Studies of the Prothrombin Activation Pathway Utilizing Radioimmunoassays for the F₂/F₁₋₂ Fragment and Thrombin–Antithrombin Complex

By Jerome M. Teitel, Kenneth A. Bauer, Herbert K. Lau, and Robert D. Rosenberg

We have evaluated the efficacy of utilizing radioimmunoassays (RIAs) for prothrombin activation fragments (F₂/F₁₋₂) and for thrombin–antithrombin complex (TAT) in purified systems and in whole blood. During venipuncture, appropriate anticoagulants were employed in order to prevent the generation of thrombin and factor Xa. The RIAs were shown to be specific for F₂/F₁₋₂ as well as TAT and did not interact with other plasma components. Initially, thrombin generation was studied in a purified human system of prothrombin, antithrombin, factor Xa, and factor V as well as phospholipid and Ca²⁺. Under these conditions, the kinetics of F₂/F₁₋₂ and TAT generation were virtually superimposable. However, when factor V was omitted from the reaction mixture, a significantly greater amount of F₂/F₁₋₂ as compared to TAT was observable. Subsequently, prothrombin activation was monitored during the spontaneous coagulation of freshly drawn blood. Throughout the entire course of thrombin generation, the observable rate of formation of F₂/F₁₋₂ was considerably greater than that of TAT. We have examined the levels of F₂/F₁₋₂ and TAT in normal individuals. Our studies indicate that the concentrations of F₂/F₁₋₂ and TAT average 1.97 nM and 2.32 nM, respectively. We have also quantitated the concentrations of F₂/F₁₋₂ and TAT in patients with disseminated intravascular coagulation. In these individuals, the levels of both components are elevated. However, the ratio of F₁₋₂ to TAT ranges from 2.37 to 5.55. Thus, we conclude that under in vivo conditions, prothrombin activation is characterized by the accumulation of a stable precursor, such as prethrombin-2, and that this phenomenon may be related to an alteration of factor V function.

The conversion of prothrombin to thrombin is the key event in the coagulation of blood. Activation of the above zymogen takes place at an appreciable rate only in the presence of factor Xa, factor V, calcium ions, and a lipid surface (platelets). This process may occur in stepwise fashion with the cleavage of activation fragments F₁ and F₂ from the amino terminus of the parent zymogen. The above transformation results in the generation of the intermediate species prethrombin-1 and prethrombin-2. The latter component may also be formed from prothrombin by liberation of a single polypeptide, F₁₋₂. Subsequently, prethrombin-2 can be internally scissioned to yield thrombin. Once produced, this enzyme may act on its substrates, which include fibrinogen, factor V, factor VIII, factor XIII, as well as specific receptors on platelets and endothelial cells. Alternatively, this serine protease may be inhibited by its natural antagonist, the plasma protein antithrombin, via the formation of a stable inactive enzyme–inhibitor complex. Although other plasma proteins play a minor role in the inactivation of thrombin, considerable evidence favors antithrombin as the inhibitor of primary physiologic importance.

When the coagulation system is activated during pathologic conditions, only a small percentage of circulating prothrombin is transformed to thrombin (<1%). Furthermore, the resultant enzyme is rapidly neutralized by antithrombin. Therefore, direct measurements of zymogen, serine protease, or inhibitor should be unable to detect subtle degrees of thrombin generation within the blood. In contrast, quantitation of F₂/F₁₋₂ or TAT would allow one to monitor small degrees of zymogen conversion provided that these two species exhibited prolonged survival within the circulation.

We have recently reported the isolation of specific antibody populations directed against TAT and F₂/F₁₋₂. These antibody populations were utilized to construct sensitive and specific radioimmunoassays that are designed to measure these molecular species. In this article we outline studies that demonstrate that these assays are able to quantitate F₂/F₁₋₂ and TAT within the blood. In addition, we provide evidence that the conversion of prothrombin to thrombin in blood is characterized by an accumulation of intermediate species with a resultant delay in the appearance of enzyme. Furthermore, we establish the range of concentrations of F₂/F₁₋₂ as well as TAT present in normal individuals and demonstrate that elevated levels of these species are found in patients with disseminated intravascular coagulation.

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RIAs FOR PROTHROMBIN ACTIVATION

MATERIALS AND METHODS

Column Chromatographic Materials

DEAE-Sephadex was purchased from Bio-Rad (Richmond, Calif.). Sephadex G-25 and Sepharose 4B were obtained from Pharmacia Fine Chemicals (Piscataway, N.J.). Prothrombin, F1, and antithrombin were individually bound to cyanogen bromide-activated Sepharose 4B by the method of Porath et al. with modifications as described in earlier communications from this laboratory. In all cases, approximately 0.3 mg protein/ml of "packed" gel was coupled to the affinity matrix.

Chemicals

All chemicals were reagent grade or better.

Leech Protein and Snake Venoms

Factor X activating enzyme was prepared from Russell’s Viper Venom and was kindly provided by Dr. Bruce Furie (Tufts University, Boston, Mass.). Echis carinatus venom was obtained from Sigma, St. Louis, Mo. Hirudin* was purchased from Pentapharm (Basel, Switzerland).

Proteins

Bovine serum albumin (BSA) and nonimmune goat IgG were obtained from Miles Laboratories (Elkhart, Ind.). Nonimmune rabbit IgG was either provided by Miles Laboratories or isolated from crude serum by ammonium sulfate precipitation and DEAE cellulose column chromatography according to the method of McGuigan. Staphylococcal Protein A was purchased from the New England Enzyme Center, Inc., Boston, Mass.

Human prothrombin was prepared by the method of Shapiro et al. with modifications as described in a prior communication. Human factor V, human factor Xa, human TAT, human antithrombin, and human thrombin were purified by techniques established in our laboratory. Human F1, human F2, and human F1,2 were isolated as previously described.

Measurement of Protein Concentration

Protein concentrations were determined by absorbance readings at 280 nm. The molar extinction coefficients for human prothrombin, human F1, human F2, human F1,2, human thrombin, human antithrombin, and rabbit IgG were assumed to be 13.6, 11.9, 12.5, 12.9, 16.2, 6.5, and 15, respectively. Proteins whose extinction coefficients have not previously been determined, such as TAT, were calculated utilizing F1-Septharose-4B and Prothrombin-Sepharose-4B columns.

Esterolytic Assay

Thrombin was detected by its ability to release 1H from benzyloxycarbonyl arginine [3H]-ethyl ester-HCl ([3H]BzArgOEt) as described by Beaven et al. and modified by Jordan et al.

Gel Electrophoresis

The sodium dodecyl sulfate (SDS) gel electrophoretic technique of Laemmli et al. was used to establish homogeneity of protein components, to determine apparent molecular weight of various species, and to monitor conversion of one component to another.

Collection of Blood Samples

Venipunctures were performed atraumatically with 21-gauge or 23-gauge butterfly infusion sets using a two-syringe technique. When anticoagulants were employed, blood samples were drawn into plastic syringes preloaded with appropriate solutions as described below. These included: (1) ACD: citric acid 7.3 mg/ml, sodium citrate 22 mg/ml, dextrose 24.5 mg/ml. The ratio of anticoagulant to blood was used 0.15:1.0 (v/v). (2) "TAT anticoagulant": ACD, ethylene–diminietraacetic acid (EDTA) 2.234 mg/ml, adenosine 1.602 mg/ml, and hirudin 25 U/ml. The ratio of anticoagulant to blood employed was 0.2:1.0 (v/v). (3) "F1 anticoagulant": TAT anticoagulant with the addition of heparin at a final concentration of 25 U/ml. The ratio of anticoagulant to blood utilized was 0.2:1.0 (v/v). After collection of blood samples, plasma fractions were obtained by centrifugation at 4°C for 15 min at 1600 g and stored at –80°C prior to use.

Preparation of Antisera

F1/F1,2, and TAT antiserum. These antisera were raised in rabbits and processed according to techniques outlined earlier.

F1, antiserum. This antiserum was raised in rabbits according to methodologies previously described and processed in a manner similar to that outlined for F1/F1,2 except that the affinity chromatography was performed utilizing F1,-Septharose-4B and Prothrombin-Sepharose-4B columns.

Prothrombin antiserum. Crude goat serum was purchased from Atlantic Antibodies (Scarborough, Maine) and was incubated at 56°C for 30 min to remove residual fibrinogen as well as destroy complement components. The IgG fraction was then isolated by ammonium sulfate precipitation and was filtered through a prothrombin–Septharose-4B column. The relevant antiprothrombin antibody population was eluted from the column, neutralized, and dialyzed as outlined earlier.

Thrombin antiserum. Crude rabbit serum was a gift from Dr. S. Shapiro (Philadelphia, Pa.). It was heated as described above and then utilized without further purification.

Antithrombin antiserum. Crude goat serum was purchased from Atlantic Antibodies, and heated as previously outlined. The IgG fraction was then obtained by ammonium sulfate precipitation and was demonstrated to be monospecific for species containing antithrombin as outlined in a prior communication. Rabbit anti-goat IgG sera, and sheep anti-rabbit IgG sera were obtained as previously described.

Radioimmunoassays

Radiolabeling of prothrombin, F1, F2, F1,2, thrombin, and TAT with Na125I was carried out by the chloramine-T method of Greenwood et al. Thrombin was radiolabeled as described above after alkylphosphorylation with a 100-fold molar excess diisopropyl fluorophosphate (DFP). Double antibody type RIAs were performed for F1 and TAT as described in earlier reports from our laboratory. The F1,2 RIA was carried out in a similar fashion employing labeled F1,2, unlabeled F1,2 standards, as well as the specific F1/F1,2 antibody population, since this latter material cross-reacts extensively with F1,2.

F1, RIA. This assay employed labeled F1, unlabeled F1 standards, as well as the specific F1 antibody population in a manner entirely analogous to the F1/F1,2, RIA.

Thrombin RIA. This assay was conducted with labeled diisopropyl phosphorylated thrombin (DIP-thrombin), unlabeled DIP-thrombin standards, as well as the appropriate antisera using techniques outlined for the F1/F1,2, RIA.
Prothrombin RIA. This assay was carried out with labeled prothrombin, unlabeled prothrombin standards, as well as the appropriate IgG fraction according to the procedure described for the F$_2$/F$_1$$_3$, RIA, except that the second antibody system consisted of 100 µl of rabbit anti-goat IgG serum and 50 µl of the optimal concentration of goat IgG.

Analysis of Data
Estimation of relative immunoreactivity, computation of the slopes of the dose–response curves, as well as determinations of various associated indices were obtained by a least-squares fit of the RIA results to a "four parameter" model as described by Rodbard et al. Statistical analyses of data were conducted by standard techniques. In most instances, the means are provided with associated standard errors. The slopes and midpoints, respectively, of the logit-log dose–response curves for TAT, F$_2$, and F$_1$$_3$, were –0.955 ± 0.07 (SE) and 5.95 ± 0.77 (SE), –0.849 ± 0.02 and 2.85 ± 0.25, –0.867 ± 0.05 and 5.67 ± 1.22, respectively.

RESULTS
Radioimmunoassay of TAT in Human Plasma
Specificity of the antibody population has been documented in detail in an earlier communication from this laboratory. This parameter was assessed by comparing the molar concentration of a given species to that of TAT complex that displaced 50% of the immunoprecipitable $^{125}$I-TAT counts from the antibody population. Neither thrombin or antithrombin, nor any of the related hemostatic system polypeptides, were found to react to a significant extent.

In order to accurately measure ambient levels of TAT in plasma, it is essential that thrombin generation and subsequent protease inhibitor complex formation be completely inhibited at the time of venipuncture. The TAT anticoagulant (see Materials and Methods) was devised to satisfy this requirement. The efficacy of the solution was demonstrated by immediately mixing blood samples from four normal donors with the above anticoagulant mixture. Purified human thrombin (0.1 µg/ml) or buffer were then added to different aliquots of each sample, and TAT immunoreactivity was subsequently quantitated. The levels of this component in aliquots to which enzyme had been added were not significantly different from those in which buffer had been admixed (1.26 nM ± 0.01 versus 1.31 nM ± 0.01).

The estimation of plasma TAT concentrations by RIA depends on two assumptions. First, it is essential that extraneous plasma components do not contribute to the immunoreactive signal. Second, the immunoreactivity of TAT should be identical in the physiologic environment (plasma) and in the standard curve environment (RIA buffer).

To test the first assumption, we attempted to specifically remove TAT from plasma by affinity chromatography with anti-AT–Sepharose-4B (IgG directed against antithrombin bound to Sepharose 4B). The lack of any residual immunoreactive signal would provide evidence for the absence of a spurious contribution by other plasma constituents to the apparent level of TAT. Furthermore, if the material so removed could be shown to contain immunoreactive concentrations of thrombin in the predicted amount, strong presumptive independent evidence for its identity as TAT would be available.

To this end, most of the antithrombin was removed from plasma by batch adsorption with heparin–Sepharose-4B in order to prevent saturation of anti-AT–Sepharose-4B in the subsequent separation procedure. This was accomplished by first increasing the plasma sodium chloride concentration to 0.3 M, and then adding 1/10 volume of heparin–Sepharose-4B. The slurry was centrifuged at 24°C for 5 min at 1000 g and the supernatant was subjected to two further adsorption steps in an identical manner. The final supernatant was dialyzed against 0.5 M NaCl in 0.05 M Na phosphate, pH 7.5, and then filtered at 20 ml/hr through a column of anti-AT–Sepharose-4B (0.9 x 46 cm) equilibrated with the same buffer. Bound protein was eluted from the affinity matrix with 0.5 M NaCl in 1 M acetic acid, pH 2.4, and immediately neutralized with 2 M NaOH. The heparin–Sepharose-4B batch supernatant (step 1), the anti-AT–Sepharose-4B column effluent (step 2), and the material eluted from the latter column were dialyzed against RIA buffer without added BSA. Subsequently, these samples were quantitated for TAT and thrombin by specific RIAs. The results of these experiments are summarized in Table 1. The data reveal that TAT immunoreactivity is not altered by heparin–Sepharose-4B batch adsorption, but is completely removed by filtration through anti-AT–Sepharose-4B. The protein eluted from the affinity matrix was found to contain 97.5% of the initial plasma TAT, and exhibits an equivalent amount of thrombin immunoreactivity as quantitated by an independent RIA directed against this enzyme.

To examine the second assumption, we attempted to ascertain whether the immunoreactivity of TAT is altered by the plasma environment. For this purpose, we added purified TAT to both normal plasma and plasma depleted of TAT by the technique described above. These samples were then assayed by TAT RIA. The results of these studies are summarized in Fig. 1A. Linear regression analysis of the data yields a line described by the equation $y = 1.06 x + 0.13$, with an associated correlation coefficient ($r$) of 0.99. Similar examination of the results derived from addition of TAT to depleted plasma yields a line described by the equation $y = 1.01 x - 0.64$ ($r = 1.00$). Both of the above lines closely approximate the theoretical line of
constituents. The antibody population employed of the BaCl₂ precipitate, step 2 refers to the supernatant after specific immunoadsorption, and recovered material refers to the redissolved material of this component were greater than expected by a factor of 2.06 ± 0.11 (SE). Given an average hematocrit of 0.42, this might be explained on the basis of exclusion of TAT from the cellular elements of blood. This impression was confirmed by the demonstration that ¹²⁵I-TAT counts added to blood did not distribute into the cellular element fraction (data not shown).

Radioimmunoassay of F₂ and F₁₁₂ in Human Plasma

Specificity of the F₂ antibody population was assessed by comparing the molar concentration of a given species to that of F₂, which displaced 50% of the immunoprecipitable ¹²⁵I-F₂ counts from a given amount of antibody. As reported in an earlier communication from this laboratory, the related proteins F₁, thrombin, prothrombin, and prethrombin-I have minimal degrees of cross-reactivity. However, F₁₁₂ cross-reacts to a significant extent. One mole of this

100% recovery defined as y = x. However, a slight systematic overestimation of TAT is apparent when this species is added to normal plasma.

In a similar set of experiments, we added purified TAT to whole blood samples anticoagulated with the TAT anticoagulant. These mixtures were centrifuged as described in Materials and Methods to obtain aliquots of plasma and then assayed for TAT. The observed levels of this component were greater than that expected by a factor of 2.06 ± 0.11 (SE). Given an average hematocrit of 0.42, this might be explained on the basis of exclusion of TAT from the cellular elements of blood. This impression was confirmed by the demonstration that ¹²⁵I-TAT counts added to blood did not distribute into the cellular element fraction (data not shown).

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antigen has an immunoreactivity equivalent to 0.67 mole of F₂.

It has recently been reported that F₁₂⁺₂⁺ can be generated during prothrombin activation within whole blood. This species was prepared as described by Rabiet et al., and its cross-reactivity with the F₂ antibody population determined. The presence of the fragment 3 domain (mol wt - 1300) was found to have no effect on the immunoreactivity of F₁₂⁺ (data not shown). Therefore, the presence or absence of F₁₂⁺⁺ within the purified or physiologic systems utilized in these studies should not alter any conclusions presented here.

In order to accurately quantitate ambient concentrations of F₂/F₁⁺₂, it is necessary that prothrombin activation be completely suppressed after venipuncture. The “F₂ anticoagulant” was developed for this purpose. The efficacy of this technique was examined by immediately mixing blood samples from five normal donors with the above anticoagulant mixture. Purified human factor Xa (final concentration, 5 μg/ml) or buffer were added to different aliquots of each sample, and F₂/F₁⁺₂ immunoreactivity was subsequently quantitated. The levels of this component in aliquots to which enzyme had been added were not significantly different from those in which buffer had been admixed (0.793 nM ± 0.23 versus 0.756 nM ± 0.28).

The quantitation of F₂ and F₁⁺₂ in plasma by RIA requires that three conditions be satisfied. First, it must be shown that no other plasma constituent is recognized by the antibody population. Second, F₂ and F₁⁺₂ should possess identical immunoreactivities in the physiologic environment (plasma) and in standard curve environment (RIA buffer). Third, it must be possible to separate F₂ and F₁⁺₂ prior to the RIA in order to individually estimate their concentrations prior to assay.

To examine the first assumption, we attempted to specifically remove prothrombin activation fragments from plasma by immunoprecipitation. For this purpose, 1.6 M BaCl₂ was added to plasma at a final concentration of 13 mg/ml (step 1). After incubation at 4°C for 14 hr, the precipitate was removed by centrifugation at 24°C for 5 min at 1000 g, redisolved in 0.02 M EDTA, and dialyzed against RIA buffer without added BSA. Species containing the amino terminal domain of prothrombin adsorb to BaCl₂ due to the presence of γ-carboxyglutamic acid (gla) residues. Thus, F₁⁺₂ should be found in the precipitate, whereas any F₂ present in the sample should remain in the supernatant. Next, 1/10 volume of F₂ antibody at a dilution of 1:40 was added to the solubilized precipitate (step 2). This material was then incubated at 4°C for 48 hr and a rabbit IgG:sheep anti-rabbit IgG second antibody system was added (see Methods section). After a further incubation at 4°C for 18 hr, the precipitate was separated by centrifugation, washed with the RIA wash buffer, and resolubilized in 50 mM NaOH to dissociate the antigen–antibody complexes. Free antibody molecules were adsorbed by a 10% suspension of crude Staphylococcal Protein A and removed by centrifugation. The BaCl₂ supernatant, the immunoprecipitation supernatant, and the free antigen recovered as described above were dialyzed extensively against RIA buffer without added BSA and examined by F₂/F₁⁺₂ RIA. The results of these experiments are summarized in Table I. The data reveal that plasma F₂/F₁⁺₂ immunoreactivity was quantitatively adsorbed by BaCl₂, which suggests that this physiologic fluid contains F₁⁺₂, but not F₂. In addition, this finding demonstrates that only gla-containing proteins contribute to the plasma immunoreactive signal. Furthermore, the subsequent recovery from the immunoprecipitate of 53.6% of the plasma F₁⁺₂ reactivity tends to confirm the accuracy of the initial F₁⁺₂ quantitation. Given the numerous steps required to separate the above species from antigen–antibody complexes, it is not surprising that significant amounts of F₁⁺₂ were lost.

The identity of the recovered product was further documented by employing the F₁⁺₂ RIA, which recognizes F₁⁺₂ with equal avidity but prothrombin to an insignificant degree (see legend to Table I for details). It should be noted that the immunoreactivity of the recovered material as judged by the F₁⁺₂ RIA must represent F₁⁺₂, since the F₂/F₁⁺₂ antibody population used in the immunoprecipitation step does not interact with F₂. As shown in Table I, the F₁⁺₂ immunoreactivity as measured by F₁⁺₂ RIA was similar to that measured by the F₂/F₁⁺₂ RIA. This close agreement provides strong evidence that F₁⁺₂ is the only species that contributes to the F₁⁺₂ immunoreactive signal of normal plasma.

The F₂/F₁⁺₂ antibody population has previously been shown to exhibit a prothrombin molar reactivity of 1/2667 that of F₂/F₁⁺₂ when appropriately purified proteins were employed. Therefore, a normal plasma prothrombin level of approximately 1.5 μM might contribute as much as 0.56 nM to the plasma F₂/F₁⁺₂ RIA signal. This would correspond to 15.5% of the apparent F₁⁺₂ level (see below). To examine this phenomenon, the zymogen was quantitated in the

†The small differences between results obtained with the F₁⁺₂ RIA and the F₁⁺₂ RIA may be due either to experimental error or to low levels of proteolysis of the N-terminal region of F₁⁺₂, that might occur during the isolation procedure or to the loss of small numbers of γ-carboxyglutamic acid moieties within this domain that could take place with the preparative method.
systematic overestimation of plasma F,
2
added to whole blood collected with the F2 anticoagu-
linant. These mixtures were centrifuged as described in
Materials and Methods to obtain aliquots of plasma
and then assayed for F2/F1.2. The observed levels of F2
and F1.2 exceeded those expected by factors of 2.18 ±
0.19 (SE) and 2.09 ± 0.20 (SE), respectively. In
x
-36
addition to the experiment described above for TAT,
125I-F2 and 125I-F1.2 counts added to blood did not
distribute into the cellular element fraction (data not
shown).

To satisfy the third assumption, we attempted to
develop methods for separately quantitating the
plasma levels of F2 and F1.2 with the F2/F1.2 RIA. For
this purpose, the two polypeptides were added singly or
together to normal plasma. These solutions were then
subjected to adsorption with BaCl2. It was expected
that F1.2 would bind to the barium salts, whereas F2
would remain in the supernatant. The adsorption was
carried out by admixing a sufficient volume of 1.6 M
BaCl2 with the samples so that a final concentration of
0.072 M was attained. The solutions were agitated at
24°C for 30 min, incubated at 4°C for 16–18 hr, and
then centrifuged at 4°C for 30 min at 6450 g to
separate the insoluble barium salts from the supernatant
plasma. The adsorbed protein was recovered from
the insoluble barium salts by resuspending the pellet in
RIA buffer with 0.05 M EDTA, pH 7.5, and then
then adjusting the volume to equal that of the initial
plasma sample. The excess BaCl2 in the supernatant
plasma was removed by chelation with 1/4 volume of
0.25 M EDTA, pH 7.5. The various samples were
subsequently assayed with the F2/F1.2 RIA. The
results of these experiments are summarized in Table
2. It is apparent from the data that the plasma
 concentrations of F2 as well as F1.2 can be indepen-
dently quantitated with the above separation system.

Studies of Prothrombin Activation
Experiments Using Purified Reagents

In order to examine the sequence of events leading to
thrombin formation, we performed a series of experi-
ments in which purified human prothrombin was acti-

<p>| Table 2. Quantitation of F2 and F1.2 Added to Normal Plasma |
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<table>
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<th>F2 Added to Plasma (nM)</th>
<th>F1.2 Added to Plasma (nM)</th>
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<th>F1.2 Measured in BaCl2 (nM)</th>
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activated by factor Xa in the presence of factor V, cephalin, and Ca$^{2+}$ ions. The environmental conditions were established at 0.01 M CaCl$_2$, 0.118 M NaCl in 0.01 M Tris-HCl, pH 7.5, and 37°C. Aliquots were removed from the incubation mixtures at timed intervals from 0 to 60 min and mixed with an anticoagulant consisting of sodium citrate, heparin, and antithrombin at final concentrations of 3.8 mg/ml, 5 U/ml, and 0.2 mg/ml, respectively. The various samples were then analyzed by F$_2$/F$_{1+2}$ RIA and TAT RIA.

The validity of the above measurements are dependent on two assumptions. First, thrombin formed in the reaction must be quantitatively inhibited by antithrombin in the anticoagulant. In this case, the levels of thrombin generated will be equal to the concentrations of TAT measured. Second, it must be possible to accurately quantitate F$_2$/F$_{1+2}$ and TAT in the above environment. To demonstrate that thrombin is immediately incorporated into the TAT complex when the incubation mixtures are quenched with the anticoagulant, we used the synthetic substrate $[^3H]$BzArgOEt to measure free enzyme. Using this assay, we repeatedly showed that aliquots sampled from the prothrombin-activation mixture into the heparin–antithrombin anticoagulant contained no free thrombin activity at a time when prothrombin had been maximally activated as determined by F$_2$/F$_{1+2}$ RIA. However, a second aliquot obtained at the same time but delivered directly into the substrate without addition of the heparin–antithrombin anticoagulant revealed that the molar concentration of free thrombin corresponded to the initial level of prothrombin (thrombin concentration – 112% ± 4.65% of initial prothrombin concentration, n = 6).

To prove that F$_2$/F$_{1+2}$ can be measured within the above reaction mixture and that thrombin generated within this environment can be quantitated as TAT, we added F$_2$/F$_{1+2}$, free enzyme plus antithrombin, or TAT to solutions containing all of the above reagents except prothrombin and showed that each of the antigens could be accurately estimated with the appropriate RIA (data not shown).

Under most conditions tested, the generation of F$_2$/F$_{1+2}$ and TAT are tightly coupled. Figure 2A summarizes the results of experiments in which a small fraction of prothrombin was activated (mean 4.9%). Concentrations of prothrombin, factor Xa, factor V, and cephalin were 200 µg/ml, 0.055 µg/ml, 0.203 µg/ml, and 15.0 µg/ml, respectively. The levels of total activation peptide (F$_{1+2}$ represents 81%–89% of the total activation fragment) and TAT are not statistically different at any time point beyond 5 min ($p > 0.1$). Figure 2B provides data from experiments in which prothrombin conversion was complete (mean 116%). Concentrations of prothrombin, factor Xa, factor V, and cephalin were 200 µg/ml, 2.77 µg/ml, 4.88 µg/ml, and 15.0 µg/ml, respectively. The difference between levels of total activation peptide (F$_2$ constitutes 100% of the total activation peptide at times greater than 5 min) and TAT failed to achieve significance beyond 5 min ($p > 0.5$). The mean F$_2$ to TAT ratio was 1.06 ± 0.05. These findings indicate that the stepwise conversion of prothrombin to thrombin within purified systems usually proceeds without kinetic delay.

However, it is possible to establish conditions where significant discrepancies exist between the rates of
RIAs FOR PROTHROMBIN ACTIVATION

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Fig. 3. Activation of prothrombin in the absence of factor V. The concentrations of prothrombin, factor Xa, and cephalin are 200 μg/ml, 13.85 μg/ml, and 15.0 μg/ml, respectively. (○-○) The levels of total activation fragments (F2/F1+2) and (●-●) the levels of TAT. The symbols correspond to the mean of three separate experiments. Error bars indicate the associated standard errors of the mean.

appearance of F2/F1+2 and TAT. Figure 3 depicts the results of experiments of his type. The prothrombin as well as cephalin concentrations were maintained as noted above, whereas the factor Xa levels were set at 13.85 μg/ml and factor V was omitted. It is apparent that a rapid generation of F2 has occurred with little formation of TAT (F1,2 was not detected under these conditions). This indicates that the activation of prothrombin under these conditions is characterized by the marked accumulation of an intermediate species such as prethrombin-2. If this suggestion is correct, it should be possible to convert the prethrombin-2 to thrombin by incubation with *Echis carinatus* venom.22,23 Once the serine protease is produced, it would complex with added antithrombin in the presence of heparin, and the TAT concentration would be expected to rise towards that of F2.

In order to test this hypothesis, prothrombin activation was repeated under identical conditions. After an incubation period of 30 min, the reaction solution was quenched with 10% volume of 3.6% sodium citrate. One aliquot was mixed with antithrombin and heparin at final concentrations of 0.2 mg/ml and 5 U/ml, respectively. A second aliquot was incubated for 4 hr at 37°C with *Echis carinatus* venom at a concentration of 0.826 μg/ml, and then admixed with antithrombin and heparin as outlined above. Prior to the addition of venom, the levels of F2 and TAT were 2995 nM and 88.5 nM, respectively. After incubation with venom, the concentration of TAT was 3540 nM, and the ratio of F2 to TAT was reduced from 33.8 to 1.18. The transformation of prethrombin to thrombin was also documented by SDS gel electrophoresis with reducing agents added. In conjunction with the dramatic rise of TAT level, the prethrombin-2 band of molecular weight 36,000 exhibited a marked reduction in intensity (data not shown).

Experiments Using Whole Blood

After completion of the above experiments, prothrombin activation was studied in a more physiologic setting. Blood samples were obtained from normal donors by atraumatic venipuncture. After discarding the initial 3 ml, an additional 10–15 ml were drawn into a separate syringe (containing no anticoagulant) and immediately delivered into a 50-ml conical polypropylene test tube. The sample was magnetically stirred throughout the experiment, while maintained at 24°C. At varying intervals, aliquots were removed, quenched with F2 anticoagulant, centrifuged to obtain plasma, and analyzed by F2/F1+2 RIA as well as TAT RIA.

To demonstrate that thrombin is quantitatively complexed to antithrombin under the above conditions, an experiment was performed in which varying concentrations of the purified enzyme were added to citrated whole blood at levels of 220–878 nM. The samples were magnetically stirred for 5 min as previously outlined. Aliquots were then removed, mixed with F2 anticoagulant, centrifuged to obtain plasma, and examined by TAT RIA. The recovery of added thrombin as TAT was 118% ± 18.4%, n = 4. Thus, plasma levels of TAT represent valid estimates of thrombin generation within whole blood.

The results of the whole blood coagulation experiments are summarized in Fig. 4. This plot depicts the

![Fig. 4. The activation of prothrombin within whole blood. (○-○) Total activation fragment (F2/F1+2) and (●-●) TAT. The symbols correspond to the mean of three separate experiments. Error bars indicate the associated standard errors of the mean.](image-url)
levels of total activation fragment (F$_2$/F$_{1+2}$) and TAT as a function of time. It should be noted that when concentrations of total activation fragment were equal to or less than 200 nM, F$_{1+2}$ predominated as compared to F$_2$ (F$_{1+2}$ = 91% ± 1.5% of total activation fragment, n = 16). At more extensive degrees of zymogen conversion, progressively greater proportions of F$_2$ vis à vis F$_{1+2}$ were apparent. For example, when the concentrations of total activation fragment ranged from 389 nM to 693 nM, and from 921 nM to 3095 nM, F$_2$ represented 22.7% ± 6.0%, n = 4 and 59.7% ± 4.3%, n = 13 of the sum of the total activation fragments, respectively.

It is apparent from the above figure that the generation of total activation fragment at 24°C is considerably more rapid than that of TAT. Indeed, the average ratio of F$_2$/F$_{1+2}$ to TAT in blood samples obtained at 10, 20, and 30 min is 1.61 ± 0.16. Similar studies conducted at 37°C give an average ratio of F$_2$/F$_{1+2}$ to TAT of 1.71 ± 0.19, which is statistically indistinguishable from that observed at the lower temperature.

The magnitude of this discrepancy between F$_2$/F$_{1+2}$ and TAT at 24°C can also be appreciated by computing the rates of production of these species for each experiment from the time of significant prothrombin activation (10 min) to the point at which stable levels of these components are approached (~20 min). The slope of the regression line obtained from the F$_2$/F$_{1+2}$ data is 148 nM/min ± 16.1 (r = 0.88), which represents the factor-Xa-dependent rate of cleavage of the prothrombin molecule at Arg$_{227}$-Thr$_{231}$. The slope of the regression line obtained from the TAT data is 82.3 nM/min ± 14.0 (r = 0.95), which corresponds to the factor-Xa-dependent rate of scission of the prethrombin-2 molecule at Arg$_{327}$-Ile$_{329}$. The ratio of these rates is in close agreement with the ratio of the final levels of total activation fragment at TAT at 30 min. These findings suggest that under in vitro conditions the coagulation of whole blood is characterized by a relatively constant accumulation of a stable intermediate species such as prethrombin-2.

It might be hypothesized that the accumulation of thrombin precursor is dependent on the 10–15 min delay in the initiation of zymogen conversion. For this reason, an experiment was undertaken to test the effect of eliminating this lag period. To this end, blood was collected without anticoagulant as previously outlined and divided equally between two plastic tubes. Factor Xa (final concentration, 2 µg/ml) was immediately added to the first tube. After 8 min, the above enzyme was also admixed with the second tube at the same final level. Aliquots were then removed at 25-sec intervals, mixed with F$_2$ anticoagulant, centrifuged to obtain plasma, and examined by F$_2$/F$_{1+2}$ RIA as well as TAT RIA. The results of these experiments are summarized in Table 3. The data reveal that the discrepancies between the rates of generation of total activation fragment and TAT are similar in both reaction mixtures. Therefore, the observed delay in the conversion of thrombin precursor to thrombin is independent of the time course of zymogen transformation.

**Studies of F$_2$/F$_{1+2}$ and TAT Under In Vivo Conditions**

The ambient concentrations of F$_2$/F$_{1+2}$ and TAT were established in normal individuals below the age of 40. Blood samples were obtained as described in Materials and Methods with anticoagulants that prevent subsequent generation of F$_2$/F$_{1+2}$ and TAT. The mean values of F$_{1+2}$ and TAT are 1.97 nM ± 0.97 (SD) and 2.32 nM ± 0.36 (SD), respectively. The F$_2$/F$_{1+2}$ signal in normal samples is due entirely to F$_{1+2}$ as

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>TAT (nM)</th>
<th>F$<em>2$/F$</em>{1+2}$ (nM)</th>
<th>Ratio of F$<em>2$/F$</em>{1+2}$ to TAT</th>
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</thead>
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<tr>
<td>0.25</td>
<td>3.63 ± 1.64</td>
<td>10.5 ± 5.04</td>
<td>2.89 ± 0.10</td>
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<tr>
<td>0.5</td>
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<td>21.3 ± 6.89</td>
<td>1.85 ± 0.82</td>
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<td>0.75</td>
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<td>78.9 ± 30.2</td>
<td>2.51 ± 0.42</td>
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<tr>
<td>1.0</td>
<td>108 ± 36.7</td>
<td>221 ± 48.0</td>
<td>2.04 ± 0.25</td>
</tr>
<tr>
<td>8.25</td>
<td>53.3 ± 8.90</td>
<td>277 ± 156</td>
<td>5.20 ± 2.23</td>
</tr>
<tr>
<td>8.5</td>
<td>199 ± 93.3</td>
<td>304 ± 123</td>
<td>1.53 ± 0.17</td>
</tr>
<tr>
<td>8.75</td>
<td>675 ± 429</td>
<td>1,091 ± 565</td>
<td>1.62 ± 0.28</td>
</tr>
</tbody>
</table>

Each of the above values represents the mean of 3 separate experiments with associated standard errors.

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judged by differential BaCl₂ adsorption (data not shown).

In order to initiate an assessment of the sensitivity and specificity of these two RIAs, 6 patients with disseminated intravascular coagulation were studied. The syndrome was identified by characteristic abnormalities of routine clinical tests such as prolonged thrombin time (5 sec > control), diminished fibrinogen concentration (<100 mg/dl), elevated fibrinogen (fibrin) split products (≥40 μg/ml), or reduced platelet count (≤100,000/cu mm). The results are summarized in Table 4. The data reveal that F₁⁺₂/F₂⁺₂ ratios are increased to a lesser extent. In addition, the F₁⁺₂ levels are strikingly elevated, whereas TAT concentrations are increased to a lesser extent. In addition, the F₁⁺₂/F₂⁺₂ signal is solely due to F₁⁺₂ as judged by differential BaCl₂ adsorption (data not shown). Furthermore, the ratios of F₁⁺₂/TAT in the various patients with activated coagulation mechanisms ranged from 2.37 to 5.55.

**DISCUSSION**

Recent communications from this laboratory have described the isolation and characterization of specific antibody populations directed against human TAT and human F₂/F₁⁺₂. In this manuscript, we have attempted to prove that RIAs that utilize these antibody populations can be employed to quantitate TAT, F₂, and F₁⁺₂ within human blood.

First, we have shown that no plasma protein other than TAT or F₂/F₁⁺₂ interacts with the antibody populations. Second, we have demonstrated that the above RIAs can be utilized to measure TAT and F₂ within human blood. Third, we developed a procedure for individually quantitating plasma levels of F₂ and F₁⁺₂.

We have subsequently employed the F₂/F₁⁺₂ RIA and the TAT RIA to delineate molecular events that lead to thrombin generation. During activation of the zymogen, three sequential steps may take place: Prothrombin is cleaved at Arg₂₇₃-Thr₂₇₄ by factor Xa, which leads to the formation of prethrombin-2 as well as the release of F₁⁺₂. Prethrombin 2 is then scissibed at Arg₂₃₂-Ile₂₃₃ by factor Xa, which results in the generation of the two chain serine protease thrombin. The latter species complex with antithrombin on a 1:1 molar basis. Finally, the F₁⁺₂ fragment is clipped at Arg₁₅₅-Ser₁₅₆ by factor Xa or thrombin, which is responsible for the liberation of F₁ and F₂.

The time course of these specific cleavages was studied in purified systems as well as whole blood. Initially, prothrombin was activated with factor Xa in the presence of factor V, antithrombin, cephalin, and Ca²⁺ ions. The kinetics of total activation fragment (F₂/F₁⁺₂) and TAT generation were virtually superimposable under conditions of partial as well as complete zymogen transformation. The close association between the levels of these species ([(F₂ + F₁⁺₂)/TAT] = 0.97) indicates the absence of any significant delay in the two successive bond scissions at Arg₂₇₃-Thr₂₇₄, and Arg₂₃₂-Ile₂₃₃, respectively. The subsequent cleavage of F₁⁺₂ also occurs in relatively rapid fashion. Indeed, F₁⁺₂ represents only 4.4% of the total activation fragment at the point where about 20% of the zymogen has been transformed to enzyme. It is of interest that removal of factor V from the above reaction mixture results in a marked discrepancy between the amounts of F₂ and TAT generated. (F₁⁺₂ was not detected under these conditions.) At completion of zymogen conversion, the ratio of F₂ to TAT averaged 33.8. This phenomenon must reflect a delay in the cleavage of Arg₂₃₂-Ile₂₃₃ vis-à-vis scission of Arg₂₇₃-Thr₂₇₄ and should result in the accumulation of prethrombin-2. SDS gel electrophoretic analyses of the bovine system have led to similar conclusions. The above hypothesis was confirmed by adding Echis carinatus venom, which converts intermediate species to thrombin. A dramatic reduction in the ratio of F₂ to TAT was observed with the latter parameter approaching 1.18. The transformation of prethrombin-2 to thrombin was also observed by SDS gel electrophoresis. The preceding results provide compelling evidence that prothrombin activation under some conditions is characterized by the accumulation of a thrombin precursor and that this event can be detected by a ratio of F₂/F₁⁺₂ to TAT greater than unity.

Subsequently, prothrombin activation at either 24°C or 37°C was monitored during the spontaneous coagulation of freshly drawn blood as described above. After a delay of about 10 min, sufficient factor Xa was generated to initiate zymogen conversion. Throughout the entire course of prothrombin activation, the rate of generation of F₂/F₁⁺₂ was considerably greater than that of TAT. Based on these data, we calculated that the cleavage of Arg₂₇₃-Thr₂₇₄ within whole blood is
about 1.7 times more rapid than the scission of Ar322-Il323. These estimates are in reasonable accord with the final stable levels of the two components after completion of the above reaction. It is also of interest to note that \( F_{1+2} \) is the predominant form of activation fragment until about 50% of the zymogen is converted to enzyme. Thereafter, the contribution of \( F_2 \) increases in importance until it reaches 59.7% of the total activation fragment at the conclusion of prothrombin activation.

Since the transformation of zymogen within whole blood occurred after a prolonged lag phase, it was essential to show that the initial delay was not responsible for the discrepant generation of \( F_2/F_{1+2} \) and TAT. To this end, factor Xa was immediately added to freshly drawn blood so that rapid activation of prothrombin could take place, and the above conversion process was monitored as previously outlined. The ratio of \( F_2/F_{1+2} \) to TAT during thrombin generation was somewhat more discrepant than that observed with spontaneous coagulation of blood. Thus, our findings imply that under in vitro conditions, prothrombin activation within whole blood is characterized by the relatively constant accumulation of a stable precursor such as prethrombin-2. It is conceivable that this phenomenon is secondary to an alteration of factor V function.

We have also measured the levels of \( F_2/F_{1+2} \) and TAT within normal individuals. Our studies indicate that the concentrations of \( F_{1+2} \) (\( F_2 \) is not present) and TAT average 1.97 nM and 2.32 nM, respectively. Since we employ the two-syringe technique for obtaining blood samples and utilize anticoagulants able to instantaneously inhibit prothrombin activation, we suspect that the above normal values represent continuous generation of thrombin within the vascular system. However, we cannot completely exclude the possibility that these ambient levels of \( F_{1+2} \) and TAT are formed during venipuncture.

The above assays have been used to quantitate the concentrations of \( F_2/F_{1+2} \) and TAT in patients who exhibit disseminated intravascular coagulation. As expected, the levels of both components are significantly elevated during activation of the hemostatic mechanism. However, the ratio of \( F_{1+2} \) (\( F_2 \) is not present) to TAT ranged from 2.37 to 5.55. This discrepancy is somewhat more extreme than that documented for in vitro coagulation of blood. However, the above findings suggest that under in vivo conditions, prothrombin activation may be characterized by the accumulation of a stable precursor such as prethrombin-2. This phenomenon could lead to significant levels of the latter species that might play a regulatory role in zymogen conversion.

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Studies of the prothrombin activation pathway utilizing radioimmunoassays for the F2/F1 + 2 fragment and thrombin--antithrombin complex

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