Action of Water-Insoluble Trypsin Derivatives on Prothrombin and Related Clotting Factors*

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ABSTRACT: The action of two water-insoluble derivatives of trypsin, IPTT and IMET, on factor II (prothrombin) is described. The water-insoluble polytyrosyltrypsin (IPTT) was prepared by coupling polytyrosyltrypsin with a polydiazonium salt derived from a copolymer of *p*-aminophenylalanine and leucine. The water-insoluble maleic acid-ethylenetrypsin (IMET) was prepared by coupling trypsin with a copolymer of maleic anhydride and ethylene. During interaction with IPTT, factor II progressively disappeared while thrombin evolved. Factors VII and X present in the factor II preparation used were concurrently activated. Factor II could also be degraded by IMET. In this case, however, no thrombin appeared. Factor X was activated by IMET similarly to IPTT although to a lesser extent. No activation of factor VII by IMET occurred. Under the experimental conditions used thrombin could be readily degraded by IMET but not by IPTT or native trypsin. The changes recorded in the biological activity of factor II caused by IPTT or IMET were accompanied by corresponding alterations in the electrophoretic patterns of the reaction mixtures.

The complex sequence of blood clotting involves several steps which are enzymatic (Macfarlane, 1964; Davie and Ratnoff, 1964). Inert zymogens are converted to active enzymes, which in turn activate other constituents. A notable example is the conversion of factor II (prothrombin) to thrombin, a major step in this sequence. There are at least five pathways by which this may be induced: (1) in the course of spontaneous coagulation, *via* the elaboration of blood (intrinsic) thromboplastin from several sequentially interacting factors; (2) by interaction of factor II with tissue thromboplastin, Ca^{2+}, and other clotting factors; (3) by certain proteolytic enzymes; (4) by certain anions; (5) by polylysine and related compounds (for review see Alexander, 1958; Ferguson *et al.*, 1960; Alexander, 1962; Macfarlane, 1964; Davie and Ratnoff, 1964; Miller *et al.*, 1961). To what extent these mechanisms are identical with, or simulate one another, or what fundamental alteration in factor II is necessary for its activation, is obscure.

Progress in purification of factor II and availability of purified proteases led to extensive investigation of the third pathway, with the view that this approach would help elucidate the structure of this factor and its activation (Alexander and Pechet, 1962). Of many proteolytic enzymes studied, only trypsin and papain produce thrombin from factor II (Alexander, 1958). The others were either inert, or they progressively inactivated the zymogen. In contrast to papain, which apparently requires the presence of factor VII (proconvertin), factor X (Stuart factor), or both, trypsin produces thrombin directly from factor II (Alexander, 1958; Alexander and Pechet, 1958). Because of this, and the well-known ability of trypsin to activate other protease precursors (trypsinogen, chymotrypsinogen, and plasminogen), its effect on factor II and other clotting factors was studied intensively (Pechet and Alexander, 1960). The availability of water-insoluble trypsin derivatives provided an additional tool for exploring factor II activation. Two derivatives were used: (a) water-insoluble maleic acid-ethylenetrypsin (IMET)[1] (Levin *et al.*, 1964) in which trypsin is coupled to a copolymer of maleic acid and ethylene; (b) water-insoluble polytyrosyltrypsin (IPTT) (Bar-Eli and Katchalski, 1963) obtained by coupling polytyrosyltrypsin with a polydiazonium salt derived from a copolymer of *p*-aminophenylalanine and leucine.

Since by virtue of their insolubility these trypsin derivatives can be removed at will, they were used to explore various stages of the activation process. It was also deemed of interest to compare the action of IMET and IPTT on factors II, VII, and X, since it has been shown by Levin *et al.* (1964) that these trypsin derivatives act quite differently on lysozyme. Our results

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[1] Abbreviations used: IPTT, water-insoluble polytyrosyltrypsin; IMET, water-insoluble maleic acid-ethylenetrypsin.

