The sequences of events regulating thrombin generation during tissue factor--initiated clotting in whole blood at 37°C in which the contact pathway was suppressed with corn trypsin inhibitor are studied using quantitative Western blotting of factor V, prothrombin, platelet factor 4, antithrombin III, and fibrinogen. In addition, fibrinopeptide A (FPA), thrombin-antithrombin III (TAT) complex formation, and prothrombin fragment 1.2 (F1.2) were measured via commercially available enzyme-linked immunosorbent assays (ELISAs). In a typical experiment initiated with 40 pmol/L recombinant tissue factor, visual clot time (4.5 minutes), was preceded by significant cleavage of factor V resulting in 65% factor Va heavy-chain generation but only 10% light-chain formation. At this point, 50% of the platelet factor 4 is released, suggesting that half (approximately 700 pmol/L) of the platelet prothrombinase sites available have been generated. At clot time, approximately 15 nmol/L thrombin B-chain is present; however, analyses of FPA release demonstrate that only 15% of the thrombin is acting on fibrinogen. This thrombin is produced by the action of 7 pmol/L prothrombinase. The maximum rate of thrombin production is reached well after clot time and is consistent with the presence of approximately 150 pmol/L prothrombinase (at about 7 minutes). These results suggest that factor Xa is the limiting factor for thrombin generation. After 60 minutes, 75% of the initial prothrombin (1.24 µmoll/L) is consumed yielding 440 nmoll/L prothrombin 2 and 360 nmoll/L thrombin (B-chain) products. The sum of these values (800 nmoll/L) is similar to the (corrected) F1.2 concentration determined by ELISA. The incomplete cleavage of prothrombin indicates both the prothrombinase complex and the formation of prothrombinase are inhibited in the reaction. TAT complex measured by ELISA is almost equivalent to B-chain concentration, but sodium dodecyl sulfate stable thrombin-antithrombin III complexes are not observed until well after clot formation and are never equivalent to ELISA-TAT values. At the point of clot formation, 80% of the fibrinogen is depleted from the fluid phase, whereas only 35% to 46% of the FPA is released, suggesting a significant incorporation of uncleaved fibrinogen into the initial clot formed.

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a complex interaction of the TFPI-factor Xa complex with the factor VIIa-tissue factor complex.22

On an activated cell membrane, factor Xa forms a complex with factor Va, prothrombinase, which activates prothrombin to thrombin.23 Once thrombin is generated, feedback activations of factor V and factor VIII accelerate the overall reaction process.24 Additional thrombin feedback contributes to the activation of factor XI, which may accelerate the thrombin generation process by activation of factor IX.11,25 Thrombin acts on the platelet membrane receptor26 to induce platelet aggregation and upon fibrinogen to produce fibrinopeptide A and fibrin I and subsequently fibrinopeptide B and fibrin II. The fibrin monomers polymerize to form the clot.27-29

The extrinsic pathway, involving the factor VIIa-tissue factor complex, has strong evidential support as the physiological initiator of in vivo clotting events,10-13 whereas the intrinsic pathway,24-30 which requires the activation of factor XII and factor XI in reactions supported by high molecular weight kininogen, prekallikrein, and negatively charged surfaces, has a questionable role in normal hemostasis. The observations of apparently normal hemostasis in individuals deficient in factor XII, high molecular weight kininogen, and prekallikrein suggest that the intrinsic pathway is not required for normal hemostatic function; however, this pathway may play a significant role during pathologic states.30,31

In any event, in vitro, surface-dependent contact activation is initiated when blood comes in contact with virtually any artificial container. Thus, the intrinsic pathway must be suppressed if the tissue factor–mediated reactions are to be studied.

Recently, we reported a model system that employs the procoagulant components of the extrinsic pathway, with each component reconstituted at its average physiological concentration.32 In this model system, the activations of prothrombin, factor V, factor VIII, factor IX, and factor X in the presence of Ca2+ and synthetic phospholipid vesicles were monitored in response to reactions initiated by factor VIIa–tissue factor. Less than 1% of factors IX and X were converted to active enzyme during the interval in which quantitative prothrombin conversion occurred. In this purified system, no novel products of proteolytic processing of the zymogens and procofactors were observed. In addition, thrombin generation over time was characterized by an apparent “lag” followed by a rapid propagation phase. It was also observed that varying the initiator concentration (factor VIIa-tissue factor) did not significantly alter the rate of thrombin generation in the thrombin propagation phase of the reaction but primarily influenced the lag time before the propagation phase. Similar reconstructed systems have since been reported that incorporate AT-III, TFPI, and the protein C-thrombomodulin components.32-34

Most studies aimed at the evaluation of coagulant processes in quasi-physiological systems using plasma and blood commonly employ Ca2+ chelators such as citrate or EDTA. However, because many Ca2+-dependent processes, such as vitamin K–dependent protein folding and cellular Ca2+ transients, occur with rates relevant to those of clot formation, the chelation-recalcification processes may influence underlying cellular and enzymatic mechanisms.32 Finally, most kinetic evaluations of blood or plasma clotting are limited to analyses of an end point involving clot formation, although a recent study by Kessels et al33 uses chromogenic peptide substrates to monitor thrombin generation in a minimally altered whole blood system.

The present study was conducted to evaluate the relationships between protease enzymology and platelet activation that occur during the tissue factor–induced clotting of minimally altered whole blood.

**MATERIALS AND METHODS**

HEPES, Tris-HCl, rabbit brain thromboplastin, 1-palmitoyl-2-oleoyl-phosphatidyl serine (PS), 1-palmitoyl-2-oleoyl-phosphatidyl choline (PC), and 2-mercaptoethanol were purchased from Sigma Chemical Co (St Louis, MO). Benzamidine-HCl was purchased from Aldrich, Inc (Milwaukee, WI). Activated partial thromboplastin time reagent was purchased from Organon Technica (Durham, NC). Ni-trocellulose was purchased from Biorad (Hercules, CA). Horseradish peroxidase–labeled goat anti-rabbit IgG, antinunouse IgG, and antihorse IgG antibodies were purchased from Southern Biotech (Birmingham, AL). Film was purchased from Dupont (Boston, MA). o-phenylenediamine/propylarginy1-chloromethyl ketone (PFP-c), rabbit antihuman α-thrombin, human platelet factor 4 (PF4), and human antithrombin III (AT-III) were gifts from Dr Richard Jenny of Haematologics Technology, Inc (Essen Junction, VT). Anti-human tissue factor antibody S09 was a gift from Dr Thomas Edgington of Scripps Research Institute (La Jolla, CA). Prothrombin and factor V were purified as previously described.44,45 Recombinant tissue factor (residues 1 to 242) was a gift from Dr Hissids of Genentech, Inc and was relipidated in 25% phosphatidylserine, 75% phosphatidylcholine (PCPS) vesicles by a previously described protocol46,47 at 10 μmol/L PCPS and tissue factor ranging from 2.5 to 300 nmol/L. Trypsin inhibitor from corn was purchased from Fluka (Ronkonkoma, NY) and was stored as a 5 mg/mL stock in 20 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4 at −20°C. Burro antihuman-AT-III IgG and antihuman-PF4 IgG were prepared as previously described.32 Monospecific anti–AT-III IgG was absorbed to AT-III-Sepharose and eluted with 0.1 mol/L glycine, pH 2.5, and neutralized with 0.1 mol/L Tris-HCl, pH 9.0. IgG was precipitated by 70% saturation with ammonium sulfate and stored in 50% glycerol at −20°C. The IgG fraction of anti-PF4 serum was used as primary antibody in Western blotting. The monoclonal antibodies anti–HFV-6 and anti–HFV-9 are antibodies specific for the human factor Va heavy chain and light chain, respectively, and have been described previously.51,52 Monoclonal antibodies, anti–fibrinogen-3A, anti–HFV-6 and anti–HFV-9, were provided by Dr William Church (University of Vermont). Molecular weight standards for sodium dodecyl sulfate (SDS)-gel electrophoresis were purchased from Gibco-BRL (Gaithersburg, MD). All other reagents were of analytical-grade.

