} M at a constant thrombin concentration. The initial clotting times were τ = 22.1, 22.7, 23.3, and 23.6 sec, respectively. From other experiments it is known that this variation in clotting time with cyanide concentration is due to an effect of cyanide on fibrin polymerization reactions. Clotting time increments were measured over a 2-day period. Within the variation of the single clotting time, the losses in all samples were equivalent. Furthermore, the observed losses were not significantly different from the expected average loss of 1.4% per day.

In view of the importance of having a single preparation as a source of thrombin over a period of time, the stability of stock-T at 4°C has been studied extensively. This study included development of a standard thrombin-diluting technique based on prior conditioning of vessels and sampling, the standard deviation of which is 0.12 sec at τ = 20 sec. It was found that, if stock-T preparations lose activity, the loss is less than 0.1% per week over a period exceeding 8 weeks.

### Column Properties

**DEAE-cellulose Columns**—It was important to know the conditions for removing inhibitor completely, plus a maximum amount of extraneous protein, without removing thrombin. Certain properties of the fractionation procedure were inferred from experiments with either the standard column procedure or a batch procedure in which 2 g of Cellex-D, wet weight (0.4 g, dry weight), were added to 9 ml of solution containing 0.5 mg of PD-T per ml.

At τ/2 = 0.1, pH 7, the batch procedure shows that approximately 10% of thrombin activity in PD-T is reversibly adsorbed. In the column procedure, an adequate volume of wash at τ/2 = 0.1 and pH 7 is required to effect complete passage of thrombin. Under identical conditions, diluted stock thrombin is not adsorbed. This suggests that the thrombin in PD-T interacts with another component which is adsorbed to DEAE-cellulose.

At τ/2 = 0.1, as the pH is raised from 5.5 to 9, thrombin adsorption from PD-T is negligible at pH 6, is 10% at pH 7, and is 25% at pH 8, where all may be removed if the amount of Cellex-D is increased. Between pH 5.5 and pH 9 thrombin 9 a relatively constant 50% of the absorbance of PD-T is removed. In all column experiments thrombin could be recovered (~90% total recovery) by elution at pH 7 and τ/2 = 0.1.

At pH 7, the adsorption of thrombin to Cellex-D is sensitive to ionic strength. With the use of the batch procedure, as ionic strength was increased above 0.1 nannothrombin protein in PD-T was found to be preferentially eluted, while below τ/2 = 0.1, thrombin was preferentially adsorbed.

With pH 7 and τ/2 = 0.1 established as chosen conditions, an operating ratio of Cellex-D to PD-T was determined as twice the minimal amount of Cellex-D required to give maximum protein removal. For 31 preparations from seven different lots of PD-T, the average yield was 88.6 ± 14.4%, and the column, by adsorbance, removed an average of 74.3% of the impurities, the latter ranging from 55 to 85%, depending on the PD-T lot number.

An additional observation of importance is that PD-T contains a material that appears to be capable of degrading Sephadex. This material is not present in DEAE-T or stock-T.

**Cellulose Phosphate Columns**—When a standard load of 100 absorbance units of DEAE-T is applied to the standard Cellex-P column (see "Stock-T," under "Experimental Procedure"), at pH 7 and τ/2 = 0.1, thrombin is quantitatively adsorbed. On washing at τ/2 = 0.15, 71% (average of 14 runs) of the applied absorbance is removed; some passes through and some is eluted. At τ/2 = 1.0, the yield in one and one-half hold up volumes is 89.9 ± 11.2% (average of 7 determinations).

Since Shapiro and Waugh (14) have observed an inactivation of prothrombin by carboxylic acid resin columns, an investigation was made of possible inactivation of thrombin by adsorption to Cellex-P. Up to 24 hours of adsorption time, the recovery was independent of time. This indicated that there was no significant degradation of thrombin by Cellex-P.

When both columns were connected in series as described, the average yield of stock-T for 11 runs on eight different lots of PD-T was 91.1 ± 8.4% of the original activity and 9.1% of the original absorbance.

**Solubility and Concentration of Thrombin**

Stock-T is not sufficiently concentrated to permit certain kinds of anticipated physical studies. Therefore, several concentration techniques were examined with thrombin solutions at A = 0.05.

Under these conditions dialysis against polyethylene glycol at τ/2 = 0.3 and pH 7, or ammonium sulfate precipitation can be used to give thrombin levels of approximately 0.5 absorbance unit per ml with complete recovery. If concentration to a higher level is attempted, for example 5 absorbance units per ml, thrombin activity is frequently lost and Sephadex filtration reveals inactive aggregates.

At levels below approximately 5 absorbance units per ml, acetone precipitation at pH 7 has been the most reproducible of the methods giving high yields; an average of 89 ± 7% of thrombin activity (12 trials) and 94% of absorbance (6 trials) was recovered at final acetone concentrations between 30 and 70%. Precipitation was carried out at 0°C after samples were diluted to τ/2 = 0.1. If thrombin is eluted from the Cellex-F column with 1% sodium chloride, dilution to τ/2 = 0.1 is not necessary.

After 15 min of gentle stirring, the precipitate was recovered by centrifugation, air dried, and dissolved in phosphate buffer, τ/2 = 0.3. With all procedures, the specific activity of the thrombin which appeared at the expected Sephadex filtration volume (see below) was maximal.

Concentration of thrombin without aggregate formation has been obtained up to 10 absorbance units per ml. The thrombin was acetone precipitated from 0.15 M calcium chloride at pH 7, and then dissolved either in 0.15 M calcium chloride or 0.15 M sodium citrate. Thrombin in 0.15 M calcium chloride was obtained by direct elution from Cellex-P columns or by appropriate dialysis of stock-T. The former procedure requires an adjustment of pH before acetone addition. An examination of the effects of calcium chloride was undertaken, since Miller and Copeland (9) have used acetone precipitation to obtain similar human thrombin concentrations following elution from resin columns with 0.15 M CaCl₂.

**Properties of Thrombin Obtained by Filtration on Sephadex G-100**

Twelve preparations of PD-T, ten of DEAE-T, and eight of stock-T have been filtered. Between τ/2 = 0.3 and τ/2 = 1.0 the results are independent of ionic strength. The thrombin activity for all preparations was associated with a single protein peak, the average elution volume of which was 59 ± 3% of the
The molecular weight of thrombin in stock-T has been estimated to be linearly related to the logarithm of the molecular weight. The 95% confidence limits are ±1,600.

Stock-T on G-100 Sephadex

**THROMBIN PURITY—**An estimate of the impurities that might be concealed in the thrombin peak following Sephadex G-100 filtration was obtained by collecting the thrombin peak in 0.5-ml fractions and measuring the specific activity of each. Such a procedure will detect impurities the filtration volumes of which are displaced by one or more fractions. Since both optical density and clotting time were to be measured accurately, it was necessary that the highest practical thrombin concentration be used without prior concentration. As shown above, concentration techniques are not completely reliable. Studies of purity were performed by using the most concentrated 2 ml obtained during standard thrombin elution from Cellex-P.

![Fig. 4. Stock-T effluent, 1 ml selected to contain 4.5 absorbance units, was filtered on a Sephadex G-100 column, 9.5 mm x 100 cm, with 1.0 M NaCl at pH 7.0. The flow rate was 2.14 ml per hour. Each fraction of 0.43 ml was diluted with 4.5 ml of water. Ordinates, absorbance at 280 mp; abscissae, clotting time in seconds, of thrombin solutions diluted to absorbance = 0.005 A. The small peak represents that amount of impurity required to give a constant clotting time across the main peak.](http://www.jbc.org/)

### Table I

**Thrombin molecular weight by gel filtration**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Average flow rate</th>
<th>Total filtration volume</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin A</td>
<td>3.50</td>
<td>42.46</td>
<td>39,000</td>
</tr>
<tr>
<td>Thrombin B</td>
<td>3.83</td>
<td>42.04</td>
<td>36,000</td>
</tr>
<tr>
<td>Thrombin C</td>
<td>3.52</td>
<td>41.95</td>
<td>33,000</td>
</tr>
<tr>
<td>Thrombin average</td>
<td>3.73</td>
<td>42.32</td>
<td>36,600</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>3.60</td>
<td>39.90</td>
<td>45,000</td>
</tr>
<tr>
<td>Ovalbumin (monomer)</td>
<td>3.71</td>
<td>35.25</td>
<td>70,000</td>
</tr>
<tr>
<td>Serum albumin (dimer)</td>
<td>3.71</td>
<td>27.91</td>
<td>140,000</td>
</tr>
</tbody>
</table>

Thrombin molecular weight was obtained by the filtration technique of Whitaker (15) with Sephadex G-100 columns, 9.5 mm x 100 cm. When the same column is used for all samples, log molecular weight is linearly related to filtration volume (volume from application of protein to center of effluent peak). Protein was applied as 0.5 to 1.0 absorbance unit in 1 ml. Developing buffer was 1.0 M NaCl at pH 7.0. Average thrombin molecular weight was obtained from the least squares regression with the use of other protein molecular weights as known (15) and the average filtration volume of thrombin.

![Fig. 3. Continuous fluorometric recording for stock-T applied to a Sephadex G-100 column, 9.5 mm x 100 cm. The column conditions were pH 7.0; solvent, 1 M NaCl; flow rate, 3.54 ml per hour. Fluorometric measurements were made at λ = 300 mp, with incident radiation at λ = 280 mp. The thrombin peak at 42.5 ml, contained 95% of the total area, and was contained in 8.8 ml between the marks. A, void volume (24.8 ml).](http://www.jbc.org/)
of the individual fractions expressed as the average clotting time after dilution to absorbance = 0.005. The specific activity for the last 11 clotting times averaged 20.39 ± 0.35 sec. The difference between the specific activity of the applied thrombin (23.2 sec) and the average of 20.39 sec can be accounted for by the removal of 17% as impurities. Refiltration of 1 ml of the combined three most concentrated tubes yielded a series of fractions which, except for the first, were constant in specific activity.

A total of five complete filtration experiments involving three different PD-T lot numbers has been carried out during a period of 18 months by two operators. Essentially the same result has been obtained, i.e. 10 to 15% of the applied sample as an impurity consisting of larger molecules and a constant specific activity over the last 0.60 of the thrombin peak. The average of the five constant specific activities was 20.56 ± 1.05 sec. The average impurities in all stock-T preparations were obtained as follows.

The average specific activity of the 11 stock-T preparations for which data are available is 24.24 ± 2.14 sec. If this is compared by Equation 1 with \( \tau = 20.56 \), the average value for constant specific activity, stock-T preparations contain an average of 24% impurity. Since the standard deviation of ±0.14 sec is significantly larger than ±0.07 sec, the value expected from variations between assays, it is concluded that different stock-T preparations contain different amounts of impurities.

**Specific Activity**—The specific activities given above appear as the clotting time at a constant absorbance of 0.005, other conditions being held constant. Specific activity in terms of NIH units of thrombin per mg of protein was estimated by comparison with lot-B-3 NIH thrombin dissolved carefully according to National Institutes of Health specifications (16). This working standard was diluted so that \( \tau = 20.56 \) sec could be obtained by interpolation. This experiment was repeated on fibrinogen from a single lot. By interpolation, \( \tau = 20.56 \) sec converts to 5.79 ± 0.142 NIH units per ml and thus to 1160 NIH units of thrombin per absorbance unit for material of constant specific activity. The standard deviation to be expected for National Institutes of Health conversion with stock fibrinogens from different lots of thrombin per absorbance unit for material of constant specific activity.

