Further Evidence that Thrombin-Activation
of Factor VIII is an Essential Step
in Intrinsic Clotting

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Traces of thrombin markedly enhance the factor VIII (antihemophilic globulin, antihemophilia A factor) activity of plasma (Rapaport, Schiffman, Patch & Ames 1963). In addition, traces of thrombin correct the abnormal thromboplastin generation test (TGT) of incubation reagents prepared free of the significant amounts of prothrombin which usually contaminate them (Rapaport, Schiffman & Ames 1964). A 1 : 200 dilution of normal, non-adsorbed plasma added to the incubation mixture also corrects this abnormality, whereas a 1 : 200 dilution of plasma from a patient with hereditary prothrombin deficiency has no corrective effect. These observations led to the hypothesis that native factor VIII *must* be activated by minute amounts of thrombin before it can participate effectively in the generation of intrinsic prothrombinase (Rapaport, Schiffman & Ames 1964).

This hypothesis differs fundamentally from the current concept of intrinsic clotting as a one-way sequence of reactions in which factor VIII acts several steps before the first thrombin is formed (Macfarlane 1964, Davie & Ratnoff 1964). It seemed important, therefore, to subject the hypothesis to rigorous testing. Accordingly, experiments were de-signed to determine whether or not factor VIII in normal adsorbed plasma could inter-act with serum in incubation mixtures containing minute quantities of hirudin — an anti-thrombin capable of precise, stoichiometric neutralization of thrombin (Markwardt 1963). Aliquots from such mixtures were tested for their ability to clot plasma containing a powerful factor VIII anticoagulant, which served as a sensitive indicator of the generation of clotting activity beyond the need for factor VIII.

The results of these experiments are reported herein. We believe that they provide conclusive evidence that factor VIII requires activation by thrombin to interact normally with serum in the generation of intrinsic pro-thrombinase *in vitro*.

MATERIALS AND METHODS
Materials

Normal plasma. Nine parts of venous blood were mixed with one part of an anti-coagulant made by mixing 3 parts of 0.1 M sodium citrate and 2 parts of 0.1 M citric acid. Plasma was separated by centrifugation. The plasma used for the incubation mixtures was spun at 40,000 rpm for one hour in an ultracentrifuge to remove platelets. Contact

with glass surfaces was avoided in preparation and storage of all plasma. Plasma was stored frozen.

Plasma was adsorbed for use in the incubation mixtures as follows: one part of Al-(OH)$_3$ suspension (Cutter Laboratories, Berkeley, California) was diluted with one part of distilled water and 0.1 ml of the diluted suspension was added to 1 ml of plasma which had been pre-warmed for two minutes at 37°C. The mixture was allowed to stand for three minutes at 37°C, and the Al(OH)$_3$ was then removed by centrifugation. A sample treated in this manner lost no factor V activity and less than 20 per cent of its factor VIII activity. Its prothrombin content was 1 per cent of a normal plasma reference standard (method of Hjort, Rapaport & Owren 1955, modified by addition of a serum reagent to supply factor X).

Normal serum for use in the incubation mixtures was prepared by allowing blood to clot in a 16 x 100 mm glass tube at 37°C. After standing for two hours on the clot, the serum was separated by centrifugation, and 0.2 ml of the citrate anticoagulant was added per ml of serum. Its residual prothrombin activity was approximately 4 per cent of a normal plasma reference standard. The serum was stored frozen.

Hemophilia A plasma, from a patient with a severe factor VIII deficiency, was provided through the courtesy of Dr. O. Egeberg.

Plasma containing an anticoagulant active against factor VIII was obtained from a patient with a marked hemorrhagic diathesis complicating an auto-immune disease. Detailed clinical and laboratory findings will be reported elsewhere (Gorman & Rapaport, unpublished observations). Plasma from this patient was prepared as described above but stored in the lyophilized state. Its behavior in clotting test systems may be summarized as follows:

(1) Quick, P&P (Owren & Aas 1951) and thrombin times were normal. Specific prothrombin assay (Hjort, Rapaport & Owren 1955, modified by addition of a serum reagent to supply factor X) was 96 per cent; factor V assay (Borchgrevink, Pool & Stormorken 1960) was 86 per cent; factor VII assay (Aas 1952, modified by the addition of adsorbed ox plasma) was 48 per cent; factor X assay (modification of technique of Hougie 1962) was 52 per cent.

(2) The partial thromboplastin time with kaolin (Proctor & Rapaport 1961) was markedly prolonged (patient 103 seconds, control 42 seconds) and failed to shorten when the patient's plasma was mixed with an equal volume of control plasma (mixture 100 seconds). Factors XII, XI, IX, and VIII could not be measured by one-stage partial thromboplastin time assay techniques because the anticoagulant in the patient's plasma interfered with the assays even at a 1 : 50 dilution.

(3) When the patient's adsorbed plasma was added as an extra reagent in a TGT mixture containing normal adsorbed plasma, serum, cephalin, and calcium, the mixture failed to generate intrinsic prothrombinase activity (minimum substrate clotting time: 48 seconds). When only normal reagents were used in the incubation mixture and the patient's plasma was used as the substrate, the TGT was normal (minimum substrate clotting time: 8 seconds). Therefore, the anticoagulant blocked the formation of but not the activity of intrinsic prothrombinase.

(4) When normal plasma was mixed with a 1 : 200 dilution of patient's plasma and allowed to stand for 15 minutes at room temperature, the following levels of activity were found: factor XII, 50 per cent; factor XI, 55 per cent; factor IX, 60 per cent; and *factor VIII, 8 per cent.* Normal plasma which had been mixed with a 1 : 500 dilution of patient's plasma and allowed to stand for 60 minutes at room temperature, had a factor IX level of 74 per cent and a *factor VIII level of 6 per cent.*

(5) When dilutions of the patient's plasma were incubated with normal plasma, factor VIII was inactivated progressively with an initially rapid rate tailing off to a plateau after about 60 minutes. The residual factor VIII levels of normal plasma mixed with equal parts of varying dilutions of patient's plasma and allowed to stand for 75 minutes at room temperature were as follows: dilution 1 : 350 — residual factor VIII, 7 per cent; dilution 1 : 600 — residual factor VIII, 26 per cent; dilution 1 : 750 — residual factor VIII, 43 per cent.

We conclude from these studies that the plasma contained an inhibitor active against factor VIII but not active against other clotting factors.

Standard Thrombin (lot B3), containing 21.7 units per mg, was obtained from the Division of Biologic Standards, National Institutes of Health, Bethesda, Maryland. It was received in February 1964 and stored unopened at 4°C until used in October 1964.

Purified Human Thrombin was obtained through the generosity of Dr. Kent Miller of Albany, New York. It was received in April 1964 as a lyophilized preparation sealed under vacuum and containing approximately 150 Iowa units per ml when reconstituted in 3 ml. It was reconstituted in distilled water in November 1964 and stored frozen in small lots over the several days it was used. When assayed immediately after reconstitution, this stock preparation gave values of 94, 96, and 110 NIH units per ml; when assayed after storage, values of 86, 88, 90, and 91 NIH units per ml were obtained. A mean value of 89 units per ml was used in calculating the strengths of dilutions. Dilutions were made in barbital buffer.

Bovine Thrombin. The contents of one ampule of a commercial preparation, Topostasine (Roche, Basel), were dissolved in oxalated saline, adsorbed with barium sulfate as described elsewhere (Rapaport, Schiffman, Patch & Ames 1963), and stored frozen as a stock solution containing approximately 1,000 NIH units per ml. Dilutions were made in barbital buffer.

Hirudin. Pure Hirudin (lot 036044) was obtained from Veb Arzneimittelwerk, Dresden, German Democratic Republic. It was stated to contain 3,000 anti-thrombin units per ampule (1 anti-thrombin unit neutralizes 1 NIH unit of thrombin). The contents of one ampule were dissolved in 100 ml of barbital buffer and stored as a stock solution at 4°C. Its anti-thrombin activity was assayed on four occasions over an eight-week period and values of 22.0, 18.0, 20.0, and 17.5 units per ml were obtained. A mean value of 19.5 units per ml was used in calculating the strengths of dilutions.

Cephalin was prepared as described elsewhere (Rapaport, Aas & Owren 1954) and was stored frozen as a stock suspension in barbital buffer containing 42 mg per ml. It was diluted 1 : 100 in buffer before use.

Citrated-saline was made by adding 1 part of 0.1 M sodium citrate to 5 parts of isotonic sodium chloride solution. *Barbital buffer* and *calcium chloride* solutions were made as described earlier (Rapaport, Schiffman, Patch & Ames 1963).

Frozen reagents were stored at —25°C in small aliquots in capped plastic vials and were thawed by immersion in water at 37°C.

Methods

Incubation mixtures usually consisted of 0.5 ml aliquots of normal adsorbed plasma diluted 1 : 5 in buffer, serum diluted 1 : 10 in buffer, hirudin diluted in buffer or buffer alone (control), and 30 mM calcium chloride solution. In some experiments, 0.1 ml of dilute purified human thrombin or buffer (control) was added to an incubation mixture after 40 minutes.

The incubation mixtures stood at 37°C in 10 × 75 glass tubes. At intervals, 0.1 ml subsamples were transferred to the *clotting mixtures*. Two clotting mixtures were used. In each, 0.1 ml aliquots of substrate plasma and cephalin were pre-warmed together for three minutes at 37°C before the addition, in rapid succession, of 0.1 ml of 20 mM calcium chloride solution and 0.1 ml of the subsample from the incubation mixture. The clotting time was measured from the addition of the latter.

The substrate plasma in the clotting mixtures differed. In one mixture (control) hemophilia A plasma was used, whereas in the second mixture factor VIII anticoagulant plasma was used. Thus, although both substrate plasmas lacked factor VIII, one of the substrate plasmas was also capable of inhibiting added factor VIII.

Thrombin concentrations were determined from reference curves made with the standard thrombin. On two occasions, 20.7 and 75.8 mg, respectively, of standard thrombin were added to exactly 25 ml of barbital buffer in a plastic tube. Brief warming at 37°C and gentle mixing with a plastic Pasteur pipette brought the thrombin completely into solution. The thrombin solution was then placed in an ice bath, and dilutions were made using chilled buffer and plastic tubes. The activity of these dilutions was assayed immediately, using a thrombin time system consisting of one part of thrombin from the ice bath added to two parts of human plasma which had been pre-warmed to 37°C. The two reference curves corresponded very closely. The thrombin concentrations of the working stock solutions of the bovine and purified human thrombins were determined by doing thrombin times on appropriate dilutions and converting these times to NIH units from the reference curves.

The *hirudin was standardized* in the following manner. A working stock solution of thrombin in barbital buffer was made up to contain approximately 100 NIH units per ml. Aliquots of 0.2 ml of this thrombin solution were mixed with from 0.1 to 0.7 ml of stock hirudin solution; thirty seconds later, enough chilled barbital buffer was added to the mixture to bring its total volume to 2 ml. This gave a series of 1 : 10 dilutions of thrombin in mixtures of varying proportions of stock hirudin solution and barbital buffer. The thrombin times of these dilutions were determined and converted to thrombin units as described above. The following equation was used to calculate the hirudin concentration of the stock solution:

$$C_h = \frac{2 \text{ ml}}{V_h} (T_e - T_o),$$

where C_h is the hirudin concentration in units per ml, V_h is the volume of stock hirudin solution added to the mixture, T_e is the thrombin concentration of a $1:10$ dilution made with buffer alone, and T_o is the thrombin concentration in the mixture containing hirudin.

Factor VIII was assayed by a modified partial thromboplastin time technique in which 0.1 ml of cephalin $1:100$ in a 10 mg per ml kaolin suspension was pre-incubated at $37°C$ with 0.1 ml of hemophilia A substrate plasma for 9 minutes. Then, 0.1 ml of test material and 0.1 ml of 40 mM $CaCl_2$ were added.

RESULTS

1. *Generation of a clotting intermediate which bypasses the factor VIII anticoagulant*

As Table I illustrates, early subsamples from an incubation mixture of adsorbed plasma, serum, and calcium clot factor VIII anticoagulant plasma much more slowly than they clot hemophilia A plasma. In clear contrast, later aliquots clot both plasmas equally rapidly. An intermediate is formed during incubation which bypasses the factor VIII anticoagulant; i.e., it is beyond the need for further native factor VIII activity.

Although unlikely, one had to rule out the possibility that the 'bypass activity' was simply thrombin-activated factor VIII. Therefore, an experiment was carried out in which factor VIII in normal plasma was first activated by a minute amount of thrombin and then mixed with factor VIII anticoagulant plasma. Subsamples of the mixture were assayed for factor VIII activity. The details of this experiment are given in the footnote

T a b l e I. *The generation of clotting activity bypassing an anticoagulant against factor VIII*

Incubation time (minutes)	Clotting times (seconds)	
	Hemo. A pl.	F. VIII anticoag. pl.
3	80	190
6	65	120
9	53	80
12	35	45
15	23	27
20	14	16
30	15	16

Incubation mixture: 0.5 ml volumes of normal adsorbed plasma $1:5$, serum $1:10$, and 30 mM $CaCl_2$. At times listed, 0.1 ml subsample added to clotting mixture.

Clotting mixture: 0.1 ml volumes of *either* hemophilia A plasma *or* factor VIII anticoagulant plasma, cephalin, 20 mM $CaCl_2$, and the subsample from incubation mixture.

to Table II. The data in this Table clearly show that the anticoagulant blocks factor VIII in both its native and its thrombin-activated form.

Thus, thrombin-activated factor VIII could not account for the 'bypass activity' formed on incubating adsorbed plasma and serum; the activity had to arise from an interaction between factor VIII and serum. The stage was set, therefore, to determine i this *interaction* required prior activation of factor VIII by traces of thrombin.

2. *Failure to generate the intermediate when traces of thrombin in the incubation mixture are neutralized by hirudin*

Hirudin neutralizes thrombin unit for unit (Markwardt 1963). Figure 1 shows what was found when adsorbed plasma, serum, and calcium were incubated, first with buffer

T a b l e   I I. *Evidence that the factor VIII anticoagulant blocks both native and thrombin-activated factor VIII*

Time of subsamples (minutes)	Factor VIII activity (per cent)			
	Plasma – Buffer +		Plasma – Thrombin +	
	Hemo. A pl.	Anticoag. pl.	Hemo. A pl.	Anticoag. pl.
1	78	16	405	13
3	84	14	510	10
7	84	14	510	11
10	84	14	405	11

Aliquots of 0.3 ml of normal plasma and of *either* bovine thrombin (0.1 NIH unit per ml) *or* buffer were incubated together for 3 minutes at room temperature. Then 0.3 ml of *either* hemophilia A plasma 1 : 10 in buffer *or* factor VIII anticoagulant plasma 1 : 10 in buffer was added. Incubation was continued and at the times listed, subsamples were diluted 1 : 10 in citrated-saline and assayed for factor VIII activity. Clotting times were converted to per cent factor VIII activity from a dilution curve prepared with normal, non-thrombin activated plasma.

as an added reagent (control), and then with hirudin in a final concentration of 1.6 units per ml in the mixture. The control mixture generated 'bypass activity', which clotted both hemophilia A plasma and factor VIII anticoagulant plasma in about 20 seconds. No such activity was demonstrable in the mixture containing hirudin. Subsamples after 40 minutes of incubation gave prolonged, widely divergent clotting times for the two plasmas, very similar to the clotting times obtained with the initial subsamples.

In this experiment, hirudin was carried over from the incubation mixture into the clotting mixture to give a final concentration of hirudin in the latter of 0.40 units per ml. Therefore, an additional control experiment was carried out in which hirudin was added directly, as a separate reagent, to the clotting mixture. The details of the experiment are given in the footnote to Table III. As can

be seen from Table III, a late subsample from an incubation mixture without hirudin (i.e., a subsample containing 'bypass activity') clotted hemophilia A plasma and factor VIII anticoagulant plasma in similar times even when the clotting mixtures contained about six times more hirudin than in the experiment shown in Figure 1. Obviously, therefore, the long, divergent clotting times of Figure 1 did not result from hirudin being carried over into the clotting mixture but from a failure to generate 'bypass activity' in the presence of hirudin. Neutralizing the thrombin in the incubation mixture with hirudin prevented the formation of the intermediate.

3. The amount of thrombin required to initiate generation of the intermediate

One can estimate a maximum concentration for the thrombin which could form in

Table III. *The similar clotting times of hemophilia A plasma and factor VIII anticoagulant plasma when the clotting mixture contains both 'bypass activity' and hirudin*

Final conc. of hirudin in clotting mixture (units pr ml)	Clotting times (seconds)	
	Hemo. A plasma	Anticoag. plasma
0 (buffer)	24	24
1.3	39	41
2.5	39	41

An incubation mixture was made up of equal volumes of adsorbed plasma 1 : 5, serum 1 : 10, buffer and 30 mM $CaCl_2$. After 35 min., subsamples were added to the clotting mixtures. In the clotting mixtures, 0.1 ml of *either* hemophilia A *or* factor VIII anticoagulant plasma and 0.1 ml of cephalin were incubated together for 3 min. Then, in rapid succession, were added 0.1 ml of *either* buffer *or* hirudin to give the final conc. listed, 20 mM $CaCl_2$, and the subsample from the incubation mixture.

the incubation mixture, assuming total conversion of its prothrombin to thrombin. Markwardt's studies with hirudin (Markwardt 1963) indicate that normal plasma contains about 200 NIH units of potential thrombin (i.e., prothrombin) per ml. Our adsorbed plasma contained 1 per cent of normal prothrombin activity (potential of 2 NIH units of thrombin per ml) ; our serum contained 4 per cent residual prothrombin activity (potential of 8 NIH units of thrombin per ml). An incubation mixture contained 0.5 ml of plasma 1 : 5 (potential of 0.1 NIH unit) plus 0.5 ml of serum 1 : 10 (potential of 0.4 NIH unit) in a total volume of 2 ml. Therefore, the maximum possible thrombin concentration in the mixture would be about 0.25 NIH unit per ml.

With this theoretical upper limit in mind,

the next step was to determine how much hirudin was needed to interfere with the generation of 'bypass activity'. Incubation mixtures were made with increasingly dilute hirudin to give the final concentrations in the incubation mixture shown in Table IV. Subsamples were tested frequently over a 40- to 60-minute period, but for simplicity of presentation only the 30-minute data are given in the Table. This interval was chosen because control mixtures with buffer developed full activity within 30 minutes. Table IV shows that as little as 0.005 units per ml of hirudin interfered with the interaction between factor VIII and serum over a 30-
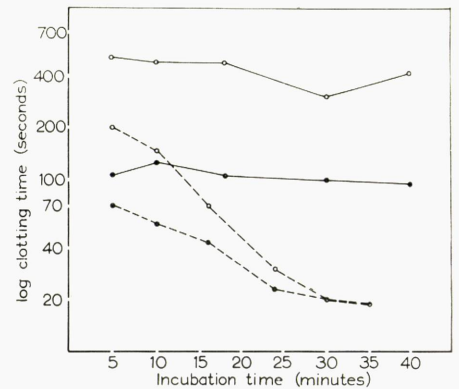

Fig. 1. Evidence that hirudin in a final concentration of 1.6 units per ml blocks the generation of clotting activity bypassing the factor VIII anticoagulant.

Incubation mixtures contained 0.5 ml volumes of normal ads. plasma 1 : 5, serum 1 : 10, *either* buffer *or* hirudin (final conc. 1.6 units per ml), and 30 mM $CaCl_2$. At times shown, 0.1 ml subsamples were added to clotting mixtures prepared as described in footnote to Table I.

	Incub. mix.	Clotting mix.
○——○	Hirudin	F.VIII anticoag. pl.
●——●	Hirudin	Hemophilia A pl.
○-----○	Buffer	F.VIII anticoag. pl.
●-----●	Buffer	Hemophilia A pl.

Table IV. *The minimum concentration of hirudin which inhibits the generation of clotting activity bypassing the factor VIII anticoagulant*

Final conc. of hirudin in incubation mixture (units per ml)	Clotting times of 30-min. subsamples (sec.)	
	Hemo. A plasma	Anticoag. plasma
None	24	24
0.005	60	120
0.01	60	135
0.02	80	195
0.07	73	235
0.16	80	240

Incubation mixtures were made up of equal aliquots of adsorbed plasma 1:5, serum 1:10, *either* buffer *or* hirudin to give the final concentrations listed, and 30 mM CaCl₂. The clotting times shown were obtained with subsamples taken at 30 minutes and added to clotting mixtures as described in the footnote to Table I.

minute incubation period. This means that as little as 0.005 NIH units per ml of thrombin (1/50th of the total theoretical thrombin potential of the mixture) may initiate the activation of factor VIII in such incubation mixtures, permitting the factor VIII then — but only then — to interact with serum to form an intermediate beyond factor VIII. These data fit well with earlier evidence that the addition of exogenous thrombin to plasma in a final concentration of 0.005 to 0.01 NIH units per ml produces a readily detectable increase in factor VIII activity (Rapaport, Schiffman & Ames 1964).

The interference produced by hirudin in concentrations up to 0.01 units per ml was overcome when mixtures were incubated for 60 minutes. The 0.02 units per ml mixture still exhibited a partial block, clotting

hemophilia A plasma in about 40 seconds and factor VIII anticoagulant plasma in about 60 seconds. The 0.07 units per ml mixture required between 90 and 120 minutes to generate enough 'bypass activity' to clot the two plasmas in the same time. The incubation mixture containing 0.16 units per ml of hirudin still gave divergent clotting times of 82 seconds for hemophilia A plasma and 135 seconds for anticoagulant plasma when tested after 110 minutes of incubation. (This incubation mixture contained enough hirudin to block approximately three-fifths of the theoretical total potential thrombin yield of the mixture). Clearly, mixtures of adsorbed plasma, serum, and calcium generate only minute amounts of thrombin before clotting intermediates beyond factor VIII are generated, but, just as clearly, these minute amounts are essential. If they are neutralized, even prolonged incubation fails to result in the generation of 'bypass activity'.

4. *Generation of the intermediate when thrombin is added to a 'hirudin-blocked' incubation mixture*

A final experiment seemed important to complete the evidence. If hirudin blocked the generation of the intermediate because it neutralized minute amounts of thrombin, then adding highly purified human thrombin to an incubation mixture containing hirudin should overcome the 'hirudin-block'. Mixtures containing hirudin were allowed to incubate for 40 minutes to establish the existence of a 'hirudin-block'. Then, 0.1 ml of either human thrombin or of buffer (control) was added to the incubation mixture. Consistently, activity bypassing the factor VIII anticoagulant developed within the incubation
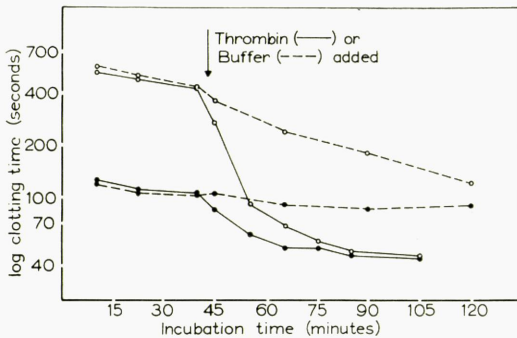
Fig. 2. The slow generation of 'bypass activity' when 0.20 units of hirudin in the incubation mixture are incompletely neutralized by the addition of 0.11 units of thrombin.

Both incubation mixtures contained 0.5 ml volumes of ads. plasma 1 : 5, serum 1 : 10, hirudin in a final concentration of 0.14 units per ml, and 30 mM calcium chloride. At the time indicated by the arrow *either* 0.11 units of thrombin (0.1 ml of a 1 : 80 dilution of the stock preparation containing 89 units per ml) *or* 0.1 ml of buffer was added to the incubation mixture (see text).

The clotting mixtures are described in the footnote to Table I. Solid circles (●) denote hemophilia A plasma clotting times; open circles (○) denote factor VIII anticoagulant plasma clotting times.

mixture receiving the thrombin. The rate of its formation varied with the amount of thrombin added.

Figure 2 illustrates an experiment in which the hirudin in the incubation mixture was incompletely neutralized by the exogenous thrombin. The incubation mixture contained hirudin in a concentration of 0.14 units per ml and a total volume of 2 ml. A volume of 0.6 ml was removed over 40 minutes to obtain the clotting times shown prior to the addition of thrombin. This left 0.20 units of hirudin in the mixture (1.4 ml x 0.14 units per ml). Thrombin, 0.11 NIH units, was then added to the mixture, leaving 0.09 units

of hirudin not neutralized by thrombin. As the Figure shows, under this condition activity bypassing the factor VIII anticoagulant developed gradually, over the next 40 to 60 minutes, in the thrombin-treated mixture but not in the control mixture. Apparently, the added thrombin reduced the excess of hirudin to an amount which could be gradually neutralized by thrombin generated within the mixture itself.

Figure 3 is a plot of the clotting times obtained when the experiment was repeated with the addition of 0.22 NIH units of thrombin to the incubation mixture. This resulted in an excess of 0.02 units of exogenous thrombin over hirudin in the incubation mixture. This amount of thrombin produced a dra-
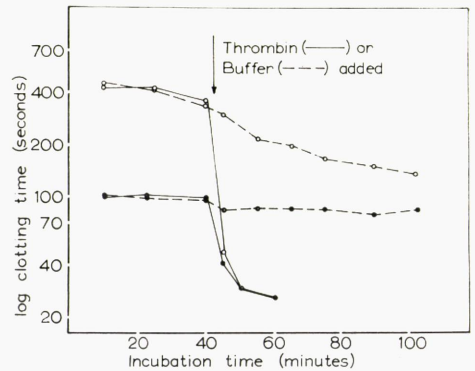

Fig. 3. The rapid generation of 'bypass activity' when 0.20 units of hirudin in the incubation mixture are completely neutralized by the addition of 0.22 units of thrombin.

Incubation mixtures as described for Fig. 2, except that at time indicated by the arrow *either* 0.22 units of thrombin (0.1 ml of a 1 : 40 dilution of the stock preparation containing 89 units per ml) *or* 0.1 ml of buffer was added to the incubation mixture.

The clotting mixtures are described in the footnote to Table I. Solid circles (●) denote hemophilia A plasma clotting times; open circles (○) denote factor VIII anticoagulant plasma clotting times.

Table V. *The failure of thrombin added directly to the clotting mixture to eliminate the difference between the clotting times of hemophilia A plasma and factor VIII anticoagulant plasma*

Incubation time of subsamples (minutes)	Clotting times (seconds) of clotting mixtures containing			
	Buffer		Thrombin	
	Hemo. A pl.	Anticoag. pl.	Hemo. A pl.	Anticoag. pl.
45	115	435	85	240
70	90	322	75	180

An incubation mixture was made up containing 0.5 ml volumes of adsorbed plasma 1:5, serum 1:10, hirudin (final conc. 0.14 units per ml), and 30 mM $CaCl_2$. At 40 minutes, 0.1 ml of buffer was added as in control experiments shown in Figs. 2 and 3. At the times indicated in the Table, 0.1 ml subsamples were added to the clotting mixtures. The clotting mixtures consisted of 0.1 ml volumes of *either* hemophilia A *or* factor VIII anticoagulant plasma plus cephalin, which were incubated together for 3 minutes. Then, in rapid succession, were added 0.1 ml aliquots of *either* thrombin (0.09 NIH units per ml) *or* buffer, 20 mM $CaCl_2$, and the subsample from the incubation mixture.

matic shortening of the clotting times of subsamples from the incubation mixture. The difference between the clotting times of factor VIII anticoagulant plasma and of hemophilia A plasma disappeared within minutes.

This striking effect was not due to the carry-over of thrombin into the clotting mixture. In the experiment summarized in Table V, the direct addition of thrombin to the clotting mixture in four times the amount added from the incubation mixture of Figure had little effect upon the widely divergent clotting times of hemophilia A plasma and factor VIII anticoagulant plasma obtained with subsamples from an incubation mixture containing hirudin. Thus, the remarkable shortening of the clotting times shown in Figure 3 must stem from a sudden generation of activity bypassing the factor VIII anticoagulant, made possible by the slight excess of thrombin in the incubation mixture.

DISCUSSION

It was shown in 1953 (Biggs, Douglas & Macfarlane 1953) that factor VIII interacts with serum in the presence of calcium to give rise to a clotting intermediate. This intermediate was called Product I (Bergsagel & Hougie 1956). Adding exogenous thrombin to Product I incubation mixtures was shown to hasten its formation (Horowitz & Spaet 1961). It would now appear that Product I is activated factor X (Spaet & Cintron 1963). At least two sequential reactions are involved in its generation in incubation mixtures of adsorbed plasma, serum and calcium. In the first, activated factor IX reacts with factor VIII to form activated factor VIII; in the second, activated factor VIII activates factor X (Macfarlane, Biggs, Ash & Denson 1964).

The data presented herein establish that mixtures of adsorbed plasma, serum, and cal-

cium fail to generate a clotting intermediate beyond the further need for factor VIII if the minute amounts of thrombin which arise in such mixtures are neutralized by hirudin. Since traces of thrombin are known to activate factor VIII but to exert no effