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Mechanism of Bovine Prothrombin Activation by an Insoluble Preparation of Bovine Factor X_a (Thrombokinase)†

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ABSTRACT: Sepharose-factor X_a (TK-resin) has been prepared and used for the investigation of the generation of thrombin from a purified bovine prothrombin. Thrombin generation was followed by assay for clotting activity and by disc gel electrophoresis for reaction products. Under the conditions employed (25% w/v sodium citrate, pH 6.5, 24°) prothrombin activation is essentially complete (80–100%) when compared to a two-stage activation assay. Autocatalysis could not be demonstrated either by withdrawing TK-resin before complete activation or by adding activation product to fresh prothrombin. Prothrombin in the presence of 25% sodium citrate alone, in the presence of activation products, or in the presence of thrombin does not generate clotting activity; however, prothrombin in the presence of TK-resin, with or without sodium citrate, will produce clotting activity. The complex activation pattern as observed by disc gel electrophoresis is best explained by two activation pathways: one initiated by factor X_a, and the other by thrombin. The factor X_a pathway leads from prothrombin to a single-chain molecule, P_3, plus an intermediate, F_X, which (in the presence of thrombin) rapidly decomposes. The thrombin-initiated pathway leads from prothrombin to a fragment, F_A, and an intermediate, P_2. The latter is further broken down to two single-chain molecules: a fragment F_B and an intermediate P_3. Both pathways appear to converge to the same thrombin precursor, P_3. Thrombin forms from P_3 only in the presence of factor X_a. The double-pathway mechanism was tested by reacting prothrombin (1) with TK-resin in the absence of thrombin (in the presence of $0.0079 M$ DFP) and (2) with thrombin in the absence of TK-resin. Results from these experiments and the estimated molecular weights of the intermediates are consistent with the proposed mechanism. The implications of a double pathway in physiological prothrombin activation are discussed.

Central to the phenomenon of hemostasis is the activation of prothrombin to thrombin, which catalyzes the polymerization of fibrinogen and thus forms the definitive blood clot. Despite its importance and an intensive investigative effort over many years the activation mechanism remains incompletely understood. This is due in part to the difficulty in preparation and stabilization of the zymogen and its intermediates and to the apparent complexity of the pathway. The chemistry of prothrombin and thrombin and a discussion of proposed activation mechanisms have been reviewed recently (Magnusson, 1971). As discussed by Magnusson it is generally accepted that bovine prothrombin, a single-chain plasma glycoprotein without clotting activity, of apparent molecular weight 68,000–74,000, liberates a double-chain serine protease with clotting activity, having a molecular weight of 33,700–40,000. From these facts one may assume that the activation mechanism entails (1) at least two proteolytic cleavages of the zymogen to yield the double-chain enzyme, and (2) the liberation of a large piece(s) of the prothrombin molecule.

Although it is not certain how prothrombin is activated *in vivo*, in the laboratory the conversion of prothrombin to

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thrombin has been produced by multiple means. The most rapid, and apparently most complete, *in vitro* production of thrombin from prothrombin occurs in the presence of a complex of calcium, phospholipid, factor V, and factor X_a[1] (the prothrombinase complex). Since prothrombin can be activated by factor X_a alone albeit slowly, and not by the other components of this system, the important thrombin-yielding agent is held to be factor X_a (Milstone, 1964) while the other components appear to facilitate activation. For many years prothrombin was believed to be activated autocatalytically by thrombin itself. This question was clarified when factor X was demonstrated in prothrombin preparations (Lechner and Deutsch, 1965). Nevertheless, many investigators have shown an effect of thrombin on prothrombin (Asada *et al.*, 1961; Lanchantin *et al.*, 1967–1969; Seegers, 1962) and though these studies did not conclusively demonstrate that thrombin led to thrombin generation, they did show that thrombin alters the electrophoretic and chromatographic properties of prothrombin.

Since Seegers' initial studies (1949) with crude prothrombin, it has been observed repeatedly that prothrombin in high concentrations of sodium citrate plus trace amounts of factor X_a (autoprothrombin C, thrombokinase) will produce thrombin. Such a system has been attractive for experimentation since it involves very few protein components and thus would appear to facilitate interpretation. From activity and electrophoretic studies of this system a molecular mechanism was suggested (Seegers, 1962) in which prothrombin progressed through two intermediates before yielding thrombin. Other experiments with bovine prothrombin in the presence of sodium citrate (Mann *et al.*, 1971b) and human prothrombin in the absence of sodium citrate (Aronson and Menache, 1966) support this general scheme.

Complicating the interpretation of prothrombin activation experiments has been the observation that prothrombin liberates more than one physicochemically distinct molecule with clotting or esterase activity (Lanchantin *et al.*, 1967; Seegers *et al.*, 1968; Mann and Batt, 1969; Batt *et al.*, 1970; Rosenberg and Waugh, 1970; Mann *et al.*, 1971a). This observation led Seegers *et al.* (1969, 1970) to postulate that prothrombin is a dimer of two thrombins. Rosenberg and Waugh (1970) explained the multiple thrombins they found by postulating the existence of at least two prothrombins and various activation pathways. Mann and Batt (1969) and Mann *et al.* (1971a) have ascribed the origin of multiple thrombins to autolysis.

For the last 3 years we have been investigating the chemistry of prothrombin activation. In this initial study we have attempted to develop a simplified system which contained at the outset (1) one soluble protein component, (2) an insoluble activator which could be added or removed at will, and (3) an ionic environment favorable to prothrombin and thrombin stability which allowed essentially complete conversion and minimal non-thrombin-producing side reactions. We have concerned ourselves with the following specific questions. (1) Does prothrombin elaborate a substance which inhibits or causes thrombin production? (2) What are the intermediates of prothrombin activation? (3) What is the mechanism of prothrombin activation?

This is an initial report of experiments with such a system. Activation studies are described which test the generation of thrombin activity and follow the production of intermediates.

The experiments reported here indicate that (1) prothrombin activation by the insoluble activator, TK-resin (at pH 6.5 in 25% trisodium citrate), occurs by one of two pathways: a thrombin-initiated or a factor X_a initiated pathway. (2) Nothing in the soluble activation mixture generates thrombin activity from prothrombin.

Materials

All salts used were of reagent grade purchased from local suppliers. Sodium citrate and sodium dodecyl sulfate were Fisher Certified. Water was doubly distilled. Sephadex G-100, DEAE-Sephadex A-50, Sepharose 4B (Pharmacia, Piscataway, N. J.), and DEAE-cellulose (Cellex D, Bio-Rad, Richmond, Calif.) were prepared as suggested by the manufacturer. Acrylamide (electrophoresis grade), *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylenediamine, riboflavine, and CNBr were obtained from Eastman Organic Chemicals (Rochester, N. Y.) and used as received. Coomassie Brillant Blue was obtained from Schwarz-Mann and Pyronin Y from Fisher. The following proteins, inhibitors, and substrates used were from Schwarz-Mann (Orangeburg, N. Y.): bovine crystallized serum albumin, porcine stomach pepsin, bovine pancreatic ribonuclease A, sperm-whale myoglobin; from Sigma Chemical Co. (St. Louis, Mo.): phosphorylase *a*, soybean trypsin inhibitor, CbzTyrONph, TosArgOMe, and DFP. Inositihin (Associated Concentrates Inc., Woodside, N. Y.), factor VII and X deficient bovine plasma (Sigma), and Russell's viper venom (Burroughs-Wellcome and Co., Tuckahoe, N. Y.) were used in the factor X assay. Cellophane dialysis tubing (Union Carbide Corp., Chicago, Ill.) was brought to a boil in 0.2 M EDTA, rinsed copiously with distilled water, and stored in water at 4°. Polystyrene pipets and test tubes (Falcon Plastics) were used for all experiments with thrombin. For prothrombin and thrombin assays fibrinogen (Sigma, 62% clottable), prothrombin two-stage reagent (Difco Laboratories, Detroit, Mich.), and AcGlobulin (Difco) were used.

Methods

Preparative centrifugation was done in a Model RC2B (Sorvall, Norwalk, Conn.). pH measurements were conducted with a Radiometer pH Meter, Model PHM 26 (Copenhagen, Denmark). Spectrophotometry was performed with a Model 2000 Gilford (Gilford Instruments Laboratories, Inc., Oberlin, Ohio) spectrophotometer.

Protein Concentration. Protein concentration was determined by measuring the optical density at 280 nm and by the procedure of Lowry *et al.* (1951) using bovine serum albumin as the standard. For prothrombin the $A_{280}^{1\%}$ of 16.5 (Ingwall and Scheraga, 1969) was used.

Fibrinogen Preparation. A 0.5% solution of bovine fibrinogen was prepared in Tris-saline, dialyzed at 4° for 12 hr and filtered with paper (Whatman 1, Balston Ltd., England). The solution was divided into 10-ml portions, frozen, and thawed as needed.

Thrombin and Prothrombin Assay. Thrombin clotting activity was measured by adding 0.1 ml of the test solution to 0.4 ml of fibrinogen which had been incubated at 37° for at

[1] Abbreviations used are: Tris-saline, 0.01 M trishydroxymethylaminomethane–HCl plus 0.9% NaCl, pH 7.4; CbzTyrONph, *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester; $DodSO_4$, sodium dodecyl sulfate; factor X_a, activated blood clotting factor X; TK-resin, Sepharose-insolubilized factor X_a made from Milstone's thrombokinase; DFP, diisopropyl fluorophosphate; TosArgOMe, *p*-tosyl-L-arginine methyl ester-HCl.

least 30 sec. Thrombin activity was expressed in NIH units by extrapolating the observed clotting time with a linear log–log plot of NIH standard thrombin concentrations (lot B-3 obtained from Dr. D. Aronson, Division of Biologics Standards, National Institutes of Health) *vs.* clotting time. A new standard curve was drawn for each lot of fibrinogen. Dilutions were made in Tris saline. All clotting assays were done in triplicate. Thrombin esterase activity was determined following the CbzTyrONph esterase assay of Martin *et al.* (1959); however, experiments were performed at 24°. Aliquots were taken from the activation mixture and diluted 25–100× with Tris-saline. A CbzTyrONph concentration of 2×10^{-5} M was used in the assay. Prothrombin was usually assayed and quantitated by the two-stage procedure of Ware and Seegers (1949). Alternatively, because of the protracted activation times of our prothrombin preparations a modified procedure similar to that described by Mann *et al.* (1971b) was used. The latter consisted of mixing at 24° 0.1 ml of diluted (1:10 with saline) defibrinated plasma, 0.1 ml of prothrombin to be assayed, 0.3 ml of Tris-saline, 0.7 ml of prothrombin two-stage reagent (Difco), and then 0.8 ml (0.02 M) of $CaCl_2$ in Tris-saline. The clotting time was followed and the shortest clotting time recorded. In a representative assay the added prothrombin yielded about 100 NIH units while the reagents contributed 1 NIH unit. Prothrombin activity was defined in terms of thrombin activity generated. One milliliter of bovine plasma contained 200 NIH units of prothrombin.

Prothrombin Preparation. The method of prothrombin preparation used was a modification of the method developed by Moore *et al.* (1965), Malhotra and Carter (1968), and Ingwall and Scheraga (1969). Because many changes were made, the preparation is described in detail.

Large volumes of fresh blood were collected in plastic containers from several cows after severing the neck vessels. The blood was immediately mixed with 2.85% sodium citrate in an 8:1 dilution. Plasma was completely prepared within 5 hr of collection by centrifuging the blood twice in plastic containers (4°, 20 min at 2000*g*) and decanting. The plasma was divided into two liter portions, poured into plastic bags, frozen, and stored at −40°. Before use, one portion of plasma (2000 ml) was thawed by chopping the solid into small pieces and placing in a 30–40° bath (approximately 45 min) until liquid. Some plasma preparations were provided by the New England Enzyme Center (Boston, Mass.); in these preparations significantly more hemolysis was present and the defoaming agent, Dow Corning Antifoam AF emulsion (Dow Corning Corp., Midland, Mich.), was used; nevertheless, no difference was detected in the final product.

All preparative steps were conducted at 4°.

The 2-l. portion of plasma was placed in a plastic beaker and mixed slowly with a magnetic stirrer. At a rate of 1 drop/sec, 160 ml of a 1 M $BaCl_2$ solution was added by means of a buret. The solution was stirred for 60 min following the complete addition, and then centrifuged for 20 min at 5800*g*. The supernatant was discarded. The yellowish precipitate was removed from the centrifuge vessel with a spatula and finely suspended in a plastic beaker containing 300 ml of 0.154 M NaCl solution. It was then centrifuged (5800*g*) for 10 min, and the supernatant was discarded. The wash procedure was repeated identically. Prothrombin was eluted by again placing the precipitate in a plastic beaker containing 300 ml of 0.2 M EDTA (pH 7.4). The preparation was mixed for 30 min; however, not all the precipitate went into solution as a small quantity of gummy-white material remained.

Without further treatment and using slow mixing, 300 ml of saturated $(NH_4)_2SO_4$ (pH 7.0) was added from a buret at the rate of one drop/sec. After all the salt solution was added, the preparation was centrifuged at 8500*g* for 12 min. The precipitate was discarded. To the clear supernatant in the same way, another 300 ml of saturated $(NH_4)_2SO_4$ was added. After complete addition, the suspension was centrifuged (8500*g*) for 12 min. The supernatant was discarded.

The precipitate was dissolved in 15 ml of distilled water and poured into 1-cm wide dialysis bags. This preparation was dialyzed against 4 l. of 0.002 M sodium phosphate (pH 7.2) for 15–20 hr.

The dialysate was poured into a glass beaker and with stirring was titrated from pH 7.2 to pH 5.2 with 0.25% HCl at the rate of 1 drop/sec. The preparation was cleared by centrifuging at 7000*g* for 30 min. In the same way the supernatant was brought to pH 4.6. The white flocculant suspension was allowed to stand 10 min at 4° and then centrifuged (2700*g*) for 30 min.

The resulting supernatant was discarded and the precipitate was dissolved in 6 ml of 0.1 M phosphate buffer (pH 6.0). This product was either used immediately for chromatography or frozen in 2-ml portions.

For chromatography, DEAE-Sephadex A-50 was packed into a Lucite column (3.5 × 10 cm). The column was equilibrated with 300–500 ml of 0.1 M phosphate buffer (pH 6.0) at 4° with a hydrostatic pressure of 20 cm. Approximately 30–45 mg of the isoelectric precipitate was placed on the column. The column was developed using a linear salt gradient arising from 250 ml of 0.1 M phosphate buffer (pH 6.0) and 250 ml of 0.1 M phosphate buffer (pH 6.0) in 1 M NaCl. The column was run at 4° with a hydrostatic head of 20 cm at a rate of 12 ml/hr. Aliquots (3–5 ml) were collected by an automatic fraction collector in glass tubes. Those fractions in the central portion of the prothrombin elution peak were pooled. The salt concentration of the eluate was determined by means of a conductance bridge (Industrial Instrument Inc., Cedar Grove, N. J.) and a standard linear plot of sodium chloride concentration *vs.* conductivity.

The elution pattern was essentially identical with that of Ingwall and Scheraga (1969); however, the appearance of an early non-prothrombin protein peak was variable. If a fresh isoelectric precipitate preparation were used, the small early peak was not seen, but if the preparation had been stored at 4° for several weeks, this early peak was present. In contrast to Ingwall and Scheraga, prothrombin eluted from the DEAE-Sephadex column at a lower salt concentration, that is, 0.2–0.4 M NaCl compared to 0.6–0.7 M NaCl.

Gel Filtration. In testing for homogeneity by gel filtration, the prothrombin preparation was chromatographed on a Lucite column (1 × 45 cm) of Sephadex G-100 in 0.1 M sodium phosphate (pH 6.0) plus 0.4 M sodium chloride at 4°. The collection procedure was the same as for the DEAE-Sephadex chromatography.

Assay of Soluble and Insolubilized Factor X. Factor X activity was defined and measured by the Bachmann *et al.* (1958) procedure. Factor X_a was measured by the procedure of Bachmann *et al.* (1958) as modified by Papahadjopoulos *et al.* (1964); however, a commercial factor VII–X deficient plasma and, instead of Cephalin, Inositin were used. The latter was prepared by homogenizing (Waring blender, full speed 15 min) 2.8 g of the lipid in 100 ml of distilled water. The suspension was frozen in 0.5-ml aliquots which were thawed as needed and diluted with Tris-saline to a final concentration of 80 μg/ml. After dilution with Tris-saline the final concentration of Russell's viper venom used was 0.8

μg/ml. One unit of factor X activity was defined as the factor X_a activity generated by the venom from 1 ml of complete bovine plasma. A log–log plot of plasma dilution (Tris-saline) *vs.* clotting time yielded a linear relationship (slope = 0.33). Factor X_a activity was interpolated from the same plot using the clotting time from an assay in which Tris-saline replaced the venom. The activity of insolubilized factor X_a (TK-resin) was determined identically. Although the activities have been reported to be equivalent (Spaet, 1964), the chemical identity of thrombokinase and factor X_a has not been established. In these experiments factor X_a and thrombokinase have been defined by the modified Bachmann *et al.* assay (1958) and thus the terms will be used interchangeably. Tos-ArgOMe esterase activity of TK-resin was determined by the method of Sherry and Troll (1954) except that the experiments were conducted at 24°, the assay suspension was mixed magnetically, and the titration was conducted with 0.001 N NaOH.

Factor X_a Preparation. In preliminary experiments factor X_a was prepared by the procedure of Yin and Wessler (1968).[2] Thrombokinase was supplied by Dr. J. H. Milstone as step VII material (Milstone, 1959; Milstone *et al.*, 1963) labeled TK col. F4 9/13/67 and 2/16/68 and received in the frozen state. All experiments described in this report were conducted with Milstone's thrombokinase.

TK-resin Synthesis. Milstone's thrombokinase (3 ml) containing approximately 550 units of factor X_a was quickly thawed, diluted to 8 ml with Tris-saline, and then immediately titrated to pH 6.8 with 1 M citric acid and 25% trisodium citrate (or 1 N NaOH). It was then set aside in ice. Following the procedure of Cuatrecasas *et al.* (1968), 20 ml of Sepharose 4B in 40 ml of distilled water was activated with 2 g of CNBr for 4 min; the resin was rapidly washed with 1 l. of cold (4°) 0.1 M $NaHCO_3$ and most of the resin was added to the protein solution. The mixture was left at 4° with slow stirring for 2–4 days. Since thrombokinase is less stable at an acid pH (Milstone *et al.*, 1963), these preparatory steps should be performed rapidly. They were usually completed within 10–15 min.

The TK-resin was washed copiously with distilled water, 25% sodium citrate, and Tris-saline until neither protein nor factor X_a activity could be detected in the filtrate. The resin was then suspended in 10–20 ml of 0.154 M NaCl solution and stored at 4°. Leaching of factor X_a activity from the resin was tested by incubating supernatant from a 4-week-old resin preparation with chromatographed prothrombin in 25% sodium citrate. Since no detectable prothrombin activation was observed (sampling after 17-, 42-, and 65-hr incubation at 24° showed clotting times longer than 5 hr), it was concluded significant factor X_a activity is not eluted from the resin. In addition, neither absorbance at 280 nm nor protein-stainable bands by $DodSO_4$ disc gel electrophoresis (Weber and Osborn, 1969) have been found in this supernatant.

The factor X_a activity of TK resin was inhibited by soybean trypsin inhibitor. This was tested by incubating with magnetic stirring TK-resin (1 ml), prothrombin (2 ml, containing 0.6 mg/ml), and the inhibitor (5 mg/ml in final concentration) in 25% sodium citrate at 24°. After 24-hr incubation and after adding a diluted aliquot to fibrinogen, the clotting times were longer than 2 hr. Thrombin activity of TK-resin was tested by incubating at 24° 0.1 ml of TK-resin in 1 ml of fibrinogen plus 5 mg of the inhibitor. No clot was observed after 41 hr even though the fibrinogen remained clottable to fresh thrombin.

Prothrombin Activation. In the usual activation experiment prothrombin protein concentration was 0.3–0.8 mg/ml. One milliliter of TK-resin suspension (containing approximately 0.006–0.01 factor X_a unit/ml) was added for each milligram of prothrombin. The solution was made 25% (w/v) sodium citrate by adding solid salt. Immediately after the salt dissolved the activation mixture was titrated, unless otherwise stated, with 1 M citric acid, to pH 6.5 (no correction in pH reading was made for the high salt concentration) and the mixture was left at 24° with slow stirring (magnetic stirrer) in a polypropylene test tube. The generation of thrombin activity was routinely measured by removing aliquots of the activation mixture, diluting in Tris-saline and recording the clotting time in the presence of the TK-resin. When required, the resin was removed by centrifugation at 24° in a clinical centrifuge (full speed 5 min) and the supernatant was removed by pipet. In any one set of activation experiments the same lot of TK-resin was used. The thrombin clotting activity generated is expressed as a percent of the clotting activity detected in the two-stage prothrombin assay of the initial prothrombin preparation. A dilutional factor of 1.15 was used for the volume change in a solution made 25% in sodium citrate.

Gel Electrophoresis. Analytical gel electrophoresis was conducted following the procedure of Davis (1964) using 7% acrylamide gels.

Unless otherwise noted, $DodSO_4$ gel electrophoresis was run following the technique of Fairbanks *et al.* (1971).[3] Acrylamide gels (5.6% Tris-acetate, pH 6.8) were made in 10 × 0.5 cm chromic–sulfuric acid-washed glass tubes and were allowed to polymerize at least 24 hr. All samples were prepared following the Fairbanks *et al.* (1971) procedure using 1% $DodSO_4$; however, glycerol (30% v/v) was used instead of sucrose. Samples were reduced by adding 5–10% v/v mercaptoethanol and incubating at 60° for 30 min. Pyronin Y was used as the tracking dye and gels were usually run to an 8-cm mark on the glass tube at a current of 6–8 Ma/tube. The front was marked by surgical wire inserts. Gels were stained with Coomassie Blue and destained by diffusion according to Fairbanks *et al.* (1971). Gels were scanned using a Gilford spectrophotometer and Model 2410 linear transport accessory; the slit width was 0.1 mm and the wavelength was 610 nm. Molecular weight estimates of the preparations were determined from the mobility of each band, as measured, in most preparations, from the densitometer tracings. Mobilities were compared to those of the following standards: phosphorylase *a*, myoglobin, bovine serum albumin, pepsin, ribonuclease, and myoglobin cyanogen bromide fragments (prepared by the method of Gross, 1967). Interpolation was made from a linear semilog plot using the molecular weights as given by Weber and Osborn (1969) and Swank and Munkres (1971).

Alternatively, $DodSO_4$–8 M urea gels (12.5% acrylamide–Tris-phosphate pH 6.8) were run using the procedure of Swank and Munkres (1971).[3] These gels contain a 1:20 ratio of *N,N'*-methylenebisacrylamide:acrylamide and allow separation of oligopeptides. The samples were prepared as for Fairbanks *et al.* (1971) gels, but they were made 8 M in urea. Gels were run at 1.6–2 mA/gel for 15–20 hr. Staining and

[2] These preparations were performed with the gracious collaboration of Dr. Daniel Deykin and Miss Frances Cochise of the Beth Israel Hospital and Harvard Medical School, Boston.

[3] This procedure was called to the authors' attention by D. S. Papermaster.

TABLE I: Summary of Prothrombin Purification Procedure.

Step	Total Act. (NIH Units)	Sp Act. (NIH Units/mg of Protein)	Act. Yield (%)
Starting plasma	414×10^3	9.9	100
Ammonium sulfate dialysate	168×10^3	100	40.6
Isoelectric precipitate fractionation to pH 5.2	72×10^3	460	17.4
Isoelectric precipitate fractionation to pH 4.6	66×10^3	747	15.9
DEAE-Sephadex chromatography (total)	66×10^3	1409 ± 64	15.9

destaining procedures were identical with those of Fairbanks *et al.* (1971).

Sampling of the activation mixture for electrophoresis was performed by adding an aliquot of the activation mixture to an equal volume of 2% $DodSO_4$ in 0.01 M sodium phosphate (pH 7.0) or, alternatively, to an equal volume of glacial acetic acid. TK-resin was removed by centrifugation. The sample was then exhaustively dialyzed against 0.1 M acetic acid and lyophilized. Protein (10–25 μg) was placed on each gel.

Some early studies were conducted with the electrophoretic and staining procedures of Weber and Osborn (1969) using 10% acrylamide gels.

In all pictures the anodic end of the gel is positioned down.

Thrombin Isolation. At chosen times following the initiation of activation the mixture was centrifuged at room temperature (clinical centrifuge full speed for 5 min) to remove the TK-resin and to stop the activation reaction. It was then placed in a cellophane dialysis bag and dialyzed at 24° *vs.* 0.005 M sodium phosphate buffer (pH 6.0) on a dialysis rod. (The latter is identical with the apparatus described by Englander and Crowe, 1965.) It holds the dialysis bag taut and rotates in the buffer bath. A 50% reduction in conductivity occurred by 45 min. Dialysis was continued 3–5 hr. The sample was concentrated in a dialysis bag against dry Sephadex G-100 resin and then placed on a DEAE-cellulose Lucite column measuring 1×12 cm which was developed using the linear gradient system of Asada *et al.* (1961) at 24°. Fractions (1.7 ml) were collected at a rate of 12 ml/hr in polypropylene test tubes.

Results

Prothrombin Purification. The purification procedure can be performed within 2.5 days from plasma to chromatographed product. Using this method the total activity yield is about 16%, which compares to yields obtained by other recent methods: 15% (Cox and Hanahan, 1970) and 30% (Mann *et al.*, 1971b). As shown in Table I, the greatest loss of activity occurs during the initial procedures, *i.e.*, there is about 60% loss of activity after dialysis of the ammonium sulfate precipitate. The specific activity of this preparation is 1200–1400 NIH units/mg which compares to 1250–1450 NIH units (Magnusson, 1965) and 1100–1300 NIH units/mg (Mann *et al.*, 1971b) obtained by others.

This procedure yields a preparation which is estimated to be 95% pure and is essentially monodisperse by analytical (Davis, 1964) and $DodSO_4$ disc gel electrophoresis (Fairbanks *et al.*, 1971; Weber and Osborn, 1969); nevertheless, several faint slower and faster moving bands are demonstrable by both methods. This preparation gives a sharp symmetrical elution peak on Sephadex G-100 chromatography and shows constant specific activity across the peak. No electrophoretic or chromatographic evidence suggests the existence of more than one prothrombin molecule, although such studies do not completely rule out this possibility. Using the modified Bachmann assay (see Methods) no factor X_a activity is detectable; however, approxmiately $^1/_{1000}$th of a unit of factor X activity is present in 1 mg of the chromatographed product. Finding such a contaminant is consistent with the observations of others (Milstone and Oulianoff, 1969) concerning the difficulty of removing factor X (venom substrate) from prothrombin preparations. This preparation does not generate thrombin in the presence of 25% sodium citrate. After incubating a 0.8-mg/ml prothrombin solution in 25% sodium citrate at 24° at pH 6.5 for 17, 42, and 65 hr, the clotting times exceeded 4 hr. Chromatographed prothrombin was stored at 4° in the eluting buffer (pH 6.0, 0.1 M PO_4, 0.3–0.4 M NaCl) and used within 1 week. During this period the specific activity and the monodispersity on sodium dodecyl sulfate disc gel electrophoresis remained essentially unchanged. In contrast to the findings of Cox and Hanahan (1970), it was found unnecessary under these conditions to store prothrombin in DFP; however, 1 day after dialysis against 0.01 M Tris-HCl (pH 8.0), the electrophoretically monodisperse prothrombin showed a prominent second band in analytical gel electrophoresis.

TK-resin Synthesis. Initial experiments using factor X_a prepared by the Yin and Wessler (1967) method indicated, as Cuatrecasas and Anfinsen (1971) note, that binding to activated Sepharose was most efficient at an acid pH. Under the experimental conditions (see Methods) the total bound activity was about 0.005% of the initial factor X_a activity. Resin suspensions contained 0.005–0.01 factor X_a unit/ml.[4] TosArgOMe esterase activity of the resin suspension varied from preparation to preparation in the range 0.10–0.30 TosArgOMe unit (Sherry and Troll, 1954) per ml. Since purified soluble thrombokinase contains 337 TosArgOMe units/mg (Milstone, 1960), it would appear that the resin suspension contains about 0.3–1.0 μg of thrombokinase/ml. Despite the small amount of bound activity, TK-resin proved to be an effective and valuable tool (see below).

The TK-resin was stored at 4° in saline and retained its activity for over 6 months. Although TK-resin could be used at least twice, in the experiments described here only virgin activator resin was employed.

Activity Studies of Prothrombin Conversion by TK-resin. Since the goal of these experiments was to establish a well-defined system of converting prothrombin to thrombin, which

[4] In contrast to its properties in the soluble state, a coupled enzyme retains much less of its activity toward large substrates than toward small substrates (Axen and Ernback, 1971). This observation has been interpreted to indicate that a bound enzyme is sterically hindered by its support and consequently interacts less efficiently with large molecules. Moreover, compared to the soluble enzyme, the insolubilized enzyme may have less affinity for a small substrate. Axen *et al.* (1970) have shown using a small synthetic substrate that insolubilized chymotrypsin has an identical V_{max} but an elevated K_m compared to the soluble enzyme, suggesting a change in protein–substrate affinity. In view of these considerations and since the factor X_a assay is believed to involve a protein–calcium–lipid complex, the assay itself may inadequately quantitate the covalently bound factor X_a activity.

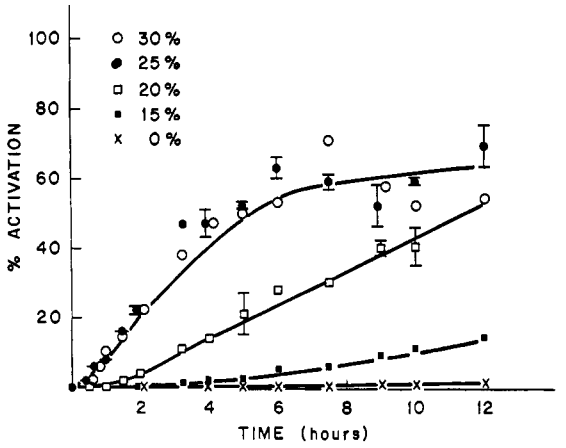


FIGURE 1: Activation of prothrombin at various sodium citrate concentrations using TK-resin at pH 6.5. At each salt concentration 3 ml of prothrombin containing 0.37 mg of protein/ml were incubated at 24° with 1 ml of TK-resin containing about 0.01 unit of factor X_a/ml. Sodium citrate concentrations were adjusted by adding solid salt to give 0, 15, 20, 25, and 30% (w/v). Clotting activity was followed with time and recorded as per cent of the initial two-stage prothrombin activity.

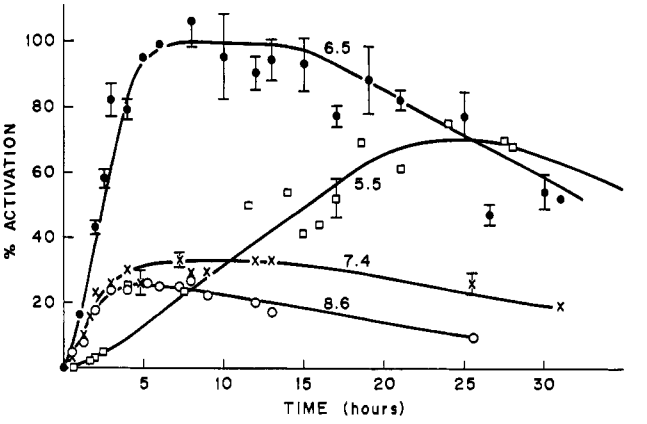


FIGURE 2: Prothrombin activation at various hydrogen ion concentrations by TK-resin in 25% sodium citrate. Except for pH adjustment experimental conditions for curves pH 5.5 (□), 7.4 (×), and 8.6 (○) are the same as for Figure 1. For curve pH 6.5 (●), to 9.0 ml of prothrombin (0.67 mg/ml) were added 3 ml of TK-resin and 3 g of sodium citrate. Hydrogen ion concentrations of pH 5.5 and 6.5 were obtained by titrating with 1 M citric acid; for pH 7.4 and 8.6 titration was performed with 1 N NaOH. Clotting activity was followed as in Figure 1.

produced minimal side products, two of the critical requirements of the system were (1) to produce as much thrombin from prothrombin as one could detect by the two-stage assay and (2) to retain maximal clotting activity in the generating system for at least twice as long as it takes to attain maximal activity.

Preliminary experiments showed that high concentrations of salt were necessary in order to measure significant prothrombin activation. As shown in Figure 1, the most rapid, and apparently most stable, activation occurred in 25 and 30% sodium citrate. In the absence of sodium citrate, prothrombin activation by TK-resin occurred very slowly and incompletely (Figures 1 and 4B). This activation pattern is qualitatively quite similar to that found by Seegers *et al.* (1950) using a completely soluble protein system. Subsequent work was done in 25% sodium citrate.

A study of several hydrogen ion concentrations showed that pH was important in realizing maximal conversion and maximal thrombin stability (Figure 2). The initial rate of generation of clotting activity was equal at pH 8.6, 7.4, and 6.5 but much slower at pH 5.5. At pH 8.6 and 7.4, however, clotting activity declined long before complete activation was observed. At pH 6.5 virtually complete activation was observed and the clotting activity remained unchanged for at least twice the generation time of maximal activity; subsequently, clotting activity slowly declined. At pH 5.5 the generation of clotting activity was slow and incomplete.

Although these kinetic data have been repeatedly observed and are qualitatively accurate, there is often a quantitative variation from experiment to experiment which can be ascribed to (1) the potency of the particular activator resin preparation, (2) the homogeneity of the resin suspension taken for a given experiment, and (3) the rate of stirring during the experiment. Effort was always made to standardize these conditions.

As a result of the experiments reported above conditions for studying prothrombin activation were defined which allow virtually complete conversion of prothrombin to thrombin (see Methods) and stable clotting activity for a reasonable period after the generation of maximal activity. Using this activation system three questions were asked.

During Activation Does Prothrombin Elaborate a Substance Which Inhibits or Causes Thrombin Production? Shown in Figure 3 is an S-shaped prothrombin activation curve. Similar kinetics have been observed by others (*e.g.*, Seegers, 1949; Lanchantin *et al.*, 1965; and Mann *et al.*, 1971b) using soluble systems. Esterase and clotting activities developed simultaneously as recorded earlier (Lanchantin *et al.*, 1967; Mann

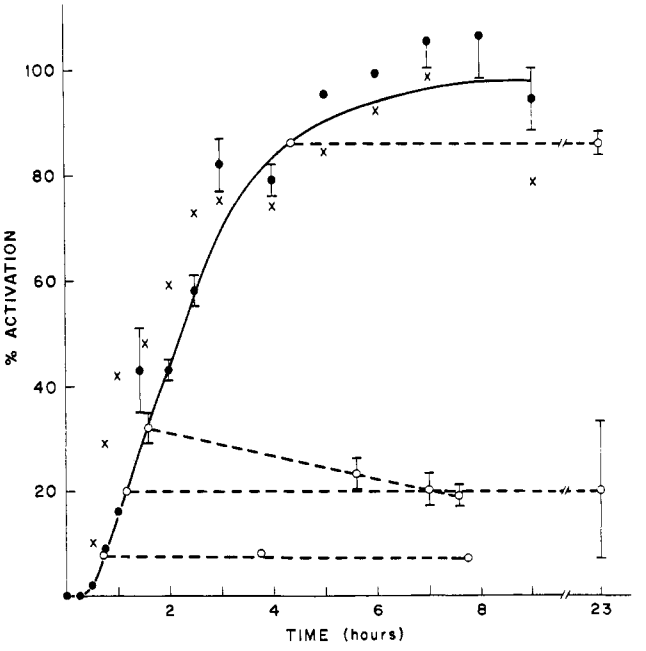


FIGURE 3: Prothrombin activation studied with respect to time by removal of the insoluble activator. From an activation mixture (same as pH 6.5, Figure 2) were taken aliquots and clotting (●) and CbzTyrONph esterase (×) activity (compared to maximal esterase activity generated) were followed (24°). At various times aliquots were centrifuged to remove the activating resin; the supernatant was withdrawn and its clotting activity followed (○).

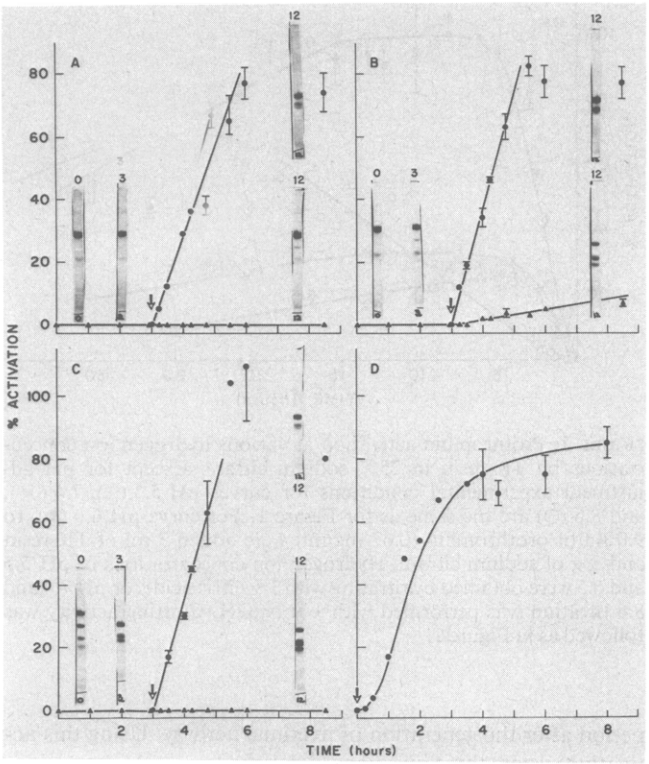


FIGURE 4: Activation characteristics of an incomplete activating system. (A) Prothrombin in concentrated salt solution but in the absence of TK-resin. Prothrombin (3 ml) (0.3 mg/ml), water (1 ml) and sodium citrate (1 g) were mixed and titrated to pH 6.4 and clotting activity was followed (lowest plot). At 3 hr 1 ml of the activation mixture was removed and 0.5 ml of TK-resin suspension plus 0.12 g of sodium citrate was added (arrow); magnetic stirring was started after the pH was adjusted to 6.5. The activation sequence of the incomplete mixture (the mixture lacking TK-resin) was followed by nonreduced $DodSO_4$ disc gel electrophoresis at 0 (left), 3 (middle), and 12 (lower right); the 3- (upper right) hr mixture plus TK-resin was also subject to electrophoresis at 12 hr. (B) Prothrombin plus TK-resin in the absence of salt. Prothrombin (3 ml) (0.3 mg/ml) plus TK-resin (1 ml) (pH 6.4) were mixed magnetically and clotting activity was followed (lowest plot). At 3-hr mixing, 1 ml of the incomplete activation mixture was removed and 0.25 g of sodium citrate was added (arrow); magnetic mixing was continued. Electrophoretic gels were run as for Figure 4A. (C) Prothrombin in concentrated salt solution plus prothrombin activation fragments in the absence of TK-resin. Prothrombin (3 ml) (0.3 mg/ml), dialyzed 19-hr prothrombin activation mixture supernatant (1 ml) (containing 224 NIH units of thrombin), and sodium citrate (1 g) (pH 6.3) were mixed and clotting activity was followed (lowest plot). After 3 hr, 1 ml of the incomplete activation mixture was added to 0.5 ml of TK-resin and 0.12 g of sodium citrate (pH 6.5) and stirred (arrow). The activation plot is corrected for added clotting activity. Electrophoretic gels were run as for Figure 4A. (D) Complete activation mixture. This mixture consisted of 3 ml of prothrombin (0.37 mg/ml), 1 ml of TK-resin, 1 g of sodium citrate (pH 6.5); clotting times were followed.

et al., 1971b). Aronson (1962) pointed out that the S-shaped prothrombin activation curve is consistent with an autocatalytic or a sequential reaction involving a slow intermediate step. Autocatalysis was tested directly in this system by removing the activator resin at various times and following clotting activity. As shown in Figure 3, additional thrombin activity did not generate after the activator was removed. In some instances clotting activity decreased with time. The latter was probably due to a surface effect since activity loss could be minimized by reducing surface exposure (*i.e.*, the number of test tube transfers). Autocatalysis was tested in addition by adding to fresh prothrombin in 25% sodium citrate the maximally activated mixture from which TK-resin had been removed. In these experiments no further clotting activity was generated (see lowest plot Figure 4C).

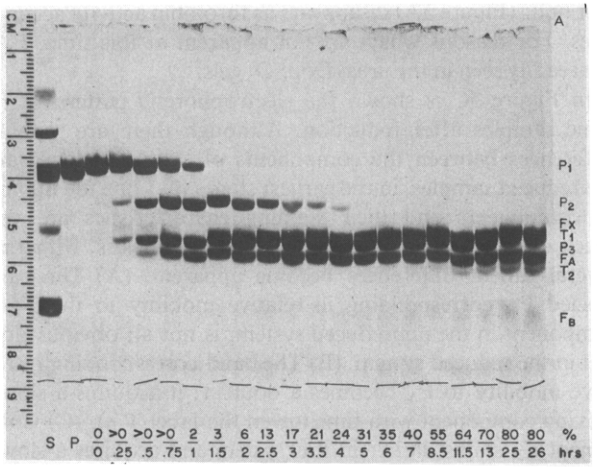
The kinetics of these experiments are given in Figure 4 where it is shown on adding sodium citrate alone (Figure 4A) or the soluble activation mixture (clotting activity plus activation fragments) to fresh prothrombin (Figure 4C) that no additional clotting activity generated; however, prothrombin plus the activator resin (Figure 4B) in the absence of sodium citrate slowly produced clotting activity. In all three experiments complete conversion would occur (after 3 hr) if the missing element of the activation mixture were added. The latter observations show that a 3-hr exposure of prothrombin to salt, factor X_a activity, or clotting activity (as well as the activation fragments) does not destroy thrombin-generation potential.

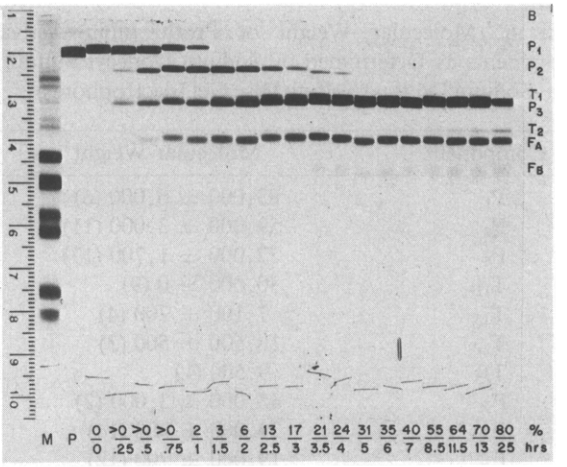
Product formation was also followed (shown by the gel patterns inserted in the figures). Over the course of 12 hr, incubating prothrombin with sodium citrate (Figure 4A) led to very little change in the electrophoretic pattern except for the appearance of a faint anodic band. On the other hand, exposing prothrombin to factor X_a (TK-resin) (Figure 4B) in the absence of salt results in a new faint anodic band by 3 hr and three new darkly staining bands by 12 hr. In the absence of factor X_a activity, incubating prothrombin plus salt plus soluble activation mixture (thrombin plus activation fragments) (Figure 4C) results in a rapid alteration of prothrombin, although no increase in clotting activity was found. In all cases, after 3 hr adding the missing element to the incomplete activation mixture resulted in the rapid development of clotting activity and the appearance of a mature 12-hr electrophoretic pattern (*cf.* 13 hr of Figure 5A).

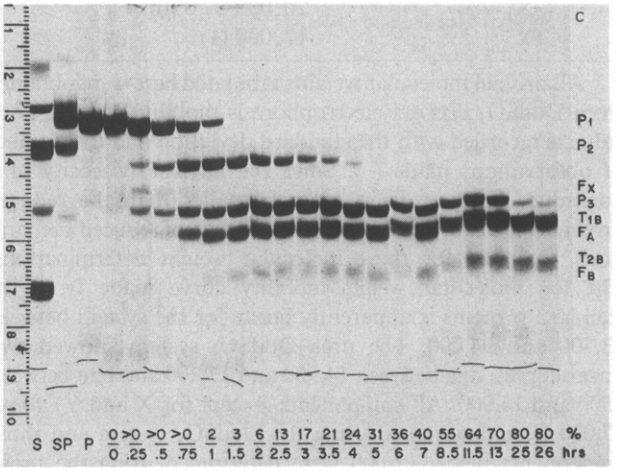
Such experiments demonstrate that under these conditions nothing in the soluble activation mixture causes or prevents the generation of clotting activity from prothrombin. These studies, however, do not explore the possible role of the salt or the activation products in altering prothrombin and thus accelerating or retarding the generation of thrombin activity. It is worthwhile noting that others (Seegers, 1962; Lanchantin *et al.*, 1967) have observed that clotting activity will accelerate prothrombin activation.

What Are the Intermediates of Prothrombin Activation? Since the activation mechanism must involve more than one step, a study of prothrombin activation products was begun using disc gel electrophoresis. For the following experiments the denaturing systems of Fairbanks *et al.* (1971) and Swank and Munkres (1971) were chosen.[5] Aware of the complicated decay patterns resulting after prolonged activation, we focused on the earliest phases of prothrombin activation. While these

[5] Studying a mixture of proteins by $DodSO_4$ disc gel electrophoresis has several serious limitations since its physical basis has not been completely elucidated. Pertinent to these experiments are the following. (1) Even after apparently adequate $DodSO_4$ saturation, charge density may play an important role in the mobility of a specific protein moiety. Recently, Tung and Knight (1971) reported that after maleylation significantly greater changes in protein mobility occur than what would be expected; this indicates $DodSO_4$ may not equalize all charge effects. Thus, if the charge characteristics of a protein moiety are unknown, its molecular weight as determined by this method can only be considered approximate. (2) A given protein component may not take up dye linearly. This is clearly shown in Figure 5, on the Standard (S) gel where pepsin stains faintly despite the fact it is present in greater concentration than phosphorylase *a* or bovine serum albumin. Thus one may not be able to state the relative concentration of a given band without knowing its staining characteristics.







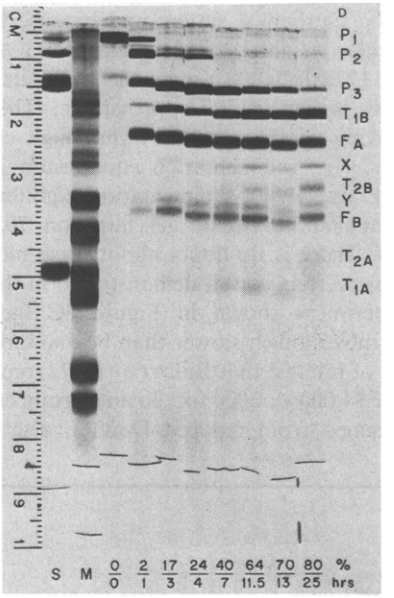


FIGURE 5: (A) $DodSO_4$ disc gel electrophoresis patterns of a nonreduced prothrombin activation mixture. The activation mixture consisted of 12 ml of prothrombin (0.45 mg/ml), 1 ml of TK-resin (0.01 unit of factor X_a) and 3.25 g of sodium citrate at pH 6.4. At various intervals 0.8-ml aliquots were removed and sreated as described in Methods. The protein standards (gel S) are phosphorylase *a* (at 30 mm, 94,000), bovine serum albumin (at 41 mm, 68,000), pepsin (at 53 mm, 35,000), and ribonuclease (at 73 mm, 13,700); each band contains about 4 μg of protein. Gel P is the prothrombin preparation used. Each gel is labeled with a fraction: the numerator refers to per cent conversion (compared to the two-stage assay) and the denominator to the incubation time of the experiment (hours). Zero per cent conversion refers to a clotting time of greater than 4 hr after a 1:10 dilution with Tris-saline. The inequality signs inserted before the zeros at 0.25, 0.50, and 0.75 hr refer to the fact thrombin activity is present though it cannot be accurately quantitated. The average clotting times at these periods after a 1:10 dilution are 35, 10, and 4 min, respectively. From 20 to 25 μg of protein was placed on each gel. The labels on the right vertical axis refer to the proposed identification and mechanism of activation (see Figure 7). (B) Urea–$DodSO_4$ disc gel electrophoresis patterns of a nonreduced prothrombin activation mixture. Conditions and labels are the same as for part A except the samples contained urea (see Methods). Gel M is the electrophoretic pattern of a reduced cyanogen bromide treated myoglobin preparation; this pattern is more fully described in the legend of part D. This picture is printed on a "soft" paper to accentuate the doublet T_1–P_3. (C) $DodSO_4$ disc gel electrophoresis patterns of a reduced prothrombin activation mixture. Aliquots of the activation mixture were reduced with mercaptoethanol; otherwise, experimental conditions were identical with part A. The protein standards in gel S are phosphorylase *a* (30 mm), bovine serum albumin (41 mm), pepsin (54 mm), and ribonuclease (74 mm). Gel PS contains phosphorylase *a*, bovine serum albumin, pepsin, and prothrombin. Gel P contains the initial prothrombin preparation. Other notation is as in part A. (D) Urea–$DodSO_4$ disc gel electrophoresis patterns of a reduced prothrombin activation mixture. Experimental conditions were identical to those used for part C except the samples contained urea (see Methods). Standards in gel S are phosphorylase *a* (9 mm), bovine serum albumin (16 mm), pepsin (25 mm), and ribonuclease (51 mm). Gel M contains cyanogen bromide fragments of myoglobin (38 mm, 17,200), myoglobin I and II (44 mm, 14,900), myoglobin I (52 mm, 8270), myoglobin II (56 mm, 6420), and myoglobin III (70 mm, 2550). Other notation is as in part A.

studies were in progress, a similar analytical approach to the study of prothrombin activation was described (Mann *et al.*, 1971b).

The generation of prothrombin activation products and their subunits was studied by two electrophoretic methods with nonreduced and reduced samples. A representative experiment is presented in Figure 5. In this particular study activation was followed over a 25-hr period at which time 80% of the potential thrombin activity had generated. Activation was less rapid in this experiment (compare to Figures 1–4) since less TK-resin was added per milligram of prothrombin in order to best observe the earliest intermediates.