The extinction coefficient of bovine thrombin is 19.5 (17), the difference between the specific activity of the applied thrombin by filtration on Sephadex G-100. It should be noted that, in addition to inhibitor, resin-T, and CP-T contain larger amounts of nonthrombin protein than stock-T.

**Stabilization of thrombin at low temperature** (4°), or high ionic strength (1.0), or both has been reported by Seegers, Lovine, and Shepard (7), Miller and Copeland (9), and Ehrenpreis and Scheraga (20). We have confirmed this result for all preparations, including PD-T, resin-T, and CP-T. It follows that stability at low temperature, or high ionic strength, or both is not sufficient to indicate stability under other conditions. The prior reports of thrombin stability at room temperature (7, 9) do not include sufficient data to permit a quantitative comparison.

It should be noted that, in addition to inhibitor, resin-T and CP-T contain larger amounts of nonthrombin protein than stock-T.

In both DEAE-T and stock-T, at yields above 90%, rapid decay is absent at pH 7, \( \Gamma/2 = 0.1 \), and 23°. A slow decay averaging 1.44% per day is present in these materials, as it is in all thrombin preparations examined. An important possibility is that the inhibitor present in PD-T has survived the fractionation procedure and is present in stock-T. This source can be eliminated. It is noted that slow decay leads to an average loss of 25% of thrombin concentration in 15 days. If this loss were due to the rapid inhibitor of PD-T, a loss of 13% should have been observed in the first 24 hours.

Certain other potential sources of slow decay are considered unlikely. Since the 2-, 4-, and 5-times concentrated samples were as stable as their controls, any mechanism requiring a concentration dependence appears to be excluded. Such mechanisms would be, for example, slowly acting inhibitors or enzymes, including thrombin autoinactivation, and the slow continuous generation of adsorptive capacity at vessel-liquid interfaces. Typical slow decay has been observed with recent assay techniques, with the use of fibrinogen stored at -90° and sampling techniques which do not introduce significant thrombin loss; therefore, time-dependent changes in the assay system are unlikely. The rate of slow inactivation is also independent of cyanide concentration up to 4 x 10^{-5} M KCN, thus eliminating the possibility of a chemical interaction with cyanide. From the above, the mechanism which appears most likely at pH 7, \( \Gamma/2 = 0.1 \), and 23° is a zero order reaction, that is, a denaturation.

**Stock-T** contains nonthrombin protein, an average of 24% in terms of absorbance at 280 mg. Most nonthrombin protein appears to be larger than thrombin and can be separated from thrombin through filtration on Sephadex G-100. It should be noted
that the stock-T preparative procedure and filtration of stock-T through Sephadex G-100 both give a yield over 90%. Thus, 80% of the activity in PD-T appears after gel filtration in a single peak the asymmetry of which is not significantly different from that of peaks given by other proteins. The molecular weight of 36,600 obtained here is to be compared with the molecular weights for bovine thrombin, obtained by ultracentrifugation, of 33,700 (Harmison, Landaburu, and Seegers (21)) and 40,000 at pH 7.0 (Winzor and Scheraga (17)). Whether or not these estimates are significantly different remains to be determined.

Although the asymmetry of the thrombin peak is not significantly different from those of other proteins, a determination of specific activity across the peak shows that the leading 0.4 has a lower specific activity than the remaining 0.6. If an inactive impurity is present, it must account for 4.5% of the absorbance of the peak. A smaller peak of Fig. 4 was obtained by determining the absorbance necessary to give constant specific activity. Another possibility is suggested by the results of gel electrophoresis of the thrombin obtained by Sephadex filtration. Dr. R. D. Rosenberg* has found that gel electrophoresis reveals the presence of five zones. Preliminary experiments in which those zones have been partially fractionated show that, although all zones are active, there is progressive change in specific activity with zone position. It is thus possible that the leading edge of the peak obtained on Sephadex G-100 filtration contains thrombin molecules which are, compared to the average, slightly larger in size and lower in specific activity.

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