**Whole blood clotting experiments.** Blood was drawn from donors with informed consent and according to the guidelines of the human subjects research committee at the University of Vermont. Subjects analyzed for whole blood clotting in this study were tested for fibrinogen, prothrombin time, activated partial thromboplastin time, and a hemogram was obtained. These tests were conducted by the outpatient laboratory at Fletcher Allen Health Care, Burlington, Vermont, under the supervision of Dr Edwin Bovill and Mr Randy Messer. All of the subjects studied were in the normal range of prothrombin time, activated partial thromboplastin time, platelets, and fibrinogen.

Clotting of whole blood was carried out in 12 × 75-mm polystyrene culture tubes purchased from VWR Scientific (product no. 3433).
60818-270). The tubes were modified by drilling a 3/16-in hole in the position of polypropylene caps (VWR 60819-047) and positioned on their sides with the hole facing up on a Thermolyne spec-mix rocker table (Sybron, Dubuque, IA) and enclosed in a 37°C temperature-controlled glove box. Blood was drawn from healthy, nonsmoking, non-aspirin-using volunteers using 19-gauge butterfly needles. The first 3 mL of blood was discarded. Subsequently, 40 mL of blood was drawn directly with an Eppendorf repeater pipet equipped with a 50-mL syringe barrel. One milliliter aliquots of the nonanticoagulated blood was rapidly distributed (over ~1 minute) into 32 reagent-loaded polystyrene tubes via the hole in the side of the tube. The tubes were rocked continuously and clot times were determined by the obvious formation of “clumps” on the sides of the tube. Clot times are expressed as the average of duplicate determinations and time zero is taken as the time the blood contacts the tube. The effects of von trypsin inhibitor (CTI), tissue factor-PCPs, PCPs alone, and anti–tissue factor antibodies were monitored by preloading the tubes with various concentrations of the effectors(s) in 20 mM/L HEPES, 150 mM/L NaCl, 5 mM/L CaCl2, pH 7.4 in a volume not exceeding 29 mL.

Analyses of whole blood clotting were performed with final plasma concentrations of 32 µg/mL CTI, 40 µmol/L tissue factor 380 µL of 31 moln Tris, pH 6.8, 150 mM/L Tris-HCl, 150 mM/L NaCl, 5 mM/L CaCl2, pH 7.4 in a volume not exceeding 29 mL.

Analyses of whole blood clotting were performed with final plasma concentrations of 32 µg/mL CTI, 40 µmol/L tissue factor and 80 µmol/L PCPs. The clotting reactions were quenched at various times by the addition of 1.0 mL of 50 mM/L EDTA, 10 mM/L benzamidine in HEPES-buffered saline followed by 10 µL of 5 mM/L FPR-ck in 0.01N HCl. The hole in the tube was sealed with tape, and the tubes were rapidly vortexed for 15 seconds to thoroughly mix the enclosed material and permit exchange of entrapped and bound elements with the fluid phase. Samples were stored on ice for a maximum of 30 minutes until they were centrifuged and the supernatant (fluid phase) was collected and analyzed. Aliquots of the fluid phase were stored at −20°C for analysis by enzyme-linked immunosorbent assay (ELISA) techniques and gel electrophoresis.

**Gel electrophoresis and Western blotting of fluid phase blood samples.** Samples for SDS-polyacrylamide gel electrophoresis (PAGE) were prepared by diluting 120 µL of fluid phase serum into 380 µL of 31 mM/L Tris, pH 6.8, 5% glycerol, 1% SDS, 0.05% bromophenol blue (SDS-PAGE buffer). Each sample was divided into two aliquots: nonreducing and reducing (1% β-mercaptoethanol). The samples were heated for 5 minutes at 95°C and either analyzed immediately or stored at −20°C. Samples equivalent to 0.38 to 0.75 µL of undiluted fluid blood were analyzed by SDS-PAGE. A separate gel was prepared for each antigen analyzed.

Transfer of proteins to nitrocellulose was performed as previously described. Membranes were blocked with 5% dried milk in 20 mM/L Tris-HCl, 150 mM/L NaCl, 0.02% Tween-20 pH 7.4 (TBST) for 1 hour. Western blotting was performed using the primary antibodies: anti-α-thrombin, anti–HFV-6, anti–HFV-9, and anti–fibrinogen-3A (all used at 5 µg/mL), anti–AT-III (at 2 µg/mL), and anti-F1.2 (at 20 µg/mL) in TBST for a 90-minute reaction. Peroxidase-conjugated secondary antibodies (antimouse, antirabbit, or antihorse, as appropriate) were used at 1/2,500 dilution in TBST for a 60-minute incubation. Membranes were rinsed after each antibody incubation with 4 x 100 mL TBST and a final 30-minute to 1-hour rinse in TBST was performed before development. Blots were developed using the Renaissance chemiluminescent reagent (Dupont, Boston, MA) for varying intervals, depending on the analyte. Film was developed in a Kodak X-omat and scanned using a Microscan 1000 scanning densitometer (TRI, Inc.)

**Fibrinopeptide A**

**Prothrombin fragment 1.2 (F1.2)**

**Thrombin-AT-III.** Fibrinopeptide A (FPA) was measured in the fluid phase samples using the Asserachrom FPA assay kit from Diagnostica Stago (American Binproducts, New Jersey) according to the manufacturer’s instructions. Serial dilutions (up to 1:1,000) of the samples were performed to accommodate the standard curve. Assays were performed in duplicate.

