Chromatographic Analysis of the Activation of Human Prothrombin with Human Thrombokinase*

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ABSTRACT: Chromatography on DEAE-cellulose was carried out to analyze the products formed from human prothrombin during the course of its conversion to thrombin by human thrombokinase. The elution patterns revealed the progressive appearance of two additional peaks with prothrombin activity. Each of the new peaks seems to be associated with the release of a nonactive fragment. There is evidence to suggest that the derivatives and fragments produced are not the results of nonspecific proteolysis but are related to the biological activation of prothrombin.

The term thrombokinase was first used by Morawitz (1904) as the name of the prothrombin activating material. Implicit in the name is the assumption of an enzymatic conversion. Milstone (1949) described the isolation from bovine plasma of a potent activator of prothrombin. This bovine thrombokinase activates prothrombin without the addition of Ca^{2+}, Factor V, or phospholipid (Milstone, 1964). The present work used DEAE cellulose chromatography to analyze the products of the reaction of human prothrombin with human thrombokinase.

Materials and Methods

Prothrombin was purified from ACD[1] bank plasma by a modification of the method of Goldstein and Zonderman (1958). The prothrombin was adsorbed from ACD bank plasma of variable age by the addition of 15 g of $BaCl_2$/l. of plasma. The resulting barium citrate precipitate was washed with 0.1 M $BaCl_2$, eluted with 40% saturated ammonium sulfate, and precipitated by raising the concentration of ammonium sulfate to 65% saturation. After dissolution of the precipitate in a minimum volume of water the salt was removed by passage through a G-25 Sephadex column previously equilibrated with distilled water. Appropriate eluate fractions were pooled and the pH was adjusted to 4.7 by addition of dilute acetic acid. After centrifugation the resulting precipitate was redissolved in 3–4 ml of 0.11 M NaCl–0.02 M cacodylate, pH 5.95. This material was chromatographed on IR-50 (Miller, 1958) equilibrated in the same buffer. These preparations had an activity of 1100–1200 U. S.[2] units/mg of protein.

Thrombokinase was first prepared from Cohn fractions III-0 or IV-1 (method 6) (Cohn *et al.*, 1946). Because of the low activity of these fractions, ACD bank plasma or ACD bank plasma with extra citric acid (0.0025 mole/l. of plasma) was used as a starting material. Figure 1 is the flow sheet for the production of thrombokinase. Figure 2 is a chromatogram of this material on DEAE-cellulose[3] (*vide infra* for experimental conditions); the TAME hydrolytic and prothrombin-converting activities are seen to be coextensive. The large amount of thrombin in the early eluting peak precluded assaying for thrombokinase in this region. Figure 3 is the chromatographic pattern obtained when the prothrombin-converting fractions from DEAE-cellulose were concentrated and placed on G-100 Sephadex.[4] Again the TAME and the prothrombin-converting activities come off the column together.

The solution of thrombokinase used in the following experiments had an optical density at 280 mμ of 1.100 and hydrolyzed 3.74 μmoles of TAME/10 min per 0.1 ml. About 50% of the TAME activity was inhibited by soy bean trypsin inhibitor[5] at a concentration of 0.1 mg/ml. The noninhibited activity presumably represented other TAME-splitting enzymes including residual thrombin. This amount of soy bean inhibitor totally inhibits the conversion of prothrombin to thrombin with the same quantity of thrombokinase.

Human fibrinogen[6] was dialyzed against 0.02 M Tris Cl–0.15 M NaCl, pH 7.4. The concentration was adjusted to either 1 or 0.5% with this buffer and aliquots were frozen at −20°. These were thawed as

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[1] Abbreviations used: ACD, acid citrate dextrose anticoagulant, NIH solution A; TAME, *p*-toluenesulfonyl-L-arginine methyl ester; RVV, Russell viper venom; DFP, diisopropyl phosphorofluoridate.

[2] One U.S. unit is identical with the so-called NIH unit.

[3] DEAE was obtained from Calbiochem, Los Angeles, Calif.

[4] Pharmacia, Piscataway, New Market, N. J.

[5] This was five times crystallized supplied by Worthington Biochemicals, Freehold, N. J.

[6] Obtained from Merck Sharp and Dohme, West Point, Pa.

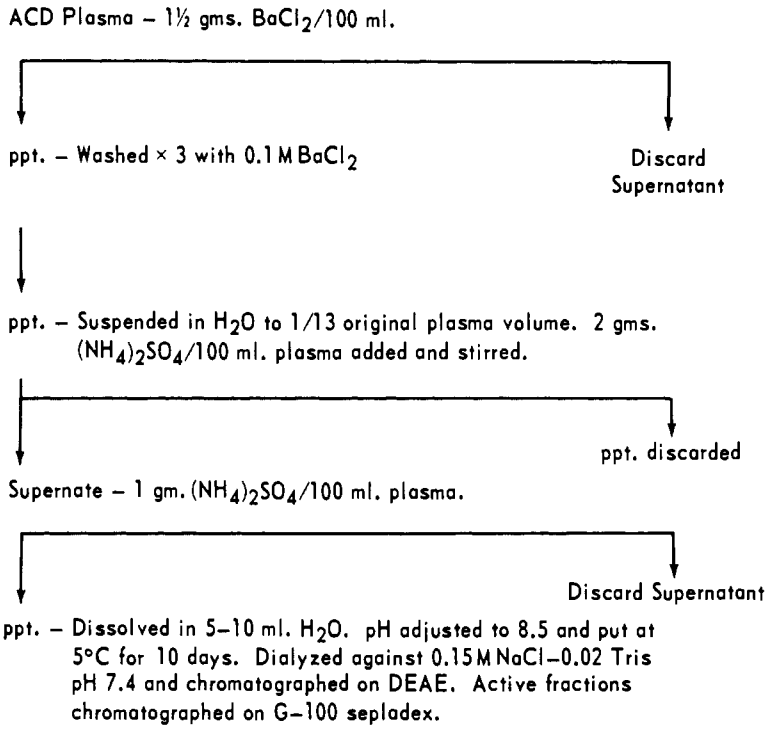

FIGURE 1: Flow sheet for the preparation of human thrombokinase.

needed for the assay of thrombin and prothrombin.

Prothrombin was measured by the two-stage prothrombin assay (Wagner *et al.*, 1955).[7] All dilutions were done in a 1:50 dilution of beef serum as a source of Factor V. This assay system was standardized against Lot B-3 U.S. standard thrombin so that 1 prothrombin unit gave 1 unit of thrombin. By this method of standardization 1 ml of human plasma contains about 160 units of prothrombin.

Thrombin was measured by adding 0.1 ml of the appropriately diluted solution to 0.4 ml of 0.25% fibrinogen. Clotting times were converted to U.S. units by reference to a dilution curve of the U.S. standard thrombin.

TAME hydrolytic activity was measured by means of a Radiometer pH-Stat. A 0.1-ml sample was added to 9.0 ml of 0.011 M TAME in 0.15 M NaCl. The pH was maintained at 8.0 by the automatic addition of 0.04 M NaOH. The TAME, thrombin, and prothrombin assays were done at room temperature (22–24°).

Thrombokinase assays were done by adding 1 ml of prothrombin (5000 units/ml) to 0.1 ml of sample to be tested for thrombokinase activity. The pH was adjusted to 7.5 and the ionic strength was 0.15. The thrombin generated was measured at various times.

For the Factor V assay 0.1 ml of Factor V[8] deficient plasma was added to 0.1 ml of test sample. After warming in a 37° bath, 0.2 ml of tissue thromboplastin[9] with $CaCl_2$ was added and the clotting time was measured.

DEAE-cellulose chromatography employed a 500-ml elution scheme utilizing a linear gradient (0.15 M NaCl–0.02 M Tris, pH 7.4, to 0.50 M NaCl–0.066 M Tris, pH 7.4) which was used to develop a 1.9 × 35 cm column. The flow rate was about 30 ml/hr for these columns. After each run the columns were flushed with 2 M NaCl and then with 10 column volumes of the starting buffer. The gradient was started immediately after application and washing in of the prothrombin samples with two 2.5-ml aliquots of starting buffer. However, during the preparation of the thrombokinase several hundred milliliters of the starting buffer was flushed through the column after the sample has been applied and before the gradient elution was started. This removed traces of thrombin to a larger extent than if the gradient had been started immediately.

Exclusion chromatography was done on G-100 Sephadex packed in a 1.8-cm column to a height of 75 cm. It was equilibrated with 0.1 M Tris–Cl, pH 8.1, and the elution was done with this same buffer. The breakthrough peak was at 65 ml.

Ultracentrifugal studies were done in a Model E Spinco ultracentrifuge with schlieren optics. All samples were run in a synthetic boundary cell and apparent diffusion coefficients were calculated from the boundary spreading. Disc electrophoresis was done on acryl-

[7] Difco two-stage reagent used as thromboplastin.

[8] Plasma from a patient deficient in Factor V kindly supplied by Dr. O. D. Ratnoff, Cleveland, Ohio.

[9] Simplastin obtained from Warner-Chilcott, Morris Plains, N. J.

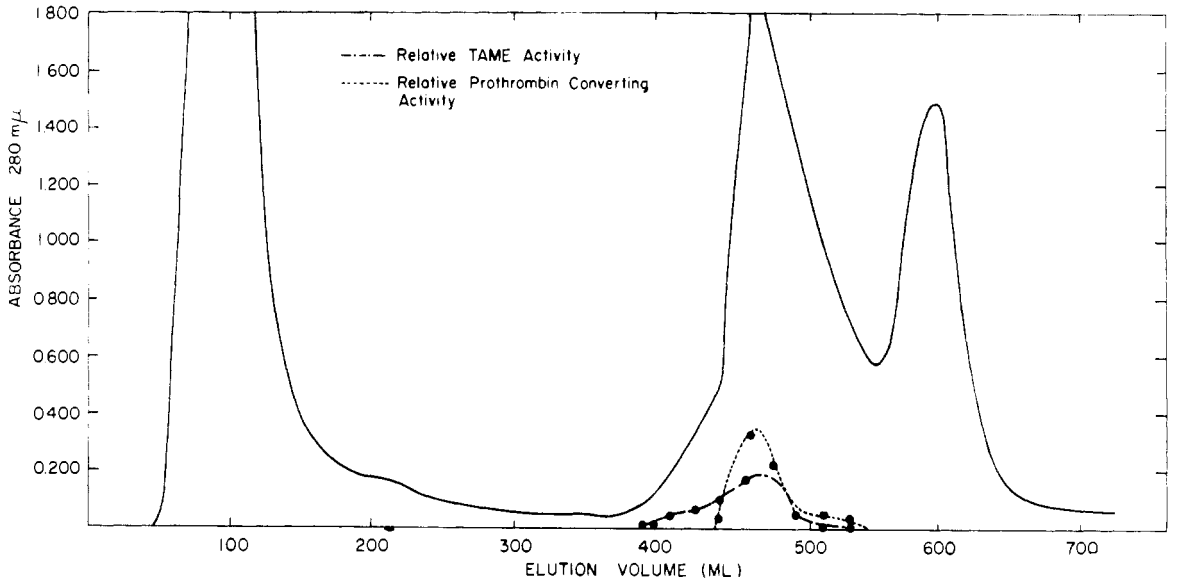

FIGURE 2: Elution of human thrombokinase from DEAE-cellulose.

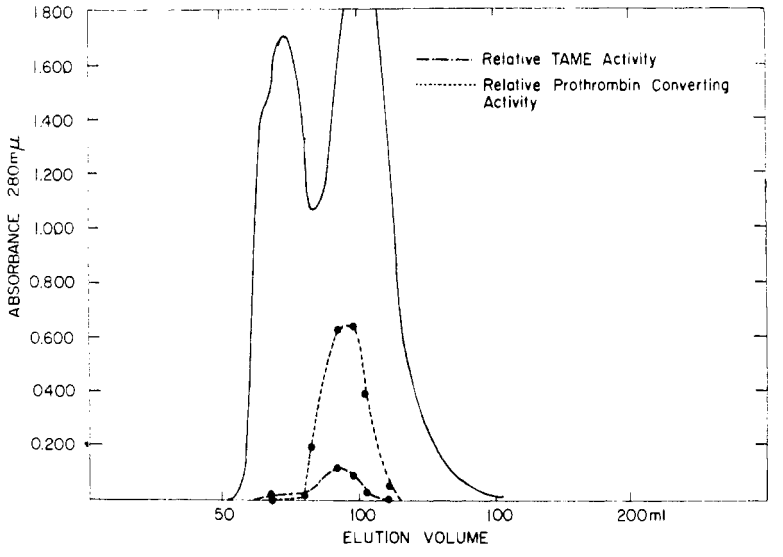

FIGURE 3: Elution of human thrombokinase from G-100 Sephadex.

amide gels at pH 8.3 with Tris–glycine buffer (Davis, 1964).

Experimental Results

The following experiments were all done using a single preparation of thrombokinase but two different batches of prothrombin, adjusted to the same concentration. To a solution of prothrombin (containing 5000 units/ml of 0.15 M NaCl–0.02 M cacodylate, pH 5.95) was added 0.1 volume of the thrombokinase (in 0.1 M Tris–Cl, pH 8.1). The pH of this mixture was adjusted to 7.5 by the addition of 1 N NaOH, and the solution was allowed to incubate at room temperature (22–24°). Controls consisted of prothrombin plus buffer.

Figure 4 shows the chromatographic pattern obtained on DEAE-cellulose after prothrombin had undergone 0.5-, 4-, and 12-hr incubations with thrombokinase, as well as a 4-hr control incubation. In all cases the treated prothrombin was added to the column immediately after incubating. At the time the samples were placed on the column they contained 20, 264,

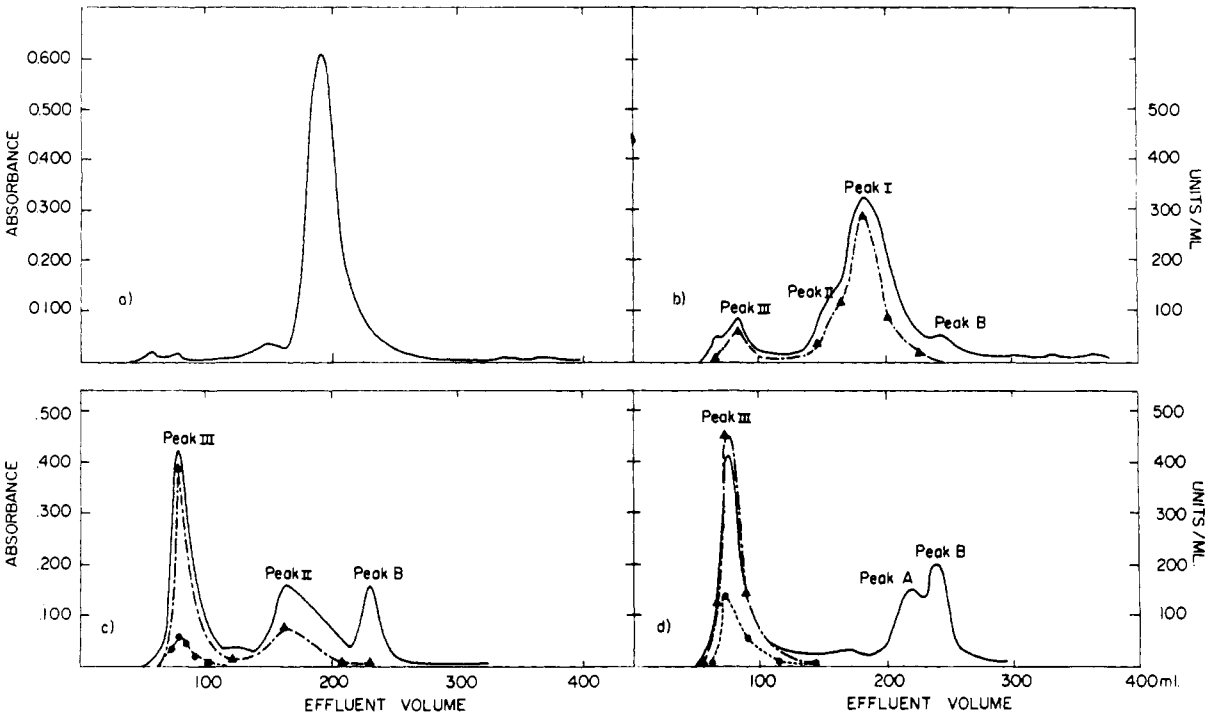

FIGURE 4: DEAE chromatographic patterns of prothrombin treated with thrombokinase for varying times. The following symbols are used: ———— A_{280}; ——·——·—— two-stage activity, *i.e.*, prothrombin plus thrombin; - - - - - - - thrombin activity; (a) 4 hr of incubation in the presence of buffer; (b) 0.5 hr of incubation with thrombokinase; (c) 4 hr of incubation with thrombokinase; (d) 12 hr of incubation with thrombokinase.

and 2100 units of thrombin/ml, corresponding to 0.4, 6, and 47% conversion to thrombin, respectively.

During the conversion of prothrombin, two new chromatographic peaks with prothrombin activity appeared. The three prothrombin peaks have been numbered I–III in inverse order of their elution. There were also two peaks which appeared during this reaction that had neither prothrombin nor thrombin activity. These have been labeled A and B, respectively (see Figure 4). The sum of the thrombin activity plus the prothrombin activity remained constant during the course of these experiments.

The untreated prothrombin was eluted in peak I at a volume of about 190 ml after starting the gradient (Figure 4a). The prothrombin used for the 0.5- and 12-hr incubations showed a small amount of chromatographic heterogeneity, whereas the material used for the 4-hr sample was homogeneous both at the start of the experiment and after the 4-hr incubation at room temperature in the absence of thrombokinase (Figure 4a). There was no increase in the heterogeneity of the prothrombin preparations during standing at room temperature.

With the addition of thrombokinase to the prothrombin there was, even in 0.5 hr, a detectable change in the chromatographic pattern. The most obvious change was the appearance of a sizable peak II which is manifested (Figure 4b) as a shoulder on the ascending side of peak I. There was a shoulder after peak I that was presumably a small amount of peak B. A detectable amount of material was also seen in the region of peak III though this is a small fraction of the total material at this incubation time.

After 4 hr there were more marked changes in the elution pattern (Figure 4c). The original prothrombin peak (peak I) had disappeared. The early peak (peak III) now contained a large portion of the optical density and prothrombin activity as well as all the thrombin activity. The shoulder appearing late in the elution pattern of the 0.5 hr sample had developed into a full-fledged peak (peak B) containing neither thrombin nor prothrombin activity.

After 12 hr of incubation with thrombokinase (Figure 4d) a stable chromatographic pattern had developed even though only 47% of the potential thrombin activity had been generated. There was a single peak (peak III) containing the thrombin and the prothrombin activity. This peak accounted for about 50% of the optical density eluted from the column. The rest of the optical density was found in two peaks (A and B) that were seen developing in the earlier chromatographs.

In order to see if this pattern was specific for the conversion by thrombokinase or was seen with other

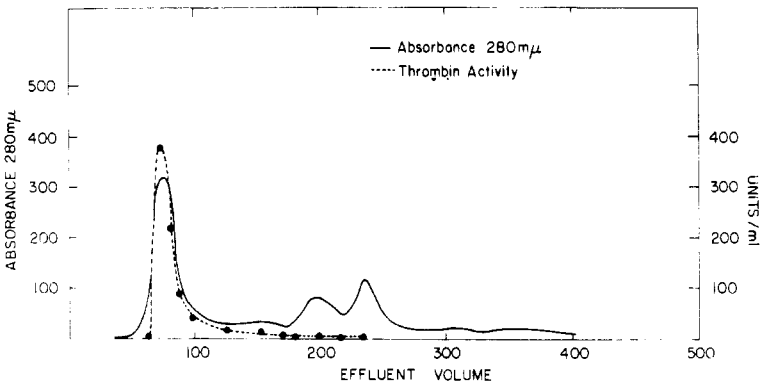

FIGURE 5: Elution pattern of prothrombin activated with plasma phospholipid reagent.

prothrombin-converting agents, a reagent was made as follows. Inosithin[10] was soaked in plasma (10 mg/ml) for 10 min and centrifuged, and the sediment was washed twice with 0.15 M $MgCl_2$. The precipitate was resuspended in 0.15 M $CaCl_2$, to the original plasma volume. This material (1 volume) was added to 5 volumes of the prothrombin. After 6 hr of incubation with this preparation there was complete conversion to thrombin. Despite this important difference, the elution pattern was comparable (Figure 5) with that of prothrombin partially converted to thrombin with the thrombokinase (Figure 4d). Acrylamide disc electrophoresis of the peaks A and B from the plasma conversion and the thrombokinase conversion showed no difference in the characteristics of these derivatives.

In all cases the stable peaks have lower sedimentation coefficients than those reported for prothrombin (Lamy and Waugh, 1953; Lanchantin *et al.*, 1965). Table I gives the physical data for peaks III, A and B, in 0.15 M NaCl–0.02 M Tris, pH 7.4. The material used for the ultracentrifuge studies was derived from thrombokinase treated prothrombin. The prothrombin/thrombin ratio for the peak III material was 2/1.

TABLE I: Physical Properties of Prothrombin Fragments.

Peak	$D_{A_{app}}$ (cm^2/sec)	$s_{20,Buffer}$ ($\times 10^{13}$ sec)	Estd Mol Wt
III	$11. \times 10^{-7}$	3.4	31,800
A	9.1×10^{-7}	2.26	26,300
B	$11. \times 10^{-7}$	1.68	16,600

The presence of both prothrombin and thrombin in the early peak prompted us to raise the question of whether both of these activities reside in the same molecule. To answer this question an aliquot of this material, containing 200 units/ml of thrombin and 800 units/ml of prothrombin, was incubated for 2 hr with 10^{-3} M DFP at pH 8.0. During the incubation period the pH was maintained constant by automatic titration with a Radiometer pH-Stat. After 2 hr there was no more acid release and there was no thrombin activity (clotting time greater than 0.5 hr). There was no change in the prothrombin activity implying that two molecular species were present in peak III. No attempt was made to remove the hydrolysis products of DFP.

Discussion

Even though the prothrombin preparation used in these studies had a specific activity 500–700 times that of plasma, it still contained other coagulation components. This was surmised since the addition of Russell viper venom (RVV)[11] to the prothrombin produces large amounts of thrombin and the type of chromatographic changes seen above. This presumably means that the preparation of prothrombin contains inactive Factor X (Esnouf and Williams, 1961). Even in the absence of RVV there is spontaneous production of thrombin after several days at room temperature.

The method of Milstone (1949, 1959a) for the preparation of bovine thrombokinase was used as a guide for the preparation of the human material. Since the human plasma contains citrate, barium sulfate cannot be used for adsorption; hence the barium chloride method of Lewis and Ware (1953) has been used. Differential elution of the prothrombin and the thrombokinase from the barium citrate precipitate has not been achieved. In contrast to the bovine thrombokinase, the preparation described could not be precipitated by lowering the pH.

The final product appears similar to the active Factor X preparation of Esnouf and Williams (1961), and to the autoprothrombin C of Marciniak and Kowarzyk (1961) and Marciniak and Seegers (1962). However, judging by both the TAME and the prothrombin-converting ability, the human thrombokinase would

[10] Soy bean phospholipid supplied by Associated Concentrates, Woodside, N. Y.

[11] Obtained from Burroughs Wellcome and Co., Tuckahoe, N. Y.

seem to have about 2% of the specific activity of Milstone's bovine material (Milstone, 1959b).

The thrombokinase preparation contained a small amount of thrombin. Treatment with 10^{-3} M DFP removes the thrombin activity with no alteration of the prothrombin activating property. There was no detectable Factor V activity.

Incubation of human prothrombin with human thrombokinase yielded two products which differ chromatographically from the original prothrombin but which contain prothrombin activity. One such chromatographic peak with prothrombin activity has been reported by Magnusson (1961) and Asada *et al.* (1961), and a second has been found in this laboratory (Aronson, 1966). These have been detected in prothrombin preparations to which no thromboplastic materials have been added.

The formation of each of these prothrombin peaks seems to be associated with the release of a nonactive fragment, *viz.*, B or A. The peak III prothrombin was eluted from DEAE along with thrombin and seems to be almost the same molecular size as thrombin. While the thrombin has not been separated from the peak III prothrombin by means of DEAE-cellulose, the results after treatment with DFP indicate that there are two different molecular species.

Despite the impurity of the preparations used, the fact that the same chromatographic pattern is obtained with Russell viper venom, a crude plasma phospholipid reagent, and the highly purified bovine thrombokinase of Milstone[12] is evidence that the derivatives and fragments formed in the above experiments are not the products of nonspecific proteolysis. The experiments of Lanchantin *et al.* (1965) on the "citrate" activation of human prothrombin also indicate several products without thrombin activity.

One possible reaction sequence would be:

$$\text{peak I} \longrightarrow \text{peak II} + \text{B}$$
$$\text{peak II} \longrightarrow \text{peak III} + \text{A}$$
$$\text{peak III} \longrightarrow \text{thrombin} + ?$$

The question of whether all these reactions are obligatory cannot be answered.

[12] Generously supplied by Dr. J. H. Milstone, New Haven, Conn.

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