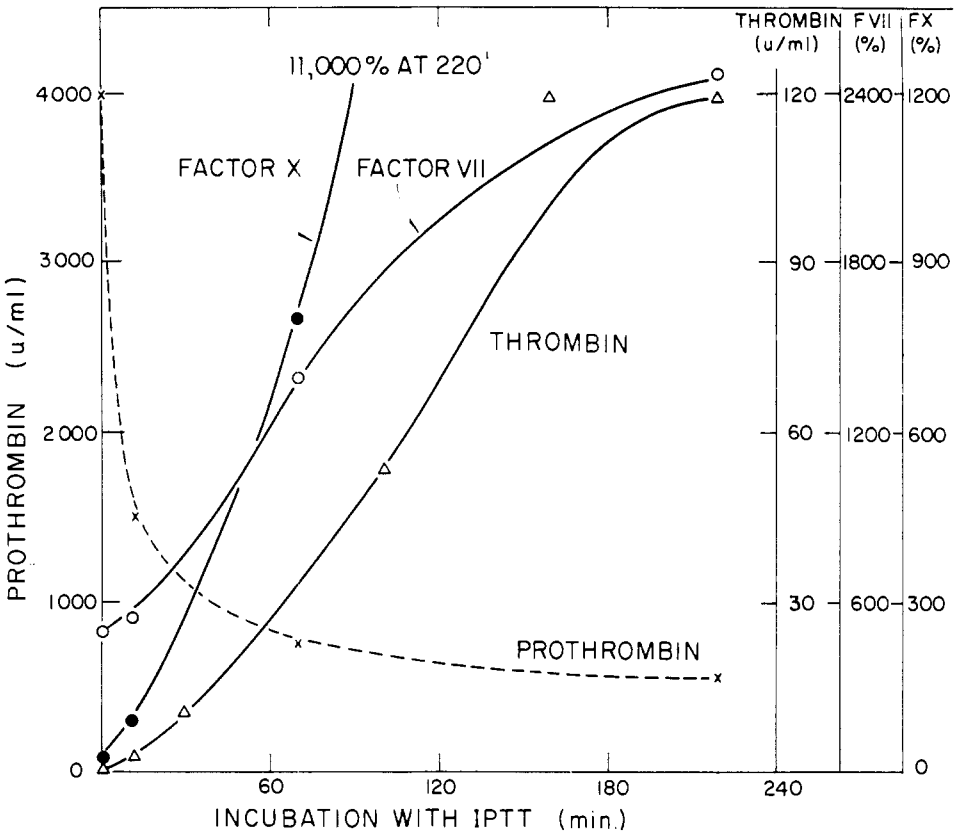

FIGURE 1: Changes in concentration of factor II, VII, X, and thrombin during incubation of a factor II preparation with IPTT under the conditions specified in the test.

indicate that in some respects they also act differently on factor II and related clotting factors.

Materials

Factor II was prepared from oxalated bovine plasma by the method of Goldstein *et al.* (1959), and kept lyophilized. The dry powder obtained by lyophilization from a saline solution of the material contained 65% protein with a factor II activity of 950 units[2] per mg of protein. It also contained some factors VII and X activity but was devoid of detectable factor V. Thrombin was obtained from Upjohn Co. Twice-crystallized salt-free lyophilized trypsin was obtained from Worthington Biochemical Corp.

IPTT was prepared by coupling polytyrosyltrypsin with a water-insoluble diazonium salt derived from a copolymer of *p*-amino-DL-phenylalanine and L-leucine (molar residue ratio 1:1), according to Bar-Eli and Katchalski (1963). The proteolytic activity of the IPTT obtained, containing about 20 mg of protein per 100 mg of water-insoluble enzyme, was assayed on casein as described by Northrop *et al.* (1948). IPTT (311 mg) had the activity of 1.0 mg of native trypsin. A stock suspension of IPTT in 0.1 M phosphate buffer, pH 7.4, possessing a caseinolytic activity of 23 μg of native trypsin per ml of suspension, was prepared and stored at 4°.

IMET was prepared by coupling trypsin with ethylene-maleic anhydride copolymer, obtained from Monsanto Chemical Co., according to the procedure of Levin *et al.* (1964). The copolymer to enzyme ratio used was 1:4 (w/w). The preparations contained about 74 mg protein (Kjeldahl) per 100 mg of the water-insoluble preparation dried to constant weight at 100°. An amount of 197 mg of IMET showed a caseinolytic activity of 1.0 mg of trypsin. A stock suspension of IMET in 0.1 M phosphate buffer, pH 7.4, possessing a caseinolytic activity of 23 μg of soluble trypsin per ml of suspension, was prepared and stored at 4°.

Methods

Assay of Thrombin. This was done by measuring the clotting time with bovine fibrinogen, determined at 37° in glass test tubes (8 × 75 mm) of uniform batch. An amount of 0.2 ml of a 2% solution of purified fibrinogen (91–98% clottable) (Laki, 1951) in 0.15 M NaCl was mixed with 0.2 ml of the thrombin solution to be tested. The tubes containing the combined ingredients were agitated for 15 sec, and gently tipped from time to time thereafter. The interval, recorded with a stop watch,

[2] Normal plasma contains approximately 250 units/ml, as determined by the method of Ware and Seegers (1949).

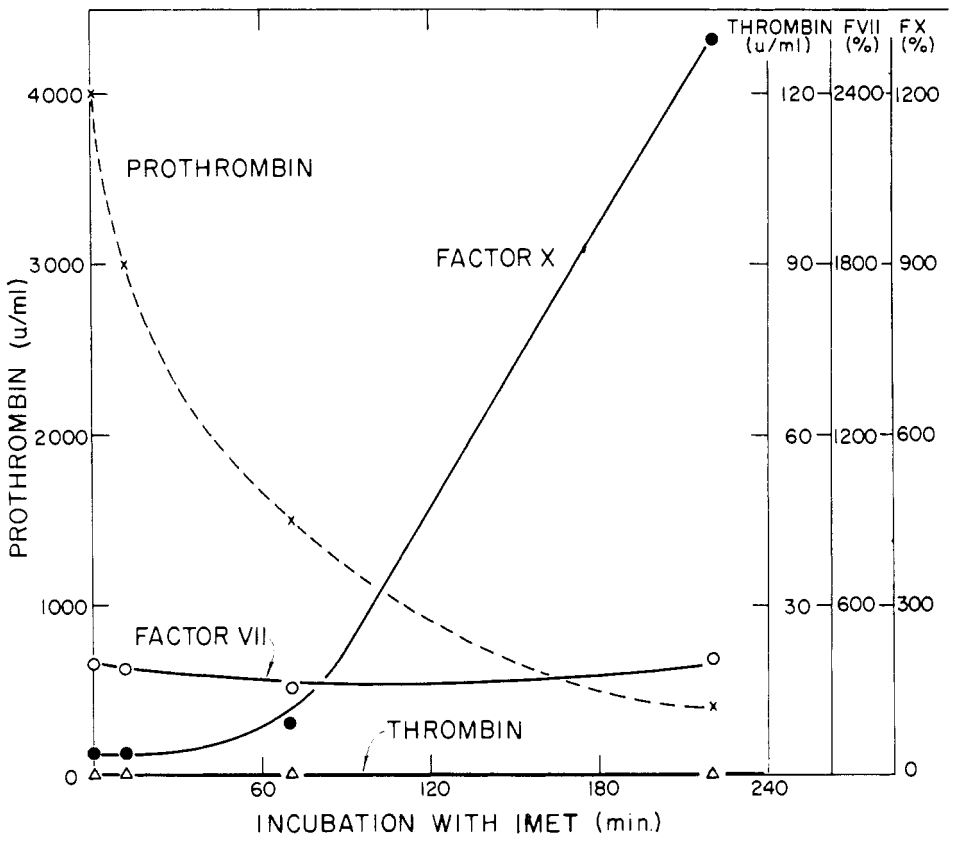

FIGURE 2: Changes in the concentration of factors II, VII, X, and thrombin during incubation of the factor II preparation used in Figure 1 with IMET under the conditions described in the text.

between the admixture and visible solid gelation was taken as the clotting time. The amount of thrombin present was interpolated from a calibration curve obtained under the same conditions with a known amount of thrombin, and was expressed in thrombin units.

Assay of Factors II, VII, and X. Factor II was measured by the two-stage method of Ware and Seegers (1949) as modified by Alexander and Goldstein (1955). Factor VII was assayed according to Owren (1953). Factor X was determined according to Bachmann *et al.* (1958).

Activation of Factor II. A solution of 200 mg of factor II in 20 ml of 0.025 M phosphate buffer pH 7.4 was prepared. Four ml was removed for control measurements, and the rest was incubated at 37° with 0.5 ml of the stock suspension of IPTT or IMET. Aliquots were withdrawn at various time intervals, filtered through Whatman No. 1 paper (for IPTT) or through a Millipore filter (Millipore Corp. HA 0.45 μ) (for IMET), and the filtrates were assayed for thrombin and factors II, VII, and X, and subjected to ultracentrifugation and electrophoretic measurements, as described below.

Sedimentation Measurements. Sedimentation runs were made in a Spinco Model E ultracentrifuge using a 12-mm Kel-F cell with 4° sector. Temperature was maintained constant during each run in the range of 21–23°.

Electrophoresis. Free boundary electrophoresis was performed in a Perkin-Elmer electrophoresis apparatus Model 38 A using the standard 2-ml cell in Veronal buffer at pH 8.6 and ionic strength 0.1.

Results

Enzymatic Studies. Incubation of purified factor II with IPTT under conditions given in Methods resulted in thrombin elaboration, as well as marked enhancement in the activities of factors VII and X that were present in the preparation (Figure 1). Noteworthy is the early sharp drop in two-stage factor II activity some time before the appearance of substantial amounts of thrombin. Under the experimental conditions used, 63% of the prothrombin had disappeared after 10 min of interaction but only trace amounts of thrombin were demonstrable. Thereafter, thrombin progressively appeared. The activities of both factors VII and X were greatly enhanced, the latter with an initial value of 33%,[3] attaining a level of 11,000% after 220 min of interaction with IPTT.

The effect of IMET, incubated with factor II under

[3] In terms of normal plasma which is arbitrarily defined as containing 100% factors VII and X.

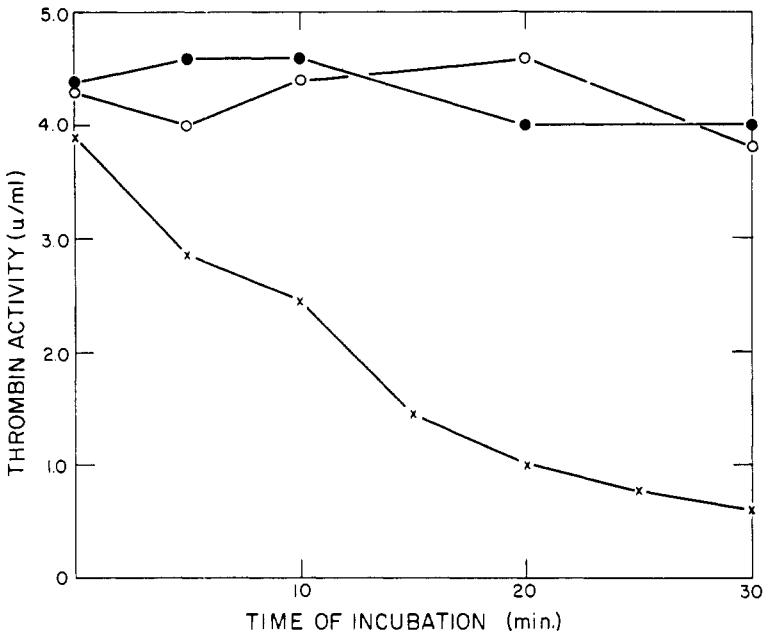

FIGURE 3: Changes in thrombin activity during its incubation with soluble trypsin (●—●), IPTT (○—○), or IMET (x—x). Incubation was run in phosphate buffer, 0.1 M, pH 7.4, at 25°. Concentration of enzyme 2 μg/ml of native trypsin and an equivalent caseinolytic activity of IPTT or IMET.

similar conditions, was quite different (Figure 2). Although factor II activity declined rapidly as with IPTT, no thrombin could be detected. As with IPTT, IMET also activated factor X, but to a much lesser extent, to a value of 1300% after 220 min of incubation. In striking contrast to the effect of IPTT, factor VII appeared to be unaffected by IMET.

Conceivably the early disappearance of factor II activity might have been referable to the elaboration of a by-product during conversion of factor II to thrombin, which, in turn, might have adversely affected the quantitative assay for thrombin. The following experiment excluded this possibility. Factor II (36 mg) was dissolved in 3.0 ml of 0.02 M phosphate buffer, pH 7.0. To this was added IMET sedimented from 2.0 ml of the stock IMET suspension. The mixture was incubated for 1 hr at 37°, and then filtered through a Millipore pad (0.22 μ). This is referred to as IMET-factor II digest. To this was added varying amounts of commercial thrombin (Parke, Davis), and the mixture was assayed for clotting activity of thrombin on fibrinogen (see Methods). As a control, the IMET-factor II digest was replaced by saline. No significant difference was observed between the two thrombin–fibrinogen calibration curves correlating thrombin activity with clotting time.

The question arose as to whether IMET failed to activate factor II, or whether it degraded thrombin as fast as it may have evolved. To explore this point, thrombin was incubated for 30 min at 37° with IMET, with IPTT, and with native trypsin, respectively, in amounts equivalent in caseinolytic activity. The results indicated that thrombin was progressively inactivated by IMET but not by IPTT or soluble trypsin (Figure 3). If incubation was extended for several hours, or if the enzyme concentration was increased, thrombin inactivation also occurred with both IPTT and native trypsin. Nevertheless, IMET was clearly more effective in this regard. It would thus appear that the failure to detect thrombin after the interaction of factor II with IMET could be explained by the prompt destruction of thrombin as it evolved. Indeed, in some experiments where high concentrations of factor II were incubated with IMET, trace amounts of thrombin were found.

Sedimentation and Electrophoretic Studies. The changes induced in factor II by IPTT and IMET were followed by observations in the ultracentrifuge and in free boundary electrophoresis. Figures 4 and 5 show the ultracentrifugal patterns of the factor II preparations exposed to IPTT or IMET. The preponderant component is reflected in a distinct peak with an average $s_{20,w}$ value of 4.57. Adjoining it was a small shoulder representing material that sedimented faster. This pattern did not change appreciably during 4 hr of incubation with either IPTT or IMET, except that in the case of IPTT the sedimentation constant of the major component decreased from 4.57 to 4.24, and in the case of IMET from 4.62 to 4.05.

The electrophoretic patterns are shown in Figures 6 and 7. The profile of the starting material, the prothrombin-rich fraction, reveals a main peak exhibiting an electrophoretic mobility of 5.5×10^{-5} cm²/v/sec, and two lesser components with mobilities of 2.2×10^{-5} cm²/v/sec and 4.1×10^{-5} cm²/v/sec, respectively. After 12 min of incubation with either enzyme derivative, and more profoundly after 70 min, the electrophoretic

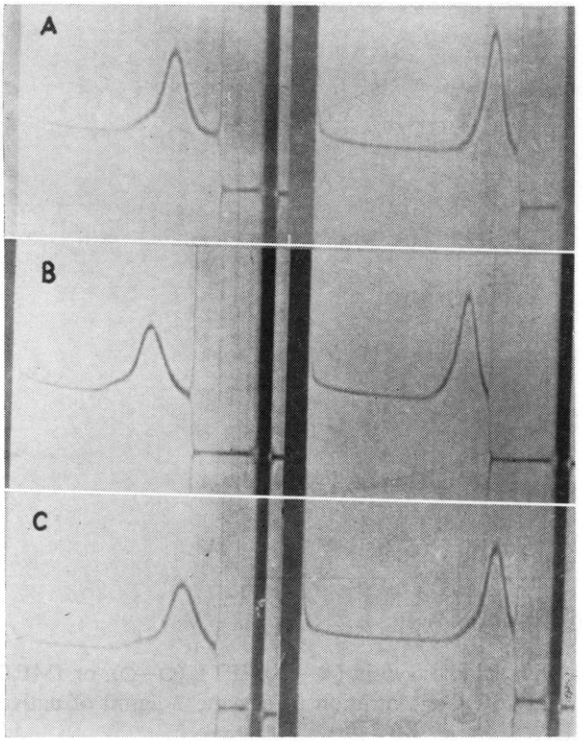

FIGURE 4: Sedimentation pattern of untreated factor II preparation, and after incubation for different time intervals with IPTT. (A) Control experiment: time of incubation, zero; the figure on the right taken 16 min, that on the left 32 min after rotor reached maximum speed. (B) Incubation with IPTT for 12 min. Figure on the right sedimentation for 16 min, that on the left for 32 min. (C) Incubation with IPTT for 70 min. Right sedimentation for 16 min, left for 32 min. Bar angle 60°, rotor speed 59,000 rpm, temperature 20.3°. Sedimentation to the left.

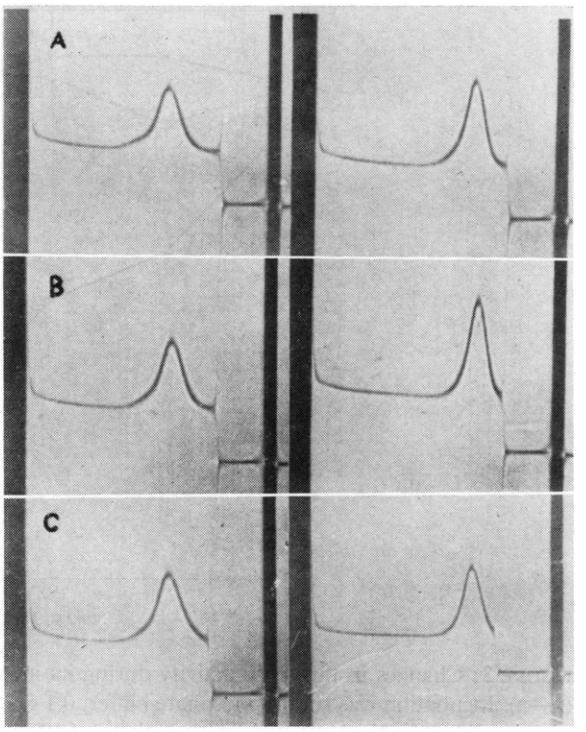

FIGURE 5: Sedimentation pattern of untreated factor II preparations, and after incubation for different time intervals with IMET. Details as for Figure 4.

pattern had altered completely. With IPTT (Figure 6), 4 peaks were observed with mobilities of 1.9×10^{-5} (A), 3.9×10^{-5} (B), 5.6×10^{-5} (C), and 6.1×10^{-5} $cm^2/v/sec$ (D). The mobility of the fastest moving component (D) was identical with that of thrombin prepared from the activation of the same prothrombin preparation with 0.85 M citrate solution (Seegers *et al.*, 1950), and run under similar conditions. Peak C probably represents the remaining factor II. Peaks A and B probably reflect contaminating proteins (perhaps factors VII and X) which are present in the fraction, and which appear in the starting material. Their areas, however, increased considerably during incubation with the enzyme.

With IMET (Figure 7), no elaboration of a fast moving entity is observed. Also as already mentioned, no detectable thrombin appears. Moreover, the area of the original major peak with 5.5×10^{-5} mobility progressively decreases, the peak of 4.1×10^{-5} disappears, and a new peak appears with a mobility of 3.3×10^{-5}, which is probably a degradation product derived from factor II by IMET.

Discussion

On the basis of previous experience with native trypsin, one would expect that the water-insoluble trypsin derivatives would activate factor II as well as factors VII (Alexander, 1958; Pechet and Alexander, 1960; Alexander *et al.*, 1962) and X (Pechet and Alexander, 1960; Alexander and Pechet, 1962; Yin,1964; Papahadjopoulos *et al.*, 1964). The data are generally in accord with earlier observations.

In scrutinizing the kinetics of factor II activation by IPTT, one is impressed by the fact that biologically demonstrable factor II rapidly disappears before a substantial amount of thrombin appears. The same phenomenon has been previously observed with soluble trypsin (Alexander, 1962), and also during the activation of factor II preparations by citrate (Seegers *et al.*, 1950; Alexander, 1962). It further confirms the view that in the transition of factor II to thrombin an intermediate substance is produced prior to the appearance of thrombin. This intermediate compound might represent the fast moving component seen in the electrophoretic pattern after 12 min of factor II incubation with IPTT (Figure 6, peak D), possessing the same electrophoretic mobility as thrombin, run under the same conditions.

In contradistinction to the results obtained with IPTT, no detectable thrombin evolved on incubating factor II with IMET. Since factor II disappeared from the reaction mixture (Figure 2), and since thrombin was readily destroyed by IMET (Figure 3), it is reason-

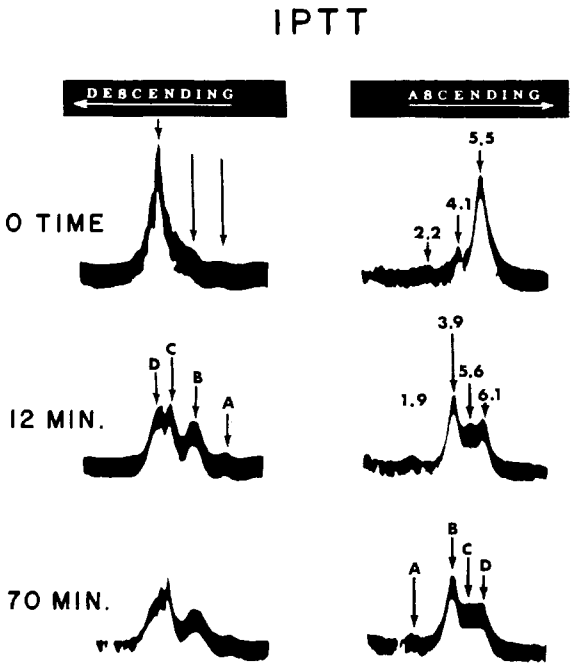

FIGURE 6: Electrophoretic patterns of untreated factor II preparation, and after 12 min and 70 min of incubation with IPTT under the conditions described in text. Protein concentration 0.65%, Veronal buffer, pH 8.6, ionic strength 0.1, current 8 ma. Photographs taken 90 min after beginning of the run. Numerals and letters described in text.

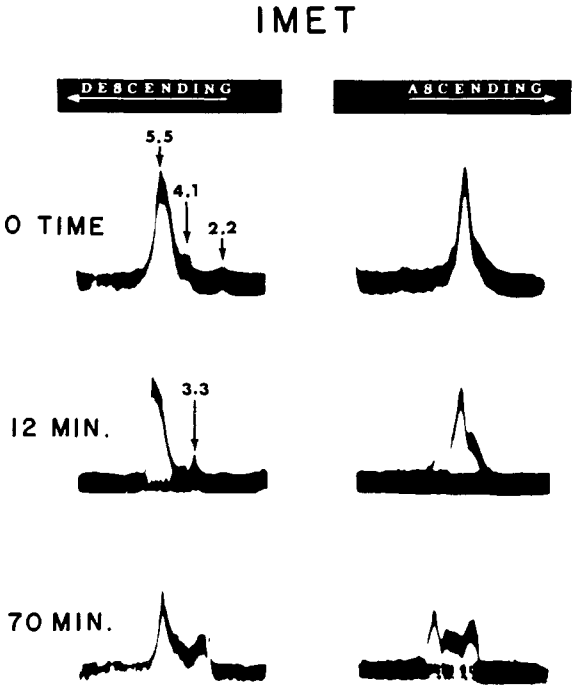

FIGURE 7: Electrophoretic patterns of factor II preparation before and after incubation with IMET. Details as for Figure 6.

able to assume that some thrombin was evolved, but it was degraded as fast as it formed. As a matter of fact, when high concentrations of factor II were interacted with IMET, traces of thrombin could be detected in the mixture. Nevertheless, thrombin elaboration by IMET at a rate that was sufficiently rapid to assure survival of some of it may have required activation of factor VII by IMET, which did not occur. This possibility is supported by the observation that the activation of factor VII and X by trypsin enhances thrombin formation from factor II (Alexander and Pechet, 1962; Ferguson *et al.*, 1960). Requirement for factor VII has already been established for thrombin formation *via* the citrate activation pathway (Alexander, 1958; Goldstein *et al.*, 1959; Streuli, 1959; Alexander, 1962; Deutsch and Lechner, 1964), or with papain (Alexander, 1958; Alexander and Pechet, 1958). Moreover, prior activation of factor VII by trypsin profoundly accelerates subsequent factor II conversion by trypsin (Alexander and Pechet, 1961; Alexander, 1962).

The electrophoretic patterns of factor II treated with IPTT or IMET (Figures 6 and 7) clearly reveal the gradual transformation of prothrombin to new molecular entities. No definite assignment can be given at this stage to the various peaks noted. A comparison of Figure 6 with Figure 7 shows, however, that different products are formed on incubation of factor II with IPTT or with IMET. It is of interest that the alteration in biological activity and electrophoretic pattern recorded are not accompanied by any marked changes in the sedimentation schlieren profile (see Figures 4 and 5).