Prothrombin fragment 1.2 (F1.2) was measured using the Enzygnot F1.2 microimmunoassay kit from Behring according to manufacturer's instructions. For samples obtained after clot formation, a 100- to 1,000-fold dilution was performed to give values on the standard curve. We noted a significant discrepancy in assays of our purified samples of F1.2 using this assay. We thus performed the following analyses: Prothrombin (1.4 µmol/L) was quantitatively activated to α-thrombin with factor Xa (5 mM/L), factor Va (10 mM/L), 5 µmol/L PCPs, 5 mM/L Ca2+ in TBS buffer containing 100 µmol/L DAPA. At various time points aliquots were quenched in 100 µmol/L FPR-ck/20 mM/L EDTA, and samples were analyzed by the F1.2 immunoassay and Western blotting after SDS-PAGE. F1.2 was quantitatively and exclusively produced (equimolar with α-thrombin) by 1 minute and remained constant for 15 minutes. The value reported by the Enzygnot assay was 1.96 µmol/L F1.2. This value is 40% in excess of the true value (1.4 µmol/L) F1.2 in the sample. The values obtained using the standards provided with the immunoassay kit were therefore multiplied by 0.713 to give corrected values for F1.2. Western blots of the kit standards displayed no reaction with monoclonal anti-F2 antibodies and displayed reaction products significantly smaller than F1.2 when probed with a polyclonal anti–prothrombin-1 antibody.
Thrombin-AT-III complex (TAT) was measured in the fluid phase samples using the Enzygnost TAT microimmunoassay kit from Behring according to manufacturer's instructions. For samples after clot formation, samples were diluted up to 1/1,000 in normal plasma to give values within the standard curve supplied with the assay. Values in diluted samples were corrected for the TAT concentration of 2.76 ngl in the normal plasma used for the dilution.

Kinetic analyses. The thrombin concentration at any time was estimated from densitometry of the thrombin B-chain and by the rate of FPA generation. The progression of FPA concentration in fluid phase over the first 7 minutes was fit to a fourth degree polynomial using the program Microsoft Excel version 5. The cleavage of fibrinogen by thrombin has been reported by Mihalysi to follow first-order kinetics at all concentrations of fibrinogen. This empirical process can be described mathematically by product inhibition with a $K_e$ equal to $K_w$. Therefore, thrombin concentrations were calculated using the following Michaelis-Menten expression that accounts for competitive product inhibition:

$$[E]t = \frac{t(K_m + [S])}{t_K} + [S]/K_{ind}[S]$$

where $[E]t$ is the thrombin concentration at time $t$, $[P]$ is the concentration of free inhibitory product, and $[S]$ is the concentration of free substrate. The inhibitory product is reported to be the $\alpha$-chain of fibrin I, which is essentially equivalent in concentration to FPA. The free product concentration $[P]$ was taken as the total product concentration (FPA concentration) at time $t$, because the thrombin concentration is less than 1.0% of product concentration over the range of fibrinogen cleavage considered. Similarly, $[S]$ is taken as the fibrinogen $\alpha\alpha$-chain concentration (total fibrinogen minus FPA) at time $t$. The data were analyzed to the point of 90% FPA release. The constants $K_m = 7.2 \mu\text{mol}/\text{L}$ and $k_{ma} = 84 \text{s}^{-1}$ were used as reported for human $\alpha$-thrombin and human fibrinogen by Higgins et al. The $K_e$ was made equivalent to $K_w$ for the purposes of these calculations and in accordance with Mihalysi. It has been reported by other investigators using this assay that FPA levels go to a maximum and then are seen to decrease. We observed a similar phenomenon using the Asserachrom FPA assay (American Bioproducts). For kinetic analyses, we have calculated molar concentration of FPA generation using the maximum FPA level from the generation curve.

Estimates of $\text{prothrombinase}$ concentration were calculated from the rates of thrombin $B$-chain production determined by densitometry and by F1.2 and TAT complex formation rates determined by ELISA. The rate of thrombin $B$-chain production at clot time was estimated by a hyperbolic fit of the densitometry data between 180 and 420 seconds with Stanford Graphics version 3 (Visual Numerics, Houston, TX). The maximum rate of $B$-chain production was estimated by point-to-point interpolation between 390 and 420 seconds performed with Stanford Graphics version 3. The prothrombin concentration was calculated using the following form of the Michaelis-Menten equation:

$$[E]t = \frac{t(K_m + [S])}{t_K} + [S]/K_{ind}[S]$$

where $[E]t$ is the $\text{prothrombinase}$ concentration at time $t$ and $[S]$ is the free prothrombin concentration. Initial prothrombin concentration was measured by densitometry of Western blots. Prothrombin at time $t$ was estimated by subtracting the thrombin $B$-chain concentration at time $t$ from the initial prothrombin concentration. The $[S]$ was taken as the total prothrombin at time $t$ since $\text{prothrombinase}$ was less than 0.1% of the prothrombin concentration over the course of the reaction analyzed. The constants $K_m = 0.82 \mu\text{mol}/\text{L}$ and $k_{ma} = 19 \text{s}^{-1}$ were used as reported for $\text{prothrombinase}$ at 22$^\circ$C on platelets by Tracy et al. 7

RESULTS

The effect of CTI on the plasma prothrombin time and the activated partial thromboplastin time were evaluated. Biggs and Nossel demonstrated that at dilute thromboplastin concentrations the plasma clot time becomes dependent on factor IXa/VIIa activity. Therefore, dilute thromboplastin time assays were performed with a 1/1,000 dilution of thromboplastin and carried out in polystyrene tubes. A pool of citrated plasma from 6 normal donors was used. CTI was added to final concentrations of 20 and 100 $\mu\text{g/mL}$, and the plasma was incubated in ice for 10 minutes before assay. The results are seen in Table 1. No significant prolongation of the prothrombin time and dilute thromboplastin time was observed, demonstrating that CTI has no effect on thrombin, factor Xa, factor VIIa, and factor IXa. The activated partial thromboplastin time is significantly prolonged by increasing amounts of CTI, indicating inhibition of the contact pathway. These observations are consistent with previous reports of CTI specificity for factor XII. Blood with no additions added to polystyrene tubes clotted between 7 and 10 minutes for all subjects. Anti–tissue factor antibody 5G9 was used to examine the contribution of the tissue factor–initiated reaction to this spontaneous clotting process. This antibody at 10 $\mu\text{g/mL}$ did not prolong whole blood clot time. Because this concentration of the antibody has been previously shown to neutralize tissue factor–dependent clotting in vivo, the spontaneous clotting process was most likely contact mediated. Increasing concentrations of CTI added to whole blood gave a dose-dependent prolongation of clot time. For subject BT, prolongation of clot time from 8.5 minutes to greater than 25 minutes was achieved with 20 and 100 $\mu\text{g/mL}$ CTI, indicating inhibition of the contact pathway. Increasing concentrations of CTI added to whole blood gave a dose-dependent prolongation of clot time. For subject BT, prolongation of clot time from 8.5 minutes to greater than 25 minutes was achieved with 20 and 100 $\mu\text{g/mL}$ CTI, indicating inhibition of the contact pathway. Increasing concentrations of CTI added to whole blood gave a dose-dependent prolongation of clot time. For subject BT, prolongation of clot time from 8.5 minutes to greater than 25 minutes was achieved with 20 and 100 $\mu\text{g/mL}$ CTI, indicating inhibition of the contact pathway.

<table>
<thead>
<tr>
<th>CTI concentration (pg/mL)</th>
<th>Prothrombin time (s)</th>
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<tr>
<td>20</td>
<td>46.6 ± 1.5</td>
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<tr>
<td>100</td>
<td>124.0 ± 1.9</td>
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Values are means ± SD.

Abbreviation: CTI, corn trypsin inhibitor.

* Thromboplastin was used at a 1/1,000 dilution.
clot time from 23 to 4.5 minutes. Eighty nmol/L PCPS alone added to CTI-inhibited blood (platelet count = 1.9 to 3.4 × 10^9/mL blood) had negligible (<10 seconds) influence on the clot time. In range-finding experiments increasing the TF concentrations by fivefold from 60 to 300 pmol/L produced a 1.5-fold decrease in clot time.

Initial experiments performed with blood without added CTI gave end products of factor V and prothrombin activation that were identical in apparent mobility as those observed in the presence of CTI and TF-PCPS. The SDS and Triton X-100 extracts of cellular-clot (solid) phase material contained significantly less prothrombin and thrombin than that observed in the fluid phase when normalized to the total blood volume and no enrichment of reactants or products, indicating that no significant irreversible compartmentalization of these products to the insoluble phase (excepting fibrin) occurred.

Whole blood clotting. The product-precursor data illustrated are for a single experiment performed with a subject identified as BT and are representative of results seen for 8 different normal subjects in 14 experiments. All data are expressed as concentrations in the plasma volume. Subject BT had a hematocrit of 40%, normal prothrombin time (12.6 seconds) and activated partial thromboplastin time (29 seconds), a platelet count of 1.9 × 10^9 platelets/mL, and a fibrinogen concentration of 2.41 mg/mL (7.09 μmol/L).

Figure 1 displays the temporal processes of product formation in CTI-inhibited blood initiated to clot with 40 pmol/L tissue factor and 80 nmol/L PCPS. In the experiment illustrated, SDS-PAGE gels and ELISA analyses were performed on samples taken at 30-second intervals for the first 8 minutes. Longer intervals were selected for later time points extending to 1 hour. The clot time (indicated by the arrow in lane 10) occurred at 4.5 minutes.

Figure 1 displays transverse sections, obtained from multiple immunoblot analyses from individual gel analyses for the analytes fibrinogen, factor V, factor Va heavy chain, factor Va light chain, prothrombin/thrombin, antithrombin III, and platelet factor 4. The immunoreactive products are identified explicitly when the electrophoretic mobility corresponds to that of a known product expected from the reactant. When there is uncertainty with respect to the identification of a reaction product (apart from its immunoreactivity and its appearance over time), either lower case letters (a, b, c, or d/e) or a prime (') are used to identify the reaction products.

In addition to the quantitation of the analytes displayed in Fig 1, samples taken from the same aliquots were analyzed using commercial assays for FPA, TAT, and prothrombin fragment 1.2 (with the latter values corrected as described in Materials and Methods).

Fibrinogen cleavage and removal from the fluid phase. The initial products of thrombin cleavage of fibrinogen are FPA and fibrin monomer.27-29 Fibrinogen and FPA are soluble components that are readily detectable in the fluid phase of blood. Fig 1A demonstrates fibrinogen in the fluid phase of clotting blood at various time points. Fibrinogen is clearly observed (M, = 340,000) for the first 4 minutes, before clot time. Densitometry of this blot (Fig 2) reveals that at clot time (4.5 minutes), 80% of the fibrinogen is depleted from and does not reequilibrate with, the fluid phase, suggesting that it is incorporated into the soluble clot. The appearance of FPA in the fluid phase increases rapidly, reaching an apparent maximum of 21 μmol/L at 7.0 minutes. This maximum is followed by a decrease to 15 μmol/L. These latter values remain essentially unchanged (Fig 2). These observations suggest that either the FPA product initially formed is more immunoreactive than the stable final product or that there is some artifact associated with the assay itself. Treatment of blood from subject BT with 0.8 μmol/L α-thrombin for 5 minutes resulted in a measured value of 15.2 μmol/L FPA in the fluid phase. Additional incubation of the thrombin treated blood at 37°C for 10 minutes also resulted in a measured value of 15.2 μmol/L FPA in the fluid phase. However, prolonged incubation of serum has been reported to give an apparent reduction in FPA values as determined with this assay,37 consistent with the initial FPA product being more immunoreactive. At clot time (4.5 minutes), 9.5 μmol/L FPA is observed, which corresponds to 45% of the apparent maximum in FPA (21 μmol/L). If one uses the stable value of FPA (15.2 μmol/L), the conclusion would be that 6.9 μmol/L FPA is released at clot time, or 45.5%. In either case it appears that substantial amounts of intact fibrinogen may be incorporated into the initial clot, regardless of the FPA uncertainty.

Factor V activation. Factor V and activation products were detected by two monoclonal antibodies: one reactive toward residues 307 to 506 on the heavy chain (anti–HFV-6) and the other raised against the light chain of factor Va (anti–HFV-9). Anti–HFV-6 has also been shown to react with platelet-derived factor V, meaning that the observed immunoreactivity is not due to the sum of plasma and platelet-derived heavy chain.56 The concentration of factor V contained in platelets is described to be approximately 20% of the blood concentration. Therefore, the observed bands of factor Va heavy and light chain may consist of 20% of platelet-derived factor V after maximum platelet release (7 minutes). Staining with α-HFV-6 after resolution on a nonreducing gel is seen in Fig 1B; factor V is observed migrating at M, = 330,000 and rapidly disappears over the first 5 minutes, indicating near complete cleavage of factor V at clot time (FV, Fig 1B, lanes 1 through 11). The concomitant appearance of the heavy chain is observed migrating as a doublet at M, = 105,000/103,000 (FVahc, Fig 1C, lanes 5 through 30). This doublet is unexpected from studies in purified systems and is observed to resolve as a single band under reducing conditions (not shown). The heavy-chain doublet is observed to be reduced in intensity from 10 to 60 minutes. The disappearance of the M, = 105,000 band precedes the M, = 103,000 band, suggesting the M, = 103,000 product is more resistant to degradation. Other immunoreactive bands are observed at M, = 70,000 (a, Fig 1C, lanes 18 through 30), M, = 45,000 (b, Fig 1C, lanes 22 through 30), and M, = 30,000 (c, Fig 1C, lanes 25 through 30). The M, = 70,000 and M, = 30,000 bands migrate identically with the previously described activated protein C degradation products of factor Va heavy chain resulting from the inactivating cleavages at Arg1506 and Arg1508, respectively.63 The M,
Figure 1. Composite Western blots of fibrinogen, factor V, prothrombin, antithrombin III (AT-III), and platelet factor 4 in clotting blood. Transverse sections of immunoblots from individual gels for various analytes separated on electrophoresis gels prepared with blood/serum samples, obtained from individual BT and subjected to tissue factor–initiated coagulation in corn trypsin inhibitor–inhibited whole blood. Gels on the same samples taken sequentially during the course of the coagulation process were run under both reducing and nonreducing conditions, transferred, and probed with the appropriate monoclonal or polyclonal antibody. Fibrinogen, factor V and factor Va light and heavy chains, and PF4 are analyzed under nonreducing conditions. Prothrombin and AT-III were analyzed under reducing conditions. At the top of the figure are indicated the time intervals at which the sample was obtained, while the bottom legend indicates the lane number on the electrophoresis gel. Clot time (4.5 minutes) is indicated by bold arrow. The letters A through H on the left margin correspond to various transverse gel sections corresponding to different analytes (see text), while the legend on the right side of the gel corresponds to the various analytes. The analytes labeled a, b, c, Tla, (TAT'), II, and d/e are not yet positively identified.

= 45,000 band most likely corresponds to the product of initial APC cleavage at Arg396, or might be the result of factor Xa or plasmin cleavage at Arg348 and Arg679 in the factor Va heavy chain. However, this fragment is not well resolved because of the presence of plasma albumin, which interferes in the molecular weight (Mₐ) range between Mₐ = 45,000 and 50,000 on nonreduced gels. Staining with anti-HFV-9 shows the light chain migrating as the expected doublet at Mₐ = 74,000 to 72,000 (FVαₗc, Fig 1D, lanes 8 through 30). This product is produced at a slower rate when compared with the heavy chain.

A quantitative display of the appearance and disappearance of factor V and its reaction products obtained by densitometric analyses of the immunoblots of Fig 1B through D is presented in Fig 3. Factor V (●) is observed to disappear within the first 5 minutes of the reaction with a concomitant appearance of the heavy chain (○). At late time intervals (beyond 8 minutes) a gradual decrease in factor Va heavy-chain concentration occurs with the appearance of the fragments "a," "b," and "c." In contrast the light chain (×) is produced at a much slower rate and becomes the limiting factor with respect to complete activation of factor V. An inspection of light- and heavy-chain concentrations at any point in the reaction provides the absolute concentration of factor Va available to form prothrombinase. The factor Va heavy chain reaches 65% of maximum at 4.5 minutes (clot time) compared with only 10% of the maximum level achieved for light chain (Fig 3). Therefore, at a normal factor
Prothrombin is observed at M, at clot time, indicated by the bold arrow, FPA is 9.5 pmol/L, representing 45% of maximum (21 pmol/L). At this time, fibrinogen is approximately 80% depleted from solution.

Other reactive bands of lesser intensity are seen at M, = 72,000 and migrates identically with purified prothrombin (II, Fig 1E, lanes 1 through 30). Activation products can be seen at 3.5 minutes (Fig 1F, lane 8) and consist of a band at M, = 36,000 and a band at M, = 30,000. The M, = 30,000 band migrates identically with a-thrombin B-chain (IIaB, Fig 3, lanes 8 through 30). The M, = 36,000 band is deduced to be prethrombin 2 (Pre 2, residues 274 to 579 in prothrombin) because it displays epitopes for the a-thrombin antibody, migrates identically with prethrombin 2 and is M, = 6,000 larger than the B-chain, a difference accounted for by the size of the A-chain of a-thrombin and previously shown to be prethrombin 2.

Other reactive bands of lesser intensity are seen at M, = 97,000 and M, = 93,000 (bands TAT, Fig 1E, lanes 19 through 30) and a band at M, = 135,000 (band Tla, Fig 1E, lanes 17 through 30). The M, = 93,000 band (TAT') migrates identically with purified covalent thrombin-AT-III complex. The M, = 97,000 band appears at approximately the same time and with the same intensity as the M, = 93,000 band. This product has similar mobility to that reported for the thrombin-heparin cofactor II complex. The band accumulating at M, = 135,000 (Tla) remains unidentified but is, most likely, an SDS-stable thrombin-inhibitor complex. A reactive band (TAT') is observed at M, = 69,000, which appears at 12 minutes and remains up to 60 minutes. This component represents an SDS-stable thrombin-AT-III complex fragment (Fig 1E, lanes 20 through 30). Collectively these SDS-stable, high molecular weight components represent less than 2% of the thrombin B-chain density observed at 60 minutes; however, their intensities may underrepresent the concentrations of these inhibited thrombin species because of nonidentical reactivities of the antibody with thrombin that is present in these complexes.

The thrombin B-chain density was quantitated by comparison to a standard density curve derived from known amounts of purified a-thrombin run on the same gel. From the blot data the thrombin B-chain can first be detected at 2.5 minutes and reaches a maximum of 360 nmol/L at 60 minutes (Fig 4). The thrombin B-chain estimated at clot time (4.5 minutes) is 15 nmol/L (inset, Fig 4). A curve representing the thrombin concentration calculated from the rates of FPA generation (Fig 2, see Materials and Methods) is also presented in Fig 4 (inset) and gives 2.5 nmol/L thrombin at clot time. Based on these analyses, only 15% of the thrombin produced at clot time is acting on fibrinogen. By either determination, less than 2% of the initial prothrombin (1.24 µmol/L, see below) is converted to a-thrombin (or meizothrombins) at clot time.

By densitometry of a less exposed film and comparison to standards, prothrombin is estimated at 1.24 µmol/L at time zero and is reduced to 0.3 µmol/L at 60 minutes (Fig 1E, lane 30) corresponding to 76% prothrombin cleavage over the entire course of the reaction. The thrombin B-chain, 360 nmol/L at 60 minutes (Fig 1F, lane 30), represents 29% of the initial prothrombin concentration. The M, = 36,000 band, representing prethrombin 2 (Pre 2, Fig 1F), reaches a maximum density at 25 minutes, which does not decrease over 60 minutes. Assuming equivalent reactivity of the antibody with prethrombin 2 and thrombin B-chain, 440 nmol/L prethrombin 2 is formed at 60 minutes, which accounts for approximately 76% of the initial prothrombin concentration.
Fig 4. Concentration of thrombin B-chain in the fluid phase of clotting blood. Thrombin B-chain was quantitated by densitometry as described in Materials and Methods and is seen to appear rapidly after an initial lag phase. The B-chain concentration (▲) reaches a maximum of 360 nmol/L at 60 minutes. The inset shows the relative concentration of B-chain calculated from rates of FPA generation and by densitometry (■) over the first 350 seconds. Clot time (4.5 minutes) is indicated by the bold arrow.

for 36% of the initial prothrombin. All together, the thrombin B-chain (α-thrombin and meizothrombin) and prethrombin 2 in the fluid phase account for 65% of the initial prothrombin. Thus, of the 76% of initial prothrombin cleaved, 11% is not accounted for by these identifiable products and is likely dispersed among the uncharacterized components and SDS-stable inhibitor complexes. In addition, some reaction products may be obscured by serum proteins.

Figure 5 displays the progress curves for formation of thrombin B-chain and prethrombin 2 deduced from densitometry of Western blots and the progress curves determined by the ELISA for TAT and F1.2 (corrected, see Materials and Methods) determined on non-denatured samples. TAT concentration levels are slightly lower than thrombin B-chain levels, indicating that most of the thrombin represented by the immunoblot corresponds to inhibited thrombin present as SDS-unstable complex(es) with AT-III. The sum of the profiles of prethrombin 2 and B-chain concentrations (Fig 5, ▲) is initially similar to the F1.2 levels determined by ELISA; these diverge from approximately 8 to 20 minutes and then become almost equivalent at longer time intervals. Immunoblots conducted with a monoclonal anti-prothrombin fragment 2 antibody are largely consistent with these corrected F1.2 ELISA data (data not shown). In general F1.2 levels are similar to the sum of thrombin B-chain plus prethrombin 2 concentrations over most of the course of the reaction. The observations with respect to F1.2, however, must be tempered by the fact that a large correction was required for the commercial assay and the kit standard “F1.2” is electrophoretically quite different from genuine F1.2 (see Materials and Methods). Collectively, these data suggest that the excess prothrombin products relative to the prothrombin F1.2 produced during the early phase of the reaction are accounted for as other prothrombin fragments (see for example II(x) in Fig 1F) and stable inhibitor complexes other than unmodified AT-III. Other SDS-stable covalent thrombin-inhibitor complex products are identified by the products T1a, TAT', and possibly II(x) (Fig 1E).

AT-III complex formation. On reduced gels, AT-III is seen to migrate at M₀ = 58,000 (ATIII, Fig 1G). Upon extended exposure of the blot, an M₀ = 93,000 band is first seen at 5.5 minutes; this band reaches a plateau of density at time points between 30 and 60 minutes (TAT, Fig 1E,
lanes 26 through 30). This band migrates identically to the thrombin-AT-III complex (TAT) and with the $M_c = 93,000$ band seen with anti-$\alpha$-thrombin staining (Fig 1E) and is thus deduced to be TAT.\textsuperscript{65} This band represents less than 0.1\% of the density of the initial AT-III, indicating less than 5 nmol/L of SDS-stable TAT complex is formed. Far less SDS-stable TAT is formed than had been anticipated, especially when considering approximately 360 nmol/L thrombin B-chain has been generated. TAT complexes formed in vitro using purified AT-III and thrombin are stable in the analytical gel system used unless an agent such as hydroxylamine is used to hydrolyze the acylenzyme-inhibitor bond. Thus, the observation that significant amounts of thrombin B-chain are liberated upon denaturation with SDS leads unalterably to the conclusion that the complex relevant to the blood clotting process is not stable under the denaturing conditions used: 95°C, 1\% SDS, (see Materials and Methods). In contrast to the limited amounts of SDS-stable covalent thrombin-AT-III complexes observed on the immunoblots, it is apparent that SDS-dissociable TAT complexes are formed at levels almost equivalent to the thrombin B-chain formed (see Fig 5).

Other stable AT-III immunoreactive complexes are observed at $M_c = 76,000$ (d, Fig 1G) and 74,000 (e, Fig 1G) and represent either cleaved forms of thrombin-AT-III or AT-III complexes with other enzymes. It should again be mentioned that the concentrations of these complexes may be underestimated because of nonequivalent reactivity of the antibody with the complexed-AT-III products; however, this conclusion is less likely with polyclonal antibodies, which are reactive with multiple epitopes.

Platelet factor 4 release. The $\alpha$-granule protein, PF4, is a soluble component readily detectable in the fluid phase of clotting blood and is used to serve as an indicator of platelet activation.\textsuperscript{66,67} Fig 1H shows the appearance of an $M_c = 8,300$ band over time that migrates identically with purified PF4. Quantitation by densitometry demonstrates a rapid increase of PF4, which reaches a maximum density at 12 minutes (Fig 3, A). Comparison with standard amounts of PF4 gives approximately 4.4 $\mu$g/mL in the fluid phase of blood at the maximum, a value consistent with quantitative release of PF4 and, hence, complete platelet activation. At clot time (4.5 minutes) approximately 50\% of the maximum PF4 is released. This is consistent with either complete activation of 50\% of the platelet population or the release of 50\% of the PF4 from the whole platelet population, which has been partially activated. The time-dependent release of PF4 shares a profile similar to that observed for FPA (Fig 2, C) but is displaced in time. In the interval of 12 to 20 minutes PF4 is observed to decrease, reaching a final value of 70\% of maximum, which persists through 60 minutes. Previous studies with purified intact platelets give estimates of 2,700 prothrombinase sites per intact activated platelet;\textsuperscript{3} thus the potential concentration of platelet sites available to support prothrombinase can be estimated. Subject BT with $1.9 \times 10^8$ platelets/mL of blood, or approximately $3.2 \times 10^9$ platelets/mL plasma, has a minimum of $4.3 \times 10^{13}$ prothrombinase sites/mL (or $\sim 700$ pmol/L) (assuming 50\% platelet site activation, Fig 3).

Prothrombinase activity. Estimates of prothrombinase concentration were calculated from the rate of thrombin B-chain generation using the Michaelis-Menten expression described in the Materials and Methods section. These data are plotted in Fig 6 together with data on the concentration of fully activated factor Va, deduced from the limiting (heavy chain or light chain) component, and the molar concentration of prothrombinase sites available on intact platelets, based upon the extent of platelet activation (PF4 release, see Fig 3). Prothrombinase reaches approximately 7 pmol/L at clot time (4.5 minutes) and reaches a maximum of 155 pmol/L at 7 minutes. Prothrombinase activity is then quenched, becoming approximately 10 pmol/L by 12 minutes. This loss of activity is not due to depletion of substrate, which is still at almost 300 nmol/L after 60 minutes (see Fig 4) nor is it a consequence of APC inactivation of factor Va, which is present at 17 nmol/L (see Fig 3), nor is it due to unavailability of prothrombinase sites on activated platelets, $\sim 1.4$ nmol/L. Because neither platelet prothrombinase sites nor...
The evaluation of the interval after clot formation is useful in defining ongoing events and identifying stable end products of the reaction. The results are best used in comparative evaluations of the temporal relationships among reactants (Fig 1) when blood clots.

Concurrent analyses of the cleavage of fibrinogen, factor V, prothrombin, platelet activation, and formation of thrombin-inhibitor complexes lead to the following conclusions:

1. Significant amounts of fibrinogen are incorporated into the insoluble clot initially formed at clot time.
2. Most thrombin is formed subsequent to clot time and 25% of prothrombin remains uncleaved. Nearly equal amounts of prethrombin 2 and thrombin are ultimately formed.
3. The thrombin produced is nearly accounted for by the formation of TAT complex as measured by native ELISA. However, most thrombin is not found in SDS-stable covalent complexes with AT-III or other inhibitors.
4. Factor Va heavy chain is quantitatively generated by clot time, whereas factor Va light chain is generated at a slower rate than the heavy chain with only 10% of the possible light chain accounted for at clot time.
5. At clot time approximately 50% of the platelet α-granule stores have been released.
6. Prothrombinase is approximately 7 pmol/L at clot time and reaches approximately 155 pmol/L at maximum. Prothrombinase activity is eventually inhibited after cleavage of about 75% of the prothrombin.
7. The clot occurs after generation of approximately 15 nmol/L thrombin B-chain or about 1% to 2% of prothrombin activation. However, only 2.5 nmol/L of the thrombin generated in blood at clot time is acting on fibrinogen, suggesting that the major fraction of thrombin activity is occupied by other substrates and inhibitors present in plasma.
8. Significant amounts of prethrombin 2 are produced as a stable product either by cleavage of prothrombin at Arg 271 by factor Xa or at Arg 286 by thrombin or by both, leading to the formation of significantly more prothrombin fragment 1.2 than thrombin or TAT complex.
9. The degradation of the factor Va heavy chain gives rise to activated protein C-like digestion products suggesting either the presence of this enzyme, either because of its constitutive presence or its activation in the time subsequent to clot formation.