The electrophoretic patterns of the nonreduced activation mixture at various times is given in Figure 5A,B; several features are worth noting. (A) Before significant clotting activity generates, the initial prothrombin band (P_1) wanes and four new bands (P_2, F_x, P_3, F_A) appear (*cf.* Figure 5A, 0.5 hr). Bands P_2 and P_3 increase in intensity concomitantly during the initial stages of activation (see Figure 5A, 0.25 and 0.5 hr). (B) The T_1–P_3 band is a doublet (best seen in

TABLE II: Molecular Weight of Prothrombin Activation Components as Determined by Sodium Dodecyl Sulfate and Urea–Sodium Dodecyl Sulfate Disc Gel Electrophoresis.[a]

Component	Molecular Weight
P_1	83,000 ± 6,000 (6)
P_2	59,000 ± 3,000 (11)
P_3	37,000 ± 1,700 (17)
T_{1B}	30,000 ± 0 (9)
T_{1A}	7,100 ± 200 (4)
T_{2B}	18,500 ± 500 (3)
T_{2A}	9,500 (1)
F_X	45,000 ± 3,000 (2)
F_A	29,000 ± 900 (14)
F_B	19,000 ± 700 (12)
X	20,000 ± 0 (6)
Y	17,000 (1)

[a] All protein molecular weights tabulated here were obtained from $DodSO_4$ disc gel electrophoresis mobility studies. Each value is recorded with the standard deviation and the number of observations made ( ). Since the intrinsic viscosity of a nonreduced protein is in general smaller than the reduced protein (Fish *et al.*, 1970) only the mobility of reduced preparations was used for these molecular weight determinations. The log molecular weight–mobility curve made from the standard proteins is apparently linear for the system between 13,700 and 94,000. The urea–$DodSO_4$ system showed two linear curves, one between 14,900 and 17,200 and one between 2500 and 14,900. All components (except for X and Y) above 17,000 were determined using the $DodSO_4$ system and those components below 17,000 were determined using the urea–$DodSO_4$ system. Because components X and Y were not detected in the Fairbanks' system and because the mobility *vs.* log molecular weight curve for urea–$DodSO_4$ gels is not linear in the 17,000–20,000 range, the molecular weight of these subunits is considered approximate. The molecular weight of bovine prothrombin is rather high compared to earlier studies, *e.g.*, sedimentation equilibrium 74,000 (Ingwall and Scheraga, 1969), sedimentation equilibrium 68,000 (Cox and Hanahan, 1970) or gel filtration 70,000 (Mann *et al.*, 1971b). There is no doubt about these mobility characteristics, however, as is well demonstrated in the coelectrophoresed experiment shown in Figure 5C (gel P) where prothrombin moves much slower than bovine serum albumin (68,000). It is of interest that Bull *et al.* (1972) report a molecular weight of 84,000 ± 3000 for bovine prothrombin which was also obtained from reduced $DodSO_4$ disc gel electrophoresis.

Figure 5B, 2–13 hr). The faster component of the doublet, P_3, appears first and decreases in intensity with time. The slower component, T_1, increases in intensity with time and is most prominent in the 25- and 26-hr gels. (C) As band P_2 wanes, the T_1–P_3 doublet increases in intensity and a faintly staining band F_B appears. (D) Band T_2 generates slowly after 7 hr. In the urea–$DodSO_4$ system (Figure 5B) this band runs more slowly than F_A while it runs faster than F_A in the $DodSO_4$ system (Figure 5A). This mobility difference is not explained though it may be due to more extensive unfolding in the urea system. (E) The faint short-lived band, F_x, found within the first hour (Figure 5A) disappears as thrombin activity accumulates. For reasons which are not apparent at this time, F_x is not readily seen in the urea–$DodSO_4$ gels.

In Figure 5C is shown the electrophoretic pattern of the same samples after reduction. Although there are mobility differences between the components of the nonreduced and the reduced samples, in the earliest stages (0–1 hr), the number of components and their staining characteristics are very much alike (compare Figure 5A,C). Nevertheless, with time several salient differences become apparent. (A) The band labeled P_3, corresponding in relative mobility to the T_1–P_3 component in the nonreduced system, is not an obvious doublet in the reduced system. (B) The band corresponding in relative mobility to F_A becomes a doublet; it acquires a slower moving component with time (given the label T_{1B}). (C) Component F_B stains darker in this system and acquires a slower moving component after 8.5 hr (labeled T_{2B}).

For the reduced preparation greater resolution is achieved by the urea–$DodSO_4$ system; such experiments, as shown in Figure 5D, reveal several additional features. (A) As thrombin activity appears, P_3 decreases and T_{1B} and T_{1A} increase in intensity. (B) Two bands are apparent in this system which have not been seen in the other gels. Band Y develops early in the activation followed by band X. No change is appreciable in either band over the time studied. (C) As T_2 develops in the nonreduced gels (Figure 5A,B, 11.5–25 hr), two new bands appear in the reduced urea–$DodSO_4$ gels: these have been labeled T_{2A} and T_{2B}.

From the gels shown in Figure 5 the molecular weight of the activation components was estimated and recorded in Table II.

To establish which of the components had thrombin activity, a prothrombin preparation was activated by TK-resin in 25% sodium citrate for only 45 min. The resin was removed, and the mixture was dialyzed and subject to DEAE-cellulose chromatography (see Methods). As Asada *et al.* (1961) reported, the first peak contained thrombin activity. In this experiment the pooled thrombin peak yielded 70% of the applied activity and contained 360 NIH clotting units/absorbance unit (280 nm). The electrophoretic pattern of this thrombin sample in the absence of mercaptoethanol (shown in Figure 6, gel 1) reveals a single component with a mobility corresponding to the T_1–P_3 band (*cf.* Figure 5A). The reduced preparation is shown in Figure 6, gel 2; it reveals two bands. The slower running band corresponding to P_3 (Figure 5C) has an apparent molecular weight (M_{app}) of 37,000, and the faster band corresponding to T_{1B} has an M_{app} of 30,000.

For the following reasons it appears that the T_1–P_3 band (Figure 5A) contains two components, a double-chained molecule, T_1, and a single-chained molecule, P_3, of similar, if not identical, molecular weight, where T_1 is the first thrombin produced and P_3 is the immediate-thrombin precursor.[6] (1) The T_1–P_3 component is a doublet (Figure 5B). (2) Thrombin activity isolated from the activation mixture is associated with the T_1–P_3 electrophoretic component (Figure 6, gel 1). (3) On reduction the T_1–P_3 component yields three components P_3, T_{1B} (Figure 6, gel 2), and T_{1A} (urea–$DodSO_4$ gel, not shown). The sum of the apparent molecular weights of

[6] Confirmation of this interpretation awaits further physicochemical studies. Since the faster component (anodic) of the doublet appears first, it is labeled P_3, and is considered the immediate thrombin precursor. The slower component appears later as thrombin activity develops and has been labeled T_1, the first thrombin molecule.

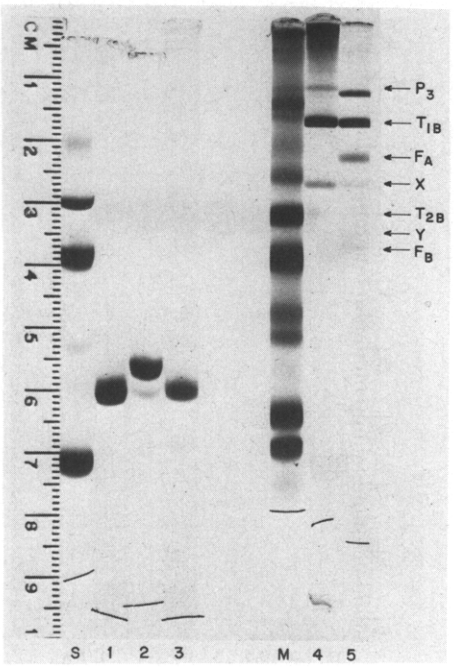


FIGURE 6: Activation of the isolated T_1–P_3 component. A mixture of 3 ml of prothrombin (0.6 mg/ml), 0.4 ml of TK-resin, 0.6 ml of distilled water, and 1 g of sodium citrate (pH 6.5) was stirred for 45 min at room temperature, then centrifuged, dialyzed, and placed on a DEAE-cellulose column (see Methods). The first eluting peak, which contained clotting activity, was pooled. $DodSO_4$ disc electrophoresis gels were run of the nonreduced (gel 1) and the reduced sample (gel 2). This eluate (5.5 ml) (A_{280} 0.042) was mixed with TK-resin (0.2 ml) and sodium citrate (pH 6.5) (1.4 g). After 24-hr mixing, the sample was denatured, dialyzed, lyophilized, and subjected to $DodSO_4$ disc gel electrophoresis without mercaptoethanol (gel 3) and with mercaptoethanol on a urea–$DodSO_4$ gel (gel 4). For comparison, gel 70%/13 hr of Figure 5D is included (gel 5). Gels S and M refer to the standards run in Figure 5.

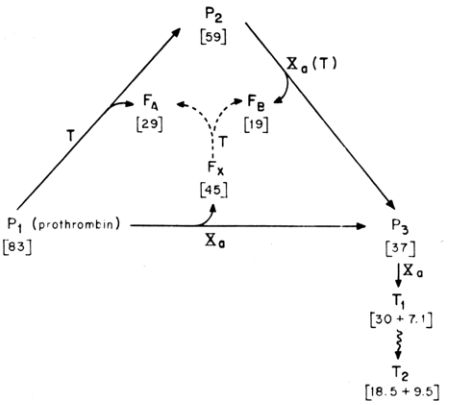


FIGURE 7: Proposed prothrombin activation scheme. T or X_a adjacent to the pathway arrow refers to thrombin or factor X_a activity respectively, which appears to catalyze the specific reaction. Dashed arrows refer to postulated steps for which direct evidence is not yet available. Bracketed numbers below labels refer to molecular weight $\times 10^{-3}$ as recorded in Table II. For a more complete description, see Text.

these three components far exceeds that of any one while the sum of the apparent molecular weights of T_{1B} and T_{1A} closely approximates the M_{app} of P_3. (4) As thrombin activity appears the electrophoretic band P_3 wanes and T_{1B} and T_{1A} grow (Figure 5D). (5) Amino acid sequence data (Magnusson, 1971) show that thrombin contains two subunits, an A chain (approximately 6000) and a B chain (approximately 30,000). In these experiments T_{1A} and T_{1B}, found after reduction, may correspond to the thrombin subunits since they generate with thrombin activity (Figure 5D), are associated with thrombin activity (Figure 6, gel 2), and have similar approximate molecular weights (Table II).

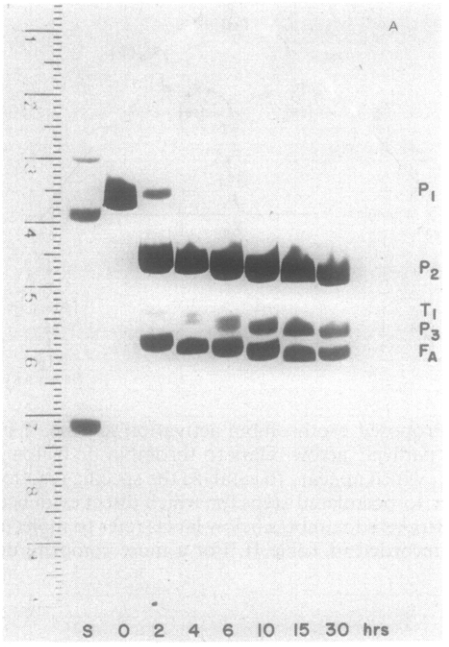
In order to distinguish which activation components are generated from T_1–P_3 and which from P_1 and P_2, chromatographed T_1–P_3 was exposed to TK-resin and sodium citrate for 25 hr. The electrophoretic pattern of the nonreduced sample is given in Figure 6 (gel 3). Two components are seen which correspond to T_1–P_3 and T_2 (the latter is very faint in this gel) (*cf.* Figure 5A); the nonreduced apparent molecular weights of these components are 36,000 and 28,000, respectively. Mann *et al.* (1971a) found that a thrombin active substance of 40,000 produces a second component of 28,000; in those studies the second component also had thrombin activity. The experiments reported here do not indicate if the T_2 component has thrombin activity but there is no doubt it is derived from T_1–P_3. The products of T_1–P_3 incubation with TK-resin were also studied after reduction. A urea–$DodSO_4$ gel (gel 4 in Figure 6) is shown adjacent to the products of the complete activation system (gel 70%/13 hr of Figure 5D). The bands observed in gel 4 of Figure 6 are P_3, T_{1B}, X, T_{2B}, T_{2A}, and T_{1A}. (The latter two bands are poorly defined in this gel.) Most striking is the absence of bands F_A and F_B. Thus T_{1B}, T_{1A}, T_{2B}, T_{2A}, and X arise from the T_1–P_3 component. In view of these results and the generation pattern of Figure 5A, it appears F_A and F_B are nonthrombogenic activation fragments where F_A is derived from P_1 and F_B from P_2. Although component Y is not seen in gel 4 of Figure 6, adequate study has not been conducted to exclude its generation from T_1–P_3.

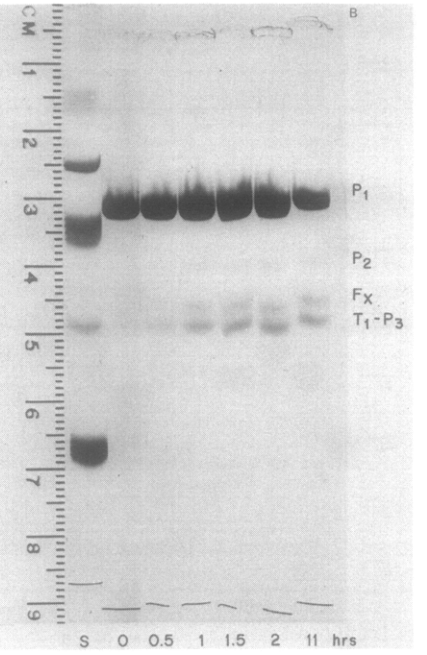
What Is the Mechanism of Prothrombin Activation? HYPOTHESIS. A unifying prothrombin activation mechanism based on the results described above is outlined in Figure 7. It is proposed that there are two pathways in the generation of thrombin activity. One pathway is initiated by factor X_a and the other by thrombin. Both pathways are assumed to yield the same immediate-thrombin precursor, P_3, which is activated only by factor X_a.

In the factor X_a-initiated pathway factor X_a cleaves prothrombin to yield two single-chain components, P_3 and F_X. Component P_3 is cleaved further by factor X_a to yield a two-chain structure, T_1, of apparently unchanged molecular weight, which has clotting activity; this molecule consists of the subunits T_{1A} and T_{1B}. With time T_1 is cleaved to the smaller molecule T_2 which consists of subunits T_{2A} and T_{2B}. Since F_X disappears once thrombin activity is generated, and since it appears stable in the absence of thrombin (see below), it is likely F_X is further cleaved by thrombin, possibly into F_A and F_B. In this pathway only one prothrombin intermediate is recognized, P_3, and the activation fragment is released as one piece, a "pro-fragment," F_X.

In the thrombin-initiated pathway prothrombin progresses through two intermediates, P_2 and P_3, before liberating thrombin. At each step an activation fragment is released: F_A and then F_B. Unique to this pathway is P_2.

TESTING THE HYPOTHESIS. Such a scheme can be tested by exposing prothrombin (a) to thrombin in the absence of factor X_a and (b) to factor X_a (TK-resin) in the absence of thrombin. One would predict that in the former case component P_2 would be prominent and F_X would be absent while in the latter case the converse would hold.





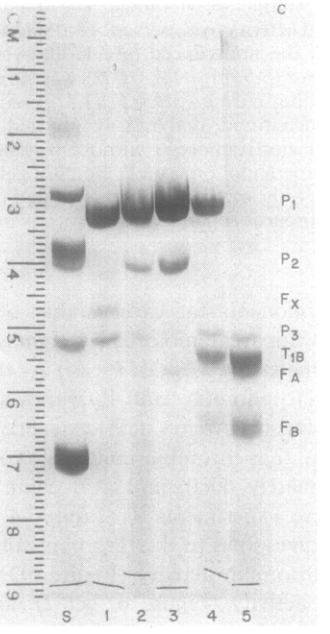


FIGURE 8: Testing the proposed mechanism of prothrombin activation. (A) Exposing prothrombin to thrombin. To 4 ml of prothrombin (0.7 mg/ml) was added 1.5 g of sodium citrate and 2 ml of thrombin prepared from a 6-hr activation mixture (see Methods) which contained 60 NIH units/ml. The pH was 6.3. The mixture was incubated at 24°. Aliquots were collected at various times and without reduction subjected to $DodSO_4$ disc gel electrophoresis. The time of assay is written on the anodic end of the gel (hours). Bands corresponding to components of Figure 5A are labeled on the right vertical axis. Gel S contains protein standards as in Figure 5A. This picture is printed on a "soft" paper to accentuate the T_1–P_3 doublet most clearly seen at 4 hr. (B) Activation mixture of prothrombin plus TK-resin plus DFP. Prothrombin (3.5 ml, 0.4 mg/ml) plus TK-resin (0.3 ml) plus DFP (0.05 ml of 0.54 M) in isopropyl alcohol plus sodium citrate (0.98 g; pH 6.4) were stirred at 24°. Aliquots were taken and without reduction subject to $DodSO_4$ disc gel electrophoresis. The gels are labeled as in part A. (C) Coelectrophoresis of various activation mixtures. All gels were run by $DodSO_4$ disc gel electrophoresis using reduced samples. Gel S contains standard proteins the same as for Figure 5. Gel 1 contains experiment "1.5 hr" of part B. Gel 3 contains a 15-hr incubate of an experiment identical with those shown in part A; however less thrombin was used (5 ml of prothrombin containing 0.4 mg/ml, 1.5 g of sodium citrate, and 1 ml of a solution containing 2 NIH units of thrombin prepared from a 9-hr activation mixture (see Methods)). Gel 2 contains an equal mixture of sample shown in gels 1 and 3. Gel 5 contains experiment "80%/25 hr" of Figure D, and gel 4 contains an equal mixture of sample shown in gels 1 and 5. All gels show a faint impurity between P_1 and P_2.

Prothrombin was incubated with T_1–P_3 as a source of thrombin in the absence of TK-resin. As shown in the nonreduced $DodSO_4$ gels of Figure 8A within 2 hr the intensity of the prothrombin band rapidly faded while prominent P_2 and F_A bands appeared. Over the course of 30 hr the P_3 band also increased slightly in intensity. Despite the increase in P_3 no additional thrombin activity was detected; in fact, there was a slow loss of clotting activity. Under these conditions it has been repeatedly observed that P_2 is rapidly produced even in the presence of minute amounts of thrombin (less than 0.3 NIH unit/ml). In these experiments component F_X was never observed. Identification of the intermediates was supported by the electrophoretic pattern of the reduced sample (not shown). In Figure 4C prothrombin was exposed to thrombin plus prothrombin activation products. In that experiment an identical generation of activation components was seen as presented in Figure 8A. This observation suggests, but does not prove, that thrombin alone is the agent causing structural alterations in prothrombin and that a second active prothrombin fragment need not be postulated to explain the observed results. Moreover, the experiment shown in Figure 4C (addition of resin at 3 hr) indicates that TK-resin efficiently catalyzes the generation of P_3 from P_2. In summary, these experiments have shown that (1) thrombin rapidly catalyzes the conversion of P_1 to P_2 but much less completely catalyzes the formation of P_3 from P_2; (2) TK-resin efficiently catalyzes the $P_2 \rightarrow P_3$ reaction; (3) F_A grows in intensity as P_1 generates P_2.

The factor X_a initiated pathway was studied by taking advantage of the differential DFP susceptibilities of thrombin and factor X_a (Leveson and Esnouf, 1969). To prothrombin was added DFP in isopropyl alcohol to give a DFP concen-

tration of 0.0079 M; then, TK-resin and sodium citrate were added. The electrophoretic pattern was followed with time. For all assays, clotting times were longer than 11 hr. These experiments are shown in Figure 8B where it is seen that (1) components with the same mobilities (*cf.* Figure 5A) as F_X and T_1–P_3 develop in intensity simultaneously and (2) although the P_2 band is present, it is very small compared to the early gels of Figure 5A (*e.g.*, 1 hr). An appropriate control containing isopropyl alcohol without DFP slowly generated thrombin activity with time and showed an electrophoretic pattern consistent with Figure 5A. If, after 24 hr, the DFP activation mixture in the presence of TK-resin was dialyzed against 25% sodium citrate at pH 6.5 for 12 hr, and then subjected to electrophoresis, the pattern showed a prominent P_2 and F_A, but no F_X component. In one experiment the TosArgOMe esterase activity of a TK-resin suspension containing 0.0079 M DFP was found to decrease by 50% after 3 hr. This observation might explain the rather slow development of the band pattern in Figure 8C after the first hour.

The existence of component F_X was initially questioned since it possibly could have been confused with either P_2 or T_1–P_3 in the presence of F_A. Therefore, the activation mixtures similar or identical with those of Figures 8A,B and 5C were coelectrophoresed. The results are presented in Figure 8C. Although the F_X band is faint, the pattern of the mixed gels clearly shows a distinction between the F_X band, P_2 and the T_1–P_3 bands. The electrophoretic patterns of gels 1 and 3 of Figure 8C differ from Figure 8A,B, respectively, since in the latter cases the samples were nonreduced.

Included in Figure 7 are the apparent catalysts for each step. The first step in each pathway is catalyzed by thrombin or factor X_a as shown by the electrophoretic pattern of the nonreduced sample in Figure 8A,B. As discussed above, the second step of the thrombin pathway appears to be catalyzed by factor X_a (though as shown in Figure 8A it may also be catalyzed by thrombin). The kinetic studies of Figures 3 and 4 have shown that thrombin is liberated only by factor X_a activity and thus for both pathways the reaction P_3 to T_1 is catalyzed by factor X_a.

It is not clear at this time which enzyme is important to the formation of T_2, X, and Y, nor through what intermediates these reactions pass. Component X clearly arises from T_1–P_3 (Figure 6, gel 4) and may be an intermediate subunit between T_1 and T_2. Component Y may also be such an intermediate; however, since it is not readily seen in the generation experiment (Figure 6, gel 4), it may also arise from P_1, P_2, or F_A.

The molecular weights recorded in Table II are consistent with the proposed activation mechanism; however, from these molecular weights we would predict that at least one additional fragment must be present in the activation mixture which has not been detected by the methods employed here. In generating T_2 from T_1 9000 daltons must be released. Such a fragment(s) could have been lost during dialysis while preparing the sample for electrophoresis. Such dialyzable fragments were not sought in these experiments.

Discussion

Crucial to the understanding of this complex activation is to resolve the possibility raised by many investigators, and again most recently by Magnusson (1971), that prothrombin activates itself by liberating its own activator. Under the conditions established here it has been possible to answer this question unequivocally since the activator was removable and the prothrombin preparation was stable. No clotting activity was generated after exposing prothrombin to high concentrations of sodium citrate, to the soluble phase of the prothrombin activation mixture, or to thrombin. In these experiments in order to generate thrombin from prothrombin, factor X_a activity had to be present. This observation concurs with the findings of others using soluble systems (*e.g.*, Milstone, 1964, and Seegers, 1967).

Our initial studies of prothrombin activation with TK-resin had suggested a simple mechanism of thrombin production. This involved a single pathway comprising two intermediates before the liberation of thrombin activity: $P_1 \rightarrow P_2 \rightarrow P_3 \rightarrow$ thrombin. A similar mechanism has been proposed by Seegers (1962), Aronson and Menache (1966), and Mann *et al.* (1971b). In the present studies, however, there are two features of the electrophoretic pattern which are inconsistent with such a simple interpretation. First, during the initial phases of activation the intensity of bands P_1 and P_3 increases simultaneously. Since the staining characteristics of both components are about equal, if only a single pathway were in effect, one would predict that P_2 would appear before P_3. Despite how early in the activation process samples were taken P_3 was present if P_2 appeared. This observation could be explained by (1) a mechanism involving two pathways or (2) a difference in rate constants where the P_2 to P_3 constant far exceeded the P_1 to P_2 constant. The latter does not appear to be the case since P_2 accumulates during activation (see Figure 5). The second feature complicating a single pathway interpretation is the presence of the faint, short-lived band, F_X, which certainly is not a contaminant nor can it be identified with one of the other activation intermediates. Since it was not possible to reconcile these two observations with a single-pathway mechanism, two pathways were proposed and tested. The idea that prothrombin is activated by several pathways has been suggested by others (Ganrot and Nilehn, 1969; Rosenberg and Waugh, 1970).

Earlier studies also support the possibility of two prothrombin activation pathways. While most investigators accept the fact that factor X_a alone will liberate thrombin activity from prothrombin (Milstone, 1964), many workers have found that thrombin accelerates prothrombin activation (*e.g.*, Seegers, 1962, 1967, and Lanchantin *et al.*, 1967, 1968) and that thrombin will generate prothrombin activation intermediates (Lanchantin *et al.*, 1968). An immediate thrombin precursor, prethrombin, has been described (Seegers and Marciniak, 1965; Seegers *et al.*, 1967) which is produced by thrombin from prothrombin. Prethrombin is catalyzed by factor X_a (autoprothrombin C) to thrombin. Studies such as these ascribe an important role to thrombin in prothrombin activation. What has not been adequately explained is the following. If thrombin is instrumental to the generation of thrombin activity, from where does the first thrombin molecule come?

The data and mechanism presented here resolve this dilemma. At least two activation pathways occur. In the absence of thrombin at a high factor X_a:thrombin ratio, that is, in the beginning stages of activation in solution, prothrombin conversion is initiated by factor X_a and would follow that pathway; however, as thrombin accumulates conversion would occur predominately by the thrombin-initiated pathway. Such would be the situation under the activation conditions used in these experiments.

Physiologically, the situation may be quite different. Prothrombin is thought to be activated by a complex of phospholipid, calcium, factor V, and factor X_a, the prothrombinase complex (Cole *et al.*, 1965; Jobin and Esnouf, 1967; Barton

and Hanahan, 1969). Prothrombin adsorbs to this complex in the presence of calcium while thrombin has no affinity for it. Thus, if prothrombin is converted physiologically by the prothrombinase complex, it would be activated in an environment of a high factor X_a:thrombin ratio. If the experiments reported here can be extrapolated to the physiological situation[7] and if the prothrombinase complex, as we presently understand it, is the important *in vivo* activator, we may then conclude that physiological activation occurs predominately along the factor X_a initiated pathway. As thrombin is produced by this complex, it would be released into the plasma and, if not inactivated, react with prothrombin. Thus, one might predict that serum should contain P_2 and, indeed, P_2 appears to have been demonstrated in serum recently by Fass and Mann (1972). Under these conditions, however, would the thrombin-initiated pathway produce significant clotting activity? Since the concentration of factor X_a is low in plasma, the thrombin pathway would probably not be productive unless the intermediates, P_2 and P_3, were to adsorb to the prothrombinase complex where the concentration of factor X_a is high.

It is not clear at this time what function the large piece F_X, liberated in the factor X_a initiated pathway, plays in the activation process. Surely, it cannot serve as the sole attachment of prothrombin to the prothrombinase complex for if it were, P_3 would be released at the same time; hence, the whole prothrombin molecule would be discharged from the physiological activator without having generated thrombin.

Intermediates of prothrombin conversion have been described by others (*e.g.*, Seegers, 1962; Seegers and Marciniak, 1965; Aronson and Menache, 1966; Lanchantin *et al.*, 1965–1968; Tishkoff *et al.*, 1968; and Mann *et al.*, 1971b). Because of the similarity of their approach, the experiments of Mann *et al.* (1971b) deserve comment. In general their results support the thrombin-initiated pathway as outlined here. Several important differences in experimental conditions may account for the absence of a second activation pathway in their data. (1) The activator they used was soluble. (2) Although they do not report hydrogen ion concentration, their activation experiment is probably at an alkaline pH since sodium citrate is an alkaline salt; as demonstrated (see Results) pH was very important in these experiments and may play a role in masking the second pathway. (3) Incubating their prothrombin in sodium citrate alone led to the formation of a prominent second electrophoretic band which appears to correspond in these experiments to P_2. This result may reflect the presence of a small but significant amount of thrombin in their prothrombin preparation. In that case, the thrombin-initiated pathway would be occurring even before their activator was added.

In conclusion, elucidating the mechanism of zymogen activation requires the identification of the activation intermediates, the reaction steps and the catalysts for each step. The experiments reported here describe electrophoretically distinct intermediates of prothrombin activation and suggest the steps and catalysts of a two-pathway mechanism leading to the generation of thrombin.

[7] It is not clear from this initial study how much of the data is applicable to *in vivo* activation. Since little is known about the physiological activator or its microenvironment, it is not yet possible to state how far these experimental conditions deviate from the physiological. Certainly, the salt concentration is higher in these experiments. Like the activator used here the physiological activator appears to be attached to a particle but we do not know if the activator in both cases functions identically.

Acknowledgments

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Kinetics of Ligand Binding in the Hemoglobin of *Lumbricus terrestris*†

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ABSTRACT: Sodium dodecyl sulfate gel electrophoresis and determinations of the per cent Fe in the hemoglobin of *Lumbricus terrestris* indicate 1 heme/17,000. This finding suggests that the double-hexagonal hemoglobin structure revealed by electron microscopy consists of 12 subunits, each containing 16 hemes. Gel filtration molecular weight studies on the hemoglobin of *Lumbricus* show a decrease in molecular weight from 2.5×10^6 at pH 7 to 0.25×10^6 (and lower) at pH 10.3. The reactions studied, CO combination and oxygen dissociation, are sensitive to changes in pH and protein concentration. Kinetic measurements strongly suggest that there is little if any cooperativity in ligand binding in the isolated 16-heme subunits. The high cooperativity in ligand binding shown by the hemoglobin from *Lumbricus* must arise from interactions among the 16-heme subunits.

A kinetic study of ligand binding in annelid hemoglobins provides an excellent opportunity to correlate structure and function in a complex protein. The intact hemoglobin from *Lumbricus terrestris*, the earthworm, has been shown (Levin, 1963; Roche, 1965) to consist of 12 subunits, arranged in two superimposed regular hexagons, 265 Å in length (measured between opposite vertices within a hexagon), 160 Å in width, and 160 Å in thickness. The intact duodecamer of mol wt 3.2×10^6 (Rossi-Fanelli *et al.*, 1970) contains approximately 192 hemes, and is one of the largest of the known respiratory proteins. The duodecamer is known to dissociate first into hexamers, splitting perpendicular to the plane of the hexagons, and then into the one-twelfth subunits (Levin, 1963). Levin further reported that at pH 10.2 the dissociation into subunits

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