Activation of Human Prothrombin by a Procoagulant Fraction from the Venom of Echis carinatus

IDENTIFICATION OF A HIGH MOLECULAR WEIGHT INTERMEDIATE WITH THROMBIN ACTIVITY

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SUMMARY

In the presence of a procoagulant fraction (Echis carinatus procoagulant) isolated from the venom of the saw-scaled viper Echis carinatus sochureki, purified human prothrombin (P1) is completely converted to thrombin. The first step is the removal of an NH2-terminal peptide (F1) representing approximately one-third of the prothrombin molecule. The remaining peptide (P") is then cleaved by the action of E.c. procoagulant to yield a two-chain, disulfide-bridged protein (P't) which has the same molecular weight as P1. P't has enzymic (thrombin) activity, as evidenced by incorporation of radiolabeled diisopropylphosphofluoridate into its heavy chain (Tb), hydrolysis of p-toluenesulfonylarginine methyl ester, and clotting of fibrinogen. Relative to thrombin, its esterolytic activity greatly exceeds its clot-promoting activity.

Examination of the polypeptide chains obtained by reducing P't has shown that its larger chain (Tb) is indistinguishable from the heavy chain of thrombin. Its other chain (F't) consists of the light chain (Tl) of thrombin bound by peptide linkage to the portion of the prothrombin molecule which had been adjacent to F1. Removal of this portion (F't) is catalyzed by thrombin (and, evidently, by P't), but not by the E.c. procoagulant. When F1 is removed from P", the remaining two-chain protein is indistinguishable from thrombin by any of the criteria applied—molecular weight, subunit chain composition, or enzymic activity.

Polyacrylamide gel electrophoresis was carried out in sodium dodecyl sulfate before and after disulfide reduction of samples generated in the presence and in the absence of diisopropylphosphorofluoridate, which inhibits thrombin but not the E.c. procoagulant. Such experiments showed that thrombin (and probably P't), as well as E.c. procoagulant, catalyzes the release of F1. Furthermore, thrombin brings about the cleavage of F1 to yield a two-chain, disulfide-bridged protein (F't).

These observations, particularly those made in the course of characterizing P", have led to the conclusion that cleavage of the peptide bond connecting the Tl and Tb portions of the prothrombin molecule (or its derivatives) produces a serine active center and, hence, a molecule possessing thrombin activity. This cleavage is catalyzed by the E.c. procoagulant but not by thrombin itself.

Considerable effort has been spent on elucidating the pathway by which the zymogen, prothrombin (P1 in the present terminology), is converted to the active enzyme, thrombin. Measurement of the appearance of new NH2-terminal amino acids has not only demonstrated the proteolytic nature of the activation but has shown that with the conversion methods used this is a multi-step reaction (1, 2). Analysis by other methods has allowed further delineation of the conversion so that a general scheme is now agreed upon (3–8). The reaction sequence seems to be the loss, first, of approximately one-half of the mass of the prothrombin molecule (in either one or two steps) followed by the splitting of the remaining peptide into a light chain (A chain) and a heavy chain (the B chain).

The currently accepted peptide structure and the nomenclature to be used in this communication are presented in Fig. 1. Table I compares this nomenclature with that used by others (4, 6, 7).

The studies cited above utilized conversion by plasma procoagulants, with the generation of thrombin being dependent on the presence of activated Factor X (Xa). Taipan snake (Oxyuranus scutellatus) venom also converts prothrombin (9) to thrombin and apparently cleaves the zymogen at the same bonds as does Xa (10).

Venom of the saw-scaled viper (Echis carinatus sochureki) has also been shown by Kornalik (11) and Schieck et al. (12) to convert prothrombin to thrombin. Unlike other venoms (e.g. Taipan snake) that activate prothrombin, the Echis carinatus venom has no calcium requirement (13). Consistent with this characteristic are the facts that (a) E.c. procoagulant can generate thrombin from the abnormal prothrombin produced by patients and animals treated with vitamin K antagonists, and (b) these abnormal prothrombins exhibit decreased calcium-ion binding. The marked difference in the cofactor and substrate requirements of plasma procoagulants and E.c. procoagulant (14, 15) induced us to employ the latter in studying the conversion of prothrombin to thrombin.

MATERIALS AND METHODS

Tris was obtained from Sigma Chemical Co., Na dodecyl-SO from Fisher Scientific, 2-mercaptoethanol from Eastman Kodak, [32P]iPr2P-Fl from Amersham/Searle, Tos-Arg-OMe from Mann

1 The abbreviations used are: iPr2P-F, diisopropylphosphorofluoridate (incorporated as iPr2P, diisopropylphosphate); Na dodecyl-SO, sodium dodecyl sulfate; Tos-Arg-OMe, p-toluenesulfonylarginine methyl ester.
A sample of dried whole venom was dissolved in 5.0 ml of 0.05 M NaCl/0.0066 M Tris-Cl to 0.15 M NaCl, and the pH was adjusted to 7.4 with NaOH. Hydrolysis was measured using a Radiometer TTTI Titrator with automatic burette. The pH was maintained constant by addition of 0.02 N NaOH; results were expressed as micromoles of Tos-Arg-OMe hydrolyzed per 100 min. All assays and incubations, unless otherwise stated, were conducted at 22°C.

Electrophoresis was carried out in 10% polyacrylamide gels

![Diagram](http://www.jbc.org/)

**Fig. 1.** Peptide structure of prothrombin (P₁) showing fragments observed during activation. The molecule is drawn to scale based primarily on the similarity of human prothrombin to bovine prothrombin. Placement of disulfide bridges was based upon known positions in bovine prothrombin (8).

| TABLE I |
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Comparison of present and previously published nomenclature

Symbol used by present authors | Symbol used by Sterin and Bise (4) | Designation used by Kiefel and Hanahan (5) and by Owen et al. (7) |
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P₁ | P₁ | Prothrombin |

P₂ | P₂ | Prothrombin |

P₃ | P₃ | Intermediate 1 |

P₄ | P₄ | Intermediate 2 |

F₁ | F₁ | Fragment 1 |

F₂ | F₂ | Fragment 2 |

F₁₃ | F₃ | Fragment 1-2* |

T₁ | T₁ | Thrombin A chain |

T₂ | T₂ | Thrombin B chain |

T | T | Thrombin A chain + B chain (covalently linked by disulfide bridge)  |

*Used only in Reference 7.

Research Laboratories, and DEAE-cellulose (DE23) from Whatman. iPr₂P-F was obtained from Calbiochem and used as a 1 M stock solution in 1-propanol. Aquasol, a xylene-based scintillation solution, was purchased from New England Nuclear; samples were counted in a Packard Tri-Carb liquid scintillation counter.

Human Factor Xₐ was prepared (16) by activation of Factor X which was isolated by a previously described procedure (17). Purified human thrombin (Lot II-1) was prepared by Dr. Kent Miller (18). Assays (16) carried out in the presence of iPr₂P-F showed that this preparation contains no detectable Xₐ. Prothrombin was prepared from human acid citrate dextrose plasma by the method of Aronson (19). The preparation was apportioned into 0.3-ml aliquots, which were stored at -15°C.

**Echis carinatus sochureki** venom (Lot ED 23ET1, obtained from Miami Serpentarium) was partially purified by an ion exchange technique employing DEAE-cellulose chromatography. A 200-μg sample of dried whole venom was dissolved in 5.0 ml of 0.05 M NaCl/0.0066 M Tris-Cl, pH 7.4, and dialyzed against two changes of 100 ml of the same buffer. It was then layered onto a DEAE-cellulose column (2.2 x 32 cm) equilibrated with this buffer and developed with a linear gradient (total volume 500 ml) from 0.06 M NaCl/0.0066 M Tris-Cl to 0.15 M NaCl/0.02 M Tris-Cl at a constant pH of 7.4 and a flow rate of approximately 30 ml per hour; 0.2-ml fractions were collected. All dialysis and chromatographic procedures were carried out at 5°C.

Fractions were assayed for procoagulant activity and fibrinolytic activity. Procoagulant activity was measured by treating 0.1 ml of human acid citrate dextrose plasma with 0.1 ml of a given chromatographic fraction and recording the time of clot formation. Fibrinolytic activity was measured by testing 0.2 ml of 0.25% human fibrinogen (Merek Sharp and Dohme) with 10 μl of thrombin (20 U.S. units/ml) and then incubating the resultant clot with 0.1 ml of the fraction being tested and recording the time of clot lysis. Those fractions which exhibited a clot formation time of 17 s or less and a clot lysis time of greater than 24 hours were pooled and used as E.c. procoagulant (Fig. 2). Two different chromatographic pools have been used throughout the course of the following experiments. The Asa of these pools was in the range of 0.090. Two bands were seen when a sample of E.c. procoagulant was concentrated 50-fold (Minicon B15, Amicon Corporation) and analyzed by Na dodecyl-S0₄-electrophoresis.

Prothrombin was measured by the two-stage prothrombin assay of Wagner et al. (20). Prothrombin two-stage reagent (Difco Laboratories) was used (21). This assay system, as well as those involving clot formation by E.c. procoagulant, was standardized with U.S. Standard thrombin (Lot B-3) so that 1 prothrombin unit gave 1 unit of thrombin.

Thrombin activity was measured by adding 0.1 ml of appropriately diluted sample (all dilutions in 0.15 M NaCl/0.02 M Tris-Cl, pH 7.4) to 0.2 ml of 0.25% human fibrinogen. Tos-Arg-OMe was used to assay the esterase activity generated by the action of E.c. procoagulant on prothrombin. The sample to be tested was added to 3.0 ml of 0.01 M Tos-Arg-OMe in 0.15 M NaCl, and the pH was adjusted to 8.0 with NaOH. Hydrolysis was measured using a Radiometer TTTI Titrator with automatic burette. The pH was maintained constant by addition of 0.02 M NaOH; results were expressed as micromoles of Tos-Arg-OMe hydrolyzed per 100 min. All assays and incubations, unless otherwise stated, were conducted at 22°C.

Electrophoresis was carried out in 10% polyacrylamide gels

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Some of the data are presented as a miniprint supplement immediately following this paper. Figs. 2-4 will be found on p. 7068. Material published in miniprint form can be easily read with the aid of a large-field reading glass of a type readily available at most opticians. For the convenience of those who prefer to obtain supplementary material in the form of full size photocopies, these same data are available as JBC Document No. 74M-1399. Orders for supplementary material should specify the title, authors, and reference to this paper and the JBC Document Number, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $1.00.
(acylamide from Canalco) in the presence of 0.1% Na dodecyl-
SO₄. A current of 6 ma per gel was applied. Gels were stained with
Coomassie blue (Brilliant blue R, Sigma Chemical Co.) in accord-
ance with Weber and Osborn (22). Sample loads were within the
range of 20 to 30 µg of protein per 5-mm diameter gel. Samples were
prepared for electrophoresis by incubation for at least 2 hours at
37° after addition of 2 volumes of either a nonreducing medium
(0.01 M Na phosphate, pH 7.0, 1.0% Na dodecyl-SO₄) or a reducing
medium (0.01 M Na phosphate, pH 7.0, 1.0% Na dodecyl-So₄,
1.0% 2-mercaptoethanol) to 1 volume of sample so as to achieve a
final protein concentration of 1 mg/ml.

EXPERIMENTS AND RESULTS

Activity Changes Produced by Treating Prothrombin with E. c.
Procoagulant—Prior to each experiment the prothrombin was
thawed, adjusted to pH 7.4 by the addition of 1 M Tris, and
diluted with 0.15 M NaCl/0.02 M Tris-C1 to a concentration of 3
mg/ml (2200 units/ml). Addition of 50 µl of E.c. procoagulant per
ml of this prothrombin solution, followed by incubation, pro-
duced maximal thrombin activity (i.e. ability to clot fibrinogen)
after approximately 150 min. However, the generation of this
activity was nonlinear; an initial lag phase was followed by a
rapid increase in thrombin activity over the 60- to 120-min
interval (Fig. 3).

The Tos-Arg-OMe hydrolytic activity followed a markedly
different pattern, with a rapid rise to a peak in 45 min followed by
a gradual decrease (Fig. 3). A comparison of the thrombin activity
with the Tos-Arg-OMe hydrolytic activity is presented in the
inset of Fig. 3. During the interval of 15 to 30 min the ratio of
Tos-Arg-OMe hydrolytic activity to thrombin activity (i.e.
micromoles hydrolyzed per 100 min/U. thrombin units) was
falling, but it averaged approximately 14; over 90 to 240 min it
was stable at 0.90 ± 0.05. For purified thrombin this ratio was
usually 0.99.

Two-stage prothrombin assays performed throughout the
activation period showed that when the conversion was carried
out in the presence of 2 mM iPrzP-F, E. c. procoagulant brought
about a complete loss of prothrombin activity within 120 min
(Fig. 4). In the absence of iPrzP-F, the prothrombin-E. c.
procoagulant mixture exhibited the “Cheshire Cat phenomenon,”
i.e. a rapid loss of two-stage activity reaching a minimum at
approximately one hour, followed by a rise to the original level as
thrombin (T) was formed.

Products Generated by Treating Prothrombin with E. c.
Procoagulant or Factor Xa—To investigate the chemical changes
occurring as these activities were generated, samples taken at
various stages during the activation of prothrombin by E. c.
procoagulant were examined electrophoretically and chromato-
graphically. The elution patterns (from DEAE-cellulose)
were indistinguishable from those observed (3) when Factor Xa
was used to catalyze the conversion. Furthermore, when the
fragments formed during conversion with the E. c. procoagulant
and those formed during activation with Xa were compared by
Na dodecyl-SO₄-electrophoresis, there was identity of all bands
(Fig. 5A). When reduction was carried out prior to Na dodecyl-
SO₄-electrophoresis, the same bands were seen in both conversion
systems (Fig. 5B), but their intensities and the temporal sequence
of their appearance differed greatly.

It has recently been reported that the nature of the fragments
found after activation of bovine prothrombin by Factor Xa is
affected by the presence of iPrzP-F (4, 23). That is, when iPzP-F
is present, activation peptides F₁ and F₂ occur as a single chain
(F₁T) rather than as the two separate peptides found in the
presence of activator thrombin. Furthermore, it had been reported
that the E. c. procoagulant activity is not inhibited by iPzP-F
(12). This was confirmed by an experiment in which 1 ml iPrzP-F
was added to E. c. procoagulant (final concentration of iPzP-F,
2 mM). After 30 min, 100 µl of this mixture was added to 0.2 ml of
human plasma. The clotting time, 25 s, was identical with that of
the control sample (i.e. involving E. c. procoagulant not treated
with iPrzP-F). Therefore, iPrzP-F was used to inhibit thrombin as
it was formed by the action of the E. c. procoagulant. A solution
of prothrombin was made 2 mM in iPrzP-F and treated with E. c.
procoagulant (50 µl of procoagulant/ml of prothrombin solution).
Under these conditions, none of the F₁T (which would have ap-
ppeared as a band migrating slightly slower than F₁) was found.
Fig. 6A shows that in the presence of 2 mM iPrzP-F, the pro-
thrombin-E. c. procoagulant mixture exhibited the “Cheshire Cat phenomenon,”
i.e. a rapid loss of two-stage activity reaching a minimum at
approximately one hour, followed by a rise to the original level as
thrombin (T) was formed.

Intermediate Pz and Its Constituent Chain F₁Ta—Upon reduc-
tion, most of the Pz band disappeared, and two new bands be-
came apparent (Fig. 6B). These corresponded to the B chain
of thrombin (Tb) and component F₁Ta, respectively. The latter
migrated somewhat faster than F₁. Since component Pz is a
single polypeptide chain, it was clear that the Pz band must have
contained a two-chain (disulfide-bridged) protein which dis-
sociated to yield Tb and F₁Ta. This two-chain intermediate will
be referred to as PzT; its structure appears to be F₁Tb--S-S-Ta.
(Additional evidence regarding its formation and structure is
presented later.) The small amount of bona fide Pz formed, disap-
ppeared as a band migrating slightly slower than Pz was found.
Fig. 6A shows that in the presence of 2 mM iPrzP-F there was
minimal formation of thrombin despite the complete disappear-
ance of the original prothrombin and the appearance of bands in the PȚ and PȚa positions.

Intermediate Pz and Subfragment α bands stained rather weakly under the load conditions used. They were
readily seen in the gels but may be difficult to discern in the photo-
graphs.
Production of Subfragments of F₁ by Thrombin—Further examination of gels obtained by the electrophoresis of reduced samples revealed that, in the absence of iPr₆P-F, the intensity of the F₁ band decreased greatly during the interval of 60 to 120 min (Fig. 6B). Simultaneously there appeared two new bands, one migrating somewhat slower than Tₐ and the other, slightly slower than F₂TA. We have designated these peptides Subfragment α and Subfragment β, respectively. Since these were seen times (numbers under gels) are in minutes. Markers (M) for unreduced samples: (left gel) unreduced human thrombin; (right gel) reduced Aα, Bβ, and γ chains of human fibrinogen. Markers (M) for reduced samples: (left gel) thrombin B chain (Tₐ); chymotrypsinogen, the 130- and 96-residue fragments of chymotrypsin, and thrombin A chain (TA); (right gel) Aα, Bβ, and γ chains of human fibrinogen.

Fig. 6. Na dodecyl-SO₄-electrophoresis of prothrombin incubated with E.c. procoagulant or with Factor Xα. When iPr₆P-F (DFP) was used, a 1 mM solution was added to the prothrombin (final iPr₆P-F concentration 2 mM) 5 min prior to addition of the E.c. procoagulant. Other experimental details are given in the text. A, unreduced samples from incubation with E.c. procoagulant. B, reduced samples from incubation with E.c. procoagulant. C, reduced samples from incubation with Factor Xα.
only after reduction, it was clear that they arose from a two-chain derivative of F1; the latter has been designated F'1 (Fig. 6; cf. Fig. 1).

The fact that Subfragments α and β were not formed in the presence of iPrzP-F (Fig. 6B) indicated that they were generated from F1 by the thrombin produced during the activation. To demonstrate this generation directly, F1 was prepared in purified form and allowed to react with thrombin. Thrombin, devoid of Xγ activity, was added to prothrombin at a level of 50 units/ml. At the end of 1 hour the mixture was made 2 mM in iPrzP-F and chromatographed on DEAE-cellulose (Fig. 7). Material eluted near the end of the final peak was pooled and concentrated on a Minicon B15 filter to a concentration of 1.6 mg/ml. Na dodecyl-SO4-electrophoresis before and after reduction showed that it consisted primarily of F1. A 1.4-ml portion of this solution was treated with 0.14 ml of a thrombin solution (final concentration 1000 units/ml), and samples were taken for Na dodecyl-SO4-electrophoresis after various periods of incubation. Electrophoresis of unreduced samples revealed no changes other than the disappearance of the small amount of Pγ contaminating the F1 preparation and the presence of the added thrombin itself (Fig. 8). Upon reduction, however, the decrease in F1 during the 0- to 240-min interval was readily apparent, as was the concomitant production of Subfragments α and β.

The positive identification of these subfragments permitted a more valid comparison between the actions of E.c. procoagulant and Factor Xγ under the present experimental conditions. Whereas Xγ brought about the production of Pγ, Pδ, Fγ, and some F1 within 120 min, no degradation of F1 to Subfragments α and β was apparent until much later (Fig. 6C). By contrast, little F1 remained after 120 min of incubation of prothrombin with E.c. procoagulant (Fig. 6B). Nonetheless, when iPrzP-F was absent (thus allowing the thrombin generated to act on F1) the subfragments were produced in both activation systems, with the result that samples taken after appropriate incubation times (viz. 120 min with E.c. procoagulant, 480 min with Xγ) exhibited Subfragment β bands of similar intensities (Fig. 5B, gels 1 and 2).

Conversion of P1 to Pγ by E.c. Procoagulant—The foregoing experiments had demonstrated the feasibility of using isolated fragments to distinguish between reactions catalyzed by E.c. procoagulant and those catalyzed by the thrombin produced during the activation. Moreover, the chromatographic system employed for isolating the F1 cleaved from prothrombin by thrombin was suitable for obtaining Pγ (Fig. 7). Such Pγ was therefore prepared and purified, and subsequently treated with E.c. procoagulant in the presence and the absence of iPrzP-F. Thrombin, devoid of Xγ activity, was added to prothrombin (final thrombin concentration 50 units/ml) and the mixture was incubated for 1 hour. Following this, iPrzP-F was added (final concentration 2 mM) and chromatography on DEAE-cellulose was carried out. The material eluted just ahead of prothrombin was pooled and brought to a concentration of 1.8 mg/ml with a Minicon B15 filter (Fig. 7). Na dodecyl-SO4-electrophoresis before and after reduction showed that it consisted entirely of Pγ. E.c. procoagulant (final concentration 50 units/ml) was added to this solution in the presence and in the absence of iPrzP-F. In the absence of iPrzP-F, thrombin activity was generated at a rate equal to that observed when intact prothrombin was treated with E.c. procoagulant. Results of Na dodecyl-SO4-electrophoresis performed on samples taken at specified intervals are shown in Fig. 9.

In the presence of iPrzP-F no appreciable change was detected in the unreduced samples; in its absence new bands appeared in the positions of thrombin (T) and F1, respectively (Fig. 9A). Na dodecyl-SO4-electrophoresis of the reduced samples (Fig. 9B) revealed that even in the presence of iPrzP-F the E.c. procoagulant had cleaved Pγ, hence reduction resulted in the appearance of a band indistinguishable from the heavy chain of thrombin.
FIG. 8. Na dodecyl-SO₄-electrophoresis of unreduced and reduced samples of F₁, F₂; incubated with thrombin (T) for various lengths of time, and unreduced and reduced samples of prothrombin treated with E.e. procoagulant for 30 min in the absence of iPr₂P-F. Reduced samples are designated by BME (2-mercaptoethanol) below the incubation time, which is given in minutes. In a subsequent experiment the same samples were subjected to electrophoresis in Na dodecyl-SO₄ and then stained for glycoprotein by the method of Zacharius et al. (24). The following (unreduced) bands took up stain: P⁺; P⁺; P⁺; T; F⁺; F⁺. After reduction, positive staining was also seen in the Subfragment β and T_b bands, but not in Subfragment α, F₂T_A, F₁, or T_A. The upper arrow indicates a band which appeared upon reduction of the F₂ starting material and must have arisen from the P₂ band. The finding that it was converted to P₂ (cf. 0, 2-mercaptoethanol and 60, 2-mercaptoethanol) suggests that thrombin can cleave a peptide bond within a disulfide-bridged region of the F₂ moiety. The lower arrow indicates a contaminant which was present in the thrombin added.

(i.e. T_b) and a second band which migrated slower than F₂. When iPr₂P-F was absent from the incubation mixture, this second band disappeared, concurrent with the appearance of F₂. Na dodecyl-SO₄-electrophoresis of combinations of reduced samples showed it to be identical with the F₂T_A chain formed by the action of E.e. procoagulant on prothrombin (Fig. 6B). Thus E.e. procoagulant per se was responsible for cleavage of the peptide bond between F₂T_A and T_b (yielding exclusively P⁺ when the starting material was P₂), whereas the active thrombin produced in the absence of iPr₂P-F catalyzed the removal of F₂ (see Fig. 1).

Since early work (1, 2) had shown that a single cleavage (yielding an isoleucine NH₂ terminus) was associated with the generation of thrombin activity, it now became important to determine whether the two-chain protein, P₂, produced by E.e. procoagulant in the present study possessed such activity. Inasmuch as the experiments described above (Fig. 6B, inter alia) demonstrated that P₂ remains stable only under conditions in which thrombin is inhibited (e.g. in the presence of iPr₂P-F), prothrombin was treated for 2 hours with E.e. procoagulant in the presence of iPr₂P-F and immediately chromatographed on DEAE-cellulose (Fig. 10). To ensure complete removal of the E.e. procoagulant, the column was developed with 50 ml of the starting buffer (0.15 m NaCl/0.02 m Tris-Cl, pH 7.4) prior to initiating the gradient (cf. Fig. 7). The elution pattern of this sample was similar to that resulting from DEAE-cellulose chromatography of prothrombin treated with thrombin (Fig. 7). The fractions containing P₂ (which appears at the same point in the elution pattern as P₂) were pooled and concentrated on a Minicon B15 filter. This pool (P₂ in Fig. 10) and the F₂ pool (Fig. 10) were compared with the analogous derivatives resulting from the action of thrombin on prothrombin (Fig. 7). Fragments F₁ produced by E.e. procoagulant and by thrombin were found to be identical in their electrophoretic properties, as were P₂ and P₂, P₂ (Fig. 11).

When the chromatographically isolated P₂, P₂ was incubated with fibrinogen, no thrombin activity (i.e. clotting) was detected. Electrophoretic examination before and after reduction (Fig. 12, 0 min) showed that this pool consisted primarily of the two-chain species, P₂. To P₂, P₂ (concentration 3 mg/ml) was added either thrombin (final concentration 100 units/ml) or E.e. procoagulant (50 μl/ml). Incubation with the thrombin over a period of 240 min resulted in the appearance of F₂, with only a slight decrease in the P₂ band (Fig. 12B, With Thrombin) and no generation of thrombin activity. In contrast, incubation with E.e. procoagulant brought about a rapid decrease in P₂ (Fig. 12B, With E.e. Procoagulant, 30 min) concomitant with the appearance of thrombin activity, which increased somewhat during continued incubation (activity at 4 hours = 45 units/ml). Over the 30- to 240-min interval, the incubation with E.e. procoagulant produced thrombin (T), T_b, and a component migrating slightly slower than thrombin.

The E.e. procoagulant, unlike thrombin, was capable of generating thrombin activity from the P₂, P₂ which had been formed when either prothrombin or P₂ is treated with E.e. procoagulant in the presence of iPr₂P-F. The P₂ formed when either prothrombin or P₂ is treated with E.e. procoagulant in the presence of iPr₂P-F is actually iPr₂P⁺ since the iPr₂P moiety of iPr₂P-F is incorporated into the newly-formed serine active center. This was demonstrated by the experiment described in the last paragraph under “Experiments and Results.”
produced in the presence of iPr$_2$P-F and therefore initially possessed no thrombin activity. Since this generation accompanied a decrease in P$_3$ (which was readily brought about by E.c. procoagulant but not by thrombin at the levels used in this experiment (see Fig. 12B)), the active species was evidently formed from P$_3$. In view of the fact that F$_2$ appeared only after the production of thrombin activity, the early clotting activity must not have been due to thrombin (T) (which could have arisen only by removal of F$_2$ from P$_3$ or P$_2$) but rather to the P$_2'$ formed from P$_2$ by the action of E.c. procoagulant. The F$_2$ initially produced must then have been cleaved off by the enzymically active P$_2'$.  

In contrast to these results were the observations made when iPr$_2$P-F was present in the incubation mixture. Under this latter condition neither thrombin activity nor F$_2$ was generated regardless of whether the starting material was purified P$_3$ (Fig. 9A) or prothrombin itself (Fig. 6A). These findings indicated that the P$_2'$ generated by E.c. procoagulant must, like thrombin, have a serine active center which can react with iPr$_2$P-F. If this reaction (which results in the incorporation of iPr$_2$P) occurs immediately upon formation of P$_2'$, no thrombin activity should be detectable, and peptides which are released by thrombin (but not by E.c. procoagulant) should not appear.  

Activity and Active Center of P$_2'$—Although the results of the foregoing experiments could be adequately accounted for by this explanation, they did not provide direct evidence for the serine active center of P$_2'$ it presupposes. The following pair of experiments was therefore carried out. [32P]iPr$_2$P-F was dissolved in 1 M carrier iPr$_2$P-F at a concentration of 1 mCi/ml. In the first experiment this solution was added to prothrombin, allowed to stand for approximately 2 min, and mixed with E.c. procoagulant so as to achieve the following concentrations: iPr$_2$P-F, 2 mM; prothrombin, 3 mg/ml; E.c. procoagulant, 50 µl/ml. After incubation of this mixture for 0, 30, 60, 120, and 240 min, re-
then prepared for Na dodecyl-SO₄-electrophoresis. After electrophoresis aliquots of the [α³P]iPr₂P-F solution, held at 22° for 5 min, and placed in vials containing 10 ml of Aquasol and counted. Fig. 13 shows the gels and the distribution of radioactivity found. Prothrombin (P₁) bound no [α³P]iPr₂P-F. When the [α³P]iPr₂P-F was present in the incubation mixture, radioactivity appeared in the P₃,F₁ band. Upon reduction, no radioactivity was found in this position (showing that P₃, but not P₁, had become labeled); all of it was associated with T₂. When the [α³P]iPr₂P-F was admixed after various periods of incubation (gels not shown), radioactivity was found both in the P₃,F₁ region and in the thrombin (T) band. Upon reduction, all of the label migrated as T₂.

**DISCUSSION**

The major catalytic effect of E.chroprocoagulant appears to be the activation of prothrombin. Whereas Sakuragawa et al. (25) have reported that whole Echis carinatus venom can activate the Factor X in plasma, we found that the purified procoagulant exhibited no activator activity toward either Factor X or Factor IX, though it could slowly digest fibrinogen. Its ability to activate prothrombin assumed particular significance with the demonstration that treatment of defective prothrombin (induced by the administration of vitamin K antagonists) with Echis carinatus venom produced active thrombin (15). The capacity of E.chroprocoagulant to activate the abnormal prothrombin appears to be a function of its independence from the effects of ionic calcium (12). That is, Gitel et al. (26) have shown the importance of F₁ in the interaction of normal prothrombin with Factor Xₙ- phospholipid-Ca²⁺; the portion of the prothrombin molecule represented by this fragment is altered by vitamin K antagonists (14, 27). On the basis of these findings one would predict that removal of F₁ altogether should yield species which Xₙ- phospholipid-Ca²⁺ could convert to thrombin only slowly, but which an E.chroprocoagulant system could activate readily.

This prediction was verified by measuring two-stage activity during the incubation of prothrombin with E.chroprocoagulant, though additional experiments were required to delineate individual steps in the activation. With the release of F₁ (Fig. 6A) the two-stage activity dropped precipitously (Fig. 4), in agreement with the findings of Kiesiel and Hanahan (6). When iPr₂P-F was present in the E.chroprocoagulant system, this decrease continued, owing to the formation of inactivated P₃. In the absence of iPr₂P-F, however, two-stage activity reappeared (Fig. 4), coincident with a decrease in the amount of P₃, the disappearance of P₁, and the release of F₁ (Fig. 6B). This paradoxical (Cheshire cat) phenomenon arises from the facts that (a) the two-stage assay used here measures both prothrombin and thrombin activity and (b) the thrombin activity (i.e. clotting activity) of P₃ is less than that of thrombin (T). As P₃, which is formed quite early (Fig. 6) and exhibits much more Tos-Arg-OMe hydrolytic activity than clotting activity (Fig. 3), is converted to thrombin can be brought about by thrombin and, evidently, by P₃ itself; however, in the first stage of the two-stage assay (a 3- to 4-minute incubation in the method used here) catalysis of this conversion by the Factor Xₙ complex must have been minimal.

Such differences in the abilities of intermediate activation products to serve as substrates for the Xₙ complex or thrombin may underlie the observation made by Schieck et al. (13) and confirmed in our laboratory that treatment of plasma with E.chroprocoagulant produced only about 10% of the total potential thrombin activity. Moreover, after an early "burst," no progressive generation of thrombin activity occurred. Individual pro-
Fig. 12. Na dodecyl-SO₄-electrophoresis of P₃, P₂ (3 mg/ml) incubated with E. c. procoagulant (50 µl/ml) or with thrombin (100 units/ml). A, unreduced samples. B, reduced samples. Numbers under gels specify minutes of incubation. The upper arrow indicates an unidentified component which does not appear to be P₃ inasmuch as it resolved from T before reduction and was not seen after reduction. Its structure may be related to that of the component indicated by the upper arrow in Fig. 8. The lower arrow indicates a contaminant which was present in the thrombin added.
teinss were not isolated from the plasma milieu. However, if the reaction pathway was similar to that occurring when E.c. procoagulant was incubated with prothrombin (Figs. 6A and 6B), F₁ and P₂ would have been the major early products. These would have arisen via cleavage at the F₁/F₂ and Tₐ/Tₚ sites (Table II), the only peptide bonds in the prothrombin molecule known to be susceptible to E.c. procoagulant. Generation of thrombin (T) would require the action of P₂, the Factor X, complex, or (later) the thrombin produced, inasmuch as E.c. procoagulant is unable to cleave the F₂/Tₐ bond and release F₂ (Table II). As indicated above, the X, complex would be of limited effectiveness toward substrate(s) lacking the F₁ moiety (cf. Fig. 6C); similarly, thrombin concentration would probably be low during the early phases of the activation. The failure of P₂ to catalyze the production of thrombin in plasma remains unexplained; perhaps plasma antithrombins diminish (28) or modulate (29) its activity.

In contrast to the uncertainty surrounding the chain of reactions initiated by E.c. procoagulant in plasma, the present study has provided evidence for a number of discrete steps in purified systems. These are summarized in Fig. 14. Upon treatment of prothrombin with E.c. procoagulant, F₁ is released with the formation of P₂, P₂ is subsequently cleaved to the two-chain species P₂ with no change in molecular weight. Möriya et al. (30) have shown that a similar pathway is followed when bovine prothrombin is activated by E.c. procoagulant. These reactions occur in the presence of inP₂P-F (Fig. 6), showing that they are catalyzed by E.c. procoagulant itself. Formation of P₂ can also be brought about by thrombin (>10 units/ml for 4 hours) and, in all probability, by P₁, suggesting that this is an important mechanism of prothrombin conversion once these species are generated. Moreover, the F₁ released also undergoes cleavage by thrombin (>1000 units/ml for 1 hour) yielding the two-chain compound P₁ (Fig. 8).

Removal of F₂ from P₂ produces thrombin (T). This reaction does not take place when inP₂P-F is present (Fig. 6), indicating that it is not catalyzed by E.c. procoagulant. Similarly, removal of F₁ from P₁ to yield P₂ occurs only in the absence of inP₂P-F (Fig. 9 and Footnote 7). These results, as well as those obtained by treating P₂ with thrombin (Fig. 12), provide conclusive evidence that thrombin (>100 units/ml for 4 hours) catalyzes the release of F₂. This represents a striking confirmation of the observation made by Stenn and Blout (4) and later by Kusiel and Hanahan (6) but is contrary to the report of Seegers et al. (31).

P₃ does not appear as a major intermediate in the E.c. procoagulant activation system (Figs. 6 and 9). This is to be expected inasmuch as most of the P₃ molecules are cleaved at the Tₐ/Tₚ site (forming P₂) prior to the removal of F₁. However, the ability of the E.c. procoagulant to catalyze hydrolysis at this site, plus the finding that any P₂ generated subsequently disappears, indicate that E.c. procoagulant does convert P₂ to thrombin.

It can be seen from the over-all reaction scheme (Fig. 14) that the conversion of prothrombin (P₁) to thrombin (T) is not accomplished exclusively via cleavages catalyzed by E.c. procoagulant. In additional experiments (gels not shown) treatment of prothrombin with thrombin produced F₁ as well as P₂. Furthermore, to rule out the possibility that activated Factor X was responsible for the removal of F₁, the following experiment was carried out. Prothrombin was incubated with thrombin for 6 hours in the presence of soybean trypsin inhibitor and aprotinin (Trasylol, Bayer A.G., Leverkusen, Germany). Final concentrations were prothrombin, 4.1 mg/ml; thrombin, 100 units/ml; soybean trypsin inhibitor, 0.5 mg/ml; aprotinin, 200 kallikrein inactivator units/ml. After the incubation the reaction mixture was placed on a DEAE-cellulose column (2.2 x 35 cm) and developed with a linear gradient from 0.15 M NaCl/0.02 M Tris to 0.50 M NaCl/0.066 M Tris-Cl (total volume 500 ml). The peak regions were pooled separately, concentrated on a Minicon B15 filter, and the solutions prepared for Na dodecyl-SO₄-electrophoresis. F₁ was found in the last peak. It can be seen from the over-all reaction scheme (Fig. 14) that the conversion of prothrombin (P₁) to thrombin (T) is not accomplished exclusively via cleavages catalyzed by E.c. procoagulant.
coagulant. That is, an enzyme with thrombin activity is necessary for scission of the \( F_2/\alpha \) bond (Table II). However, the two-chain intermediate \( P' \) possesses this activity, as evidenced by its ability to (a) hydrolyze Tos-Arg-OMe, (b) clot fibrinogen, and (c) incorporate \([^{32}P]{\text{IP}2P}\). This evidence thus indicates that generation of thrombin activity does not require removal of the entire “pro” portion of the prothrombin molecule but absolutely requires cleavage at the \( \alpha/\beta \) sites so as to produce a serine-active center.

Note Added in Proof—After the present paper was submitted for publication, a paper by Kornalik and Blombäck (32) was submitted to Thrombosis Research. The latter confirmed the findings of Morita et al. (30) regarding the activation of bovine prothrombin by E.C. procoagulant.

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