We have previously reported the activations of the procoagulation factors presumed essential in a mixture composed of purified components at plasma concentrations system. This system used purified human prothrombin, factors X, IX, and V, recombinant human factor VIII, and PCPS vesicles. The final addition of factor VIIa and relipidated recombinant human tissue factor to the mixture served to initiate the reaction. A comparative analysis of this previously described system and the current whole blood model is instructive in defining the mechanisms of the numerous reactions involved.

As in the present whole blood system, the purified system

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration at Clot Time (4.5 min)</th>
<th>Maximum Observed</th>
</tr>
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<tbody>
<tr>
<td>Factor Va</td>
<td>2 nmol/L†</td>
<td>16 nmol/L‡</td>
</tr>
<tr>
<td>Platelet prothrombinase sites</td>
<td>700 pmol/L</td>
<td>1,400 pmol/L</td>
</tr>
<tr>
<td>Prothrombinase</td>
<td>7 pmol/L</td>
<td>155 pmol/L</td>
</tr>
<tr>
<td>Thrombin</td>
<td>15 nmol/L</td>
<td>360 nmol/L</td>
</tr>
<tr>
<td>Thrombin–AT-III§</td>
<td>ND</td>
<td>&lt;5 nmol/L</td>
</tr>
</tbody>
</table>

Abbreviations: ND, not detectable; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

* Assuming M, = 74/72,000, light chain as the limiting component and a normal (20 nmol/L) factor V concentration (50).
† Assuming 2,700 sites/platelet (45).
‡ Assumed 2,700 sites/platelet (45).
§ Covalent complex on SDS-PAGE.

DISCUSSION

In the model presented the coagulation process is studied in a system approaching native blood (in vitro) and thus, permits the investigation of reactions under near physiological conditions. Because all blood components are present in the system, the observations made with this model may be compared with those made using purified in vitro systems to define areas of consistency and incongruity and, therefore, identify molecules or mechanisms in need of further evaluation. The model has the following advantages: (1) the influence of contact activation is eliminated by specific inhibition of factor XIIa, (2) the reaction mixture is prelabeled for consistent sampling independent of physical state, and (3) analyses of the reactants and products by quantitative Western blotting and ELISA provide the physical state and quantitation of the proteins during various stages of the coagulation process.

Clot time serves as a relevant point of reference and is commonly referred to in the following discussion. A quantitative comparison of the degrees of coagulation factor activation at the time of clot formation is summarized in Table 2.

factor Va is limiting, the prothrombinase concentration must be limited by the active factor Xa concentration. These data suggest that the termination of prothrombinase activity is primarily a consequence of the inhibition of factor Xa. In addition, the data are consistent with the conclusion that factor Xa production by factor VIIIa–factor IXa and factor VIIa–tissue factor have already been terminated at this point in the reaction. This determination suggests that, assuming a normal plasma concentration of factor X (170 nmol/L), the active factor Xa participating in prothrombinase at clot time represents less than 0.004% of the zymogen and does not exceed 0.09% at the maximum. As the reaction progresses between 30 and 60 minutes, the rate of thrombin B-chain production diminishes to levels below that observed at clot time. This reduced rate occurs despite substantial remaining prothrombin substrate (II, Fig 3, lanes 26 through 30) and is therefore consistent with the almost complete downregulation of prothrombinase activity. This downregulation most likely occurs through direct inhibition of prothrombinase as well as the intrinsic and extrinsic tenase complexes.

<table>
<thead>
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displays a factor VIIa-tissue factor concentration dependent lag in thrombin generation. Subsequently, prothrombin is observed to be consumed with the ultimate rate of thrombin propagation varying only fivefold over three orders of magnitude of factor VIIa-tissue factor used. In the present whole blood experiments, performed over a fivefold range in TF concentration (60 to 300 pmol/L), the only observed difference in the generation of thrombin was a difference in lag time, whereas the maximal rates of B-chain production observed were virtually identical. Two recent studies from our laboratory have compared the influence of AT-III, TFPI, protein C, and thrombomodulin added to the synthetic mixture of procoagulants when the latter are at normal physiological concentration. These studies display many but not all of the qualities present in the whole blood system. In particular these studies emphasize how the factor VIIa-tissue factor pathway and TFPI influence the lag time of the reaction. In contrast, the influence of the activated protein C system is principally on the rate of the propagation phase whereas in the purified system AT-III appears to provide mainly a scavenger function, suppressing enzyme products subsequent to the coagulation related events.

The available sites for prothrombinase formation in both purified and whole blood systems are in excess (see Table 2 and Fig 6) and thus, not likely to be limiting. It appears that the concentration of fully activated factor Va (M, = 105,000 heavy chain, M, = 74,000 light chain) is well in excess of the prothrombinase formed. Thus, it follows that factor Xa formation is the limiting component in prothrombinase generation in the whole blood system. Yet, as mentioned above, the thrombin generation rates observed during the propagation phase vary little in response to a broad range of factor VIIa-tissue factor in the purified system, suggesting that the extrinsic tenase, factor VIIa-tissue factor does not significantly influence the ultimate level of factor X activation. This implies that the intrinsic tenase (factor IXa·factor VIIIa) is the major contributor of factor Xa in both systems. It should be appreciated from the present system that picomolar concentrations (or less) of factor IXa·factor VIIIa complex are all that is required for the factor Xa generation observed to be associated with clot formation.

By Western blotting it was shown previously that activation of the various zymogens and cofactors produced no unexpected major products in the purified systems. In contrast, as discussed above, prothrombin 2 represents a major prothrombin cleavage product not predicted from purified in vitro analyses. Prethrombin 2-like product(s) are formed by a single cleavage of prothrombin at Arg284, by factor Xa or at Arg284 by thrombin or both. It follows that prethrombin 2 could arise in whole blood by three possible mechanisms. Free factor Xa acting in solution could cleave at Arg284. This reaction is unlikely since factor Va and platelet prothrombinase sites are in excess of prothrombinase; hence, nearly all the factor Xa would be in this complex. Alternatively, the relative rates of cleavage at Arg271 andArg320 in prothrombin by prothrombinase could be altered in the whole blood environment. A precedent for this exists in the observations of Kung et al71 in which PCPS vesicles containing saturated acyl side chains gave rise to near equal proportions of prethrombin 2 and P-thrombin. Thus, the composition of the natural cell membranes present in whole blood may support a similar change in specificity of prothrombinase. Third, P-thrombin or meizothrombin produced during the reaction could perform feedback cleavage at Arg284 in prothrombin. Although initial cleavage at Arg284 by P-thrombin is preferential in vitro, the whole blood conditions may favorably influence the susceptibility of the Arg284 thrombin cleavage site in prothrombin. Although these three mechanisms remain a possibility, further experimentation in this system and in purified systems is warranted to resolve the mechanisms directing these observed cleavage products of prothrombin. In either case, prethrombin 2 has no known enzymatic activity or biological activation, other than as a potential intermediate in P-thrombin formation. These tentative conclusions are supported by previously reported indirect evidence for prethrombin 2 formation in blood and plasma. Rabiet et al72 reported generation of “prothrombin fragment 1.2.3” (residues 1 to 284) in clotting plasma that would potentially generate prethrombin 2 as the other product. In addition, Teitel et al73 reported that the rate and end point level of F1.2 formation exceeds that of TAT formation in spontaneously coagulating blood. These authors conclude that F1.2 is produced without corresponding thrombin activity and therefore, a significant amount of stable prethrombin 2 must be formed in clotting blood. In addition, the present study demonstrates that SDS-stable TAT complexes represent only a small fraction of the thrombin B-chain that is ultimately generated. As a whole, the above conclusions and the present observations should caution the researcher in using the F1.2 and TAT determinations as a direct index of thrombin activity.

Other prothrombin cleavage products not yet identified have been observed in the present studies. These include a potential thrombin-heparin cofactor II complex, which is formed almost equivalently with the TAT SDS-stable complex. Other products requiring analysis include the product II(x), which either represents an additional inhibitor-thrombin product or an additional cleavage product of prothrombin, and the high molecular weight inhibitor complex Ti a.

We had anticipated that the covalent thrombin-AT-III (TAT) or modified complex (TAT') would be major products based on previous work in plasma and purified systems in vitro. As seen in Fig 5 the ELISA for TAT suggests that nearly all thrombin B-chain is accounted for by complex with AT-III; however, SDS-stable TAT complexes account for less than 2% of the thrombin B-chain and 0.1% of the AT-III, suggesting that thrombin activity is not accounted for by SDS-stable covalent complex formation with AT-III. It is unlikely the complex is disrupted during analysis because it has been previously established to be stable to the SDS gel reducing conditions used. Furthermore, the noncovalent thrombin-AT-III complex has too weak an association (K = 1.4 mmol/L) to account for thrombin inhibition in the current system. The stable form of TAT complex observed on an SDS gel is most likely the acylenzyme, which can be dissociated into the products P-thrombin and modified AT-III by hydroxylamine. This complex must be preceded by a tetrahedrally adduct between the enzyme and inhibitor,
which may not be stable in the SDS gel reducing conditions used. It is therefore possible that the TAT tetrahydroxyl complex was generated and subsequently dissociated by the stopping reagents before the formation of the more stable inhibitor-acyl enzyme complex. It is also important to consider that the characterization of thrombin inhibition by AT-III has been conducted with a-thrombin almost exclusively. This is despite evidence that a-thrombin and F1.2 associate tightly ($K_d = 10^{-19}$) and are in complex when thrombin is generated in situ.76 Furthermore, fragment 2 has been reported to reduce the rate of TAT complex formation threefold.77 Therefore, the lack of SDS-stable TAT complex formation in whole blood may be a result of a contribution of F1.2 to the inhibition process. A reevaluation of AT-III interactions with the various forms of thrombin in purified systems and in this system is critical to resolving the role of AT-III in attenuating thrombin activity in clotting blood.

Factor V activation clearly plays a major role in controlling the coagulation process. An additional complexity not previously recognized is the identification of a new heavy chain of 103,000 molecular weight. The previously unidentified $M_r = 103,000$ factor Va heavy-chain species appears to show greater resistance to degradation, suggesting it is a poorer substrate for APC than the larger product. The difference of the $M_r = 105,000$ and $M_r = 103,000$ may be explained by differences in posttranslational modification. However, if the difference lies in the polypeptide, it is most likely in the COOH terminus because multiple NH$_2$-terminal amino acid sequence determination of the heavy chain has not resulted in two sequences.

The factor Va heavy-chain products a, b, and c appear to be APC associated. The activation of protein C by thrombin is quite slow in the absence of thrombomodulin.8 Although the major fraction of thrombomodulin is contributed by the endothelium, platelet- and plasma-derived thrombomodulin have been described75,74 and most likely contribute to the apparent thrombin activity in the present system. Explanations of the present system to include various endothelial cells as an additional source of thrombomodulin will clarify the contributions of various sources of this thrombin cofactor.

Our studies must be compared with two recent investigations that have employed clotting of minimally altered blood. Thrombin generation in whole blood has recently been described by Kessels et al.75 In this system, blood clotting was initiated with human brain thromboplastin as a source of tissue factor. The blood was maintained in an unaltered condition at 37°C and thrombin was monitored by chromogenic substrate activity in dilute, fluid phase samples. These investigators showed that thrombin activity reaches a maximum of 163 nmol/L and is followed by a decrease to a plateau of 50 nmol/L. In addition, these authors reported that the cleavage of prothrombin is quantitative during the clotting of whole blood. However, this analysis is limited in that the thrombin concentration measured is based on activity toward a small chromogenic substrate. The various thrombin activities are only selective toward physiological macromolecular substrates. The present observations indicate that thrombin activity is almost quantitatively inhibited based on the native TAT ELISA and incomplete cleavage of prothrombin. The discrepancy between Kessels et al and the current observations may be attributed to inhibitors of thrombin that do not occupy the active site, such as $\alpha_2$-macroglobulin, as was shown by Tans et al75 in a plasma system in which thrombin generation was initiated by thromboplastin. In addition, the protocol of Kessels et al75 calls for removal of the clot from the reaction mixture at the point it obstructs sampling. This is despite evidence from their group and others that clot-associated thrombin influences thrombin generation and fibrinogen cleavage.76

Bierdermann et al86 have described a model of spontaneously clotting of contact-activated whole blood for the purpose of investigating the effects of endothelial cells on thrombin activation. In this system, in the absence of endothelial cells, FPA generation proceeds to 14.5 $\mu$mol/L, prothrombin cleavage, judged by F1.2 assay, goes to 68% (950 nmol/L) after 60 minutes of clotting, and TAT reaches 383 nmol/L. These data are consistent with those of the present model TAT = 317 nmol/L, F1.2 = 1014 nmol/L, and FPA = 15.2 $\mu$mol/L at 60 minutes.

In summary, the model presented here examines extrinsic pathway initiated clotting of whole blood in a dynamic system at physiological temperature. This model, in conjunction with previous purified model systems, permits insights into the relevant proteolytic mechanisms of in vivo coagulation events. This model also provides opportunities for precise, holistic examinations of the fitness of blood from patients displaying thrombotic and hemorrhagic tendencies. Finally, evaluation of natural and synthetic modulators of coagulation in a relatively inexpensive, noninvasive para-vivo setting can be conducted in this system.

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