IPTT and IMET showed striking differences also in their action on factors VII and X. Whereas both factors are greatly activated by IPTT, only factor X is activated by IMET, and this to a lesser extent than with IPTT (Figures 1, 2). These noteworthy differences in two preparations derived from the same enzyme are most likely attributable to the different nature of the polymer carriers used for the preparation of these water-insoluble trypsin derivatives. In the case of IPTT the trypsin is bound to a neutral carrier possessing many hydrophobic groups, whereas in IMET the bound trypsin molecules are surrounded at neutral pH by many negatively charged hydrophilic carboxylic groups. It might, therefore, be expected that the affinity of the water-insoluble enzyme derivatives for susceptible amide bonds in a high molecular weight substrate would be different for IMET and IPTT. Peptide bonds of factors II, VII, and X essential for biologic activity might therefore show different susceptibility to IPTT or IMET.

The above consideration might be extended to explain the selective specificity toward high molecular weight substrates of enzymes such as trypsin, thrombin, and plasmin possessing the same activity toward low molecular weight substrates such as tosyl- or benzoyl-arginyl esters. Assuming identical catalytic sites in these enzymes, one has to postulate that the ultimate speci-

ficity of the enzymes toward their corresponding protein substrate is predicated upon a high affinity between distinct noncatalytic sites of the enzymes and well-defined regions of the high molecular weight substrates.

References

Alexander, B. (1958), Fourth International Congress of Biochemistry, Vol. 10, Vienna, Austria, p 37.

Alexander, B. (1962), *Thromb. Diath. Haemorrhag. 9*, Suppl. 2, 179.

Alexander, B., and Goldstein, R. (1955), The Coagulation of Blood. Methods of Study. L. M. Tocantins, Ed., New York, Grune and Stratton, pp 90–97, 141–148.

Alexander, B., and Pechet, L. (1958), *Federation Proc. 17*, 179.

Alexander, B., and Pechet, L. (1962), Proceeding of the VIIIth Congress of European Society of Hematology. Vol. II, p 404.

Bachmann, F., Duckert, F., and Koller, F. (1958), *Thromb. Diath. Haemorrhag. 2*, 24.

Bar-Eli, A., and Katchalski, E. (1963), *J. Biol. Chem. 223*, 1690.

Davie, E. W., and Ratnoff, O. D. (1964), *Science 145*, 1310.

Deutsch, E., and Lechner, K. (1964), Xth Congress of the International Society of Haematology, pG:19.

Ferguson, J. H., Wilson, E. G., Iatridis, S. G., Rierson, H. A., and Johnston, B. R. (1960), *J. Clin. Envest. 39*, 1942.

Goldstein, R., Le Bolloc'h, A. G., Alexander, B., and Zonderman, E. (1959), *J. Biol. Chem. 234*, 2857.

Laki, K. (1951), *Arch. Biochem. Biophys. 32*, 317.

Levin, Y., Pecht, M., Goldstein, L., and Katchalski, E., (1964), *Biochemistry 3*, 1905.

Macfarlane, R. G. (1964), *Nature 202*, 495.

Miller, K. D., Copeland, H. W., and McGarrahaw, J. F. (1961), *Proc. Soc. Exptl. Biol. Med. 108*, 117.

Northrop, J. H., Kunitz, M., and Herriot, R. M. (1948), Crystalline Enzymes, 2nd ed, New York, Columbia University.

Owren, P. (1953), *Am. J. Med. 14*, 201.

Papahadjopoulos, D., Yin, E. T., and Hanahan, D. J. (1964), *Biochemistry 3*, 1931.

Pechet, L., and Alexander, B. (1960), *Federation Proc. 19*, 64.

Seegers, W. H., McClaughry, R. I., and Fahey, J. L. (1950), *Blood 5*, 421.

Streuli, F. (1959), *Thromb. Diath. Haemorrhag. 3*, 194.

Ware, A. G., and Seegers, W. H. (1949), *J. Clin. Pathol. 19*, 471.

Yin, E. T. (1964), *Thromb. Diath. Haemorrhag. 12*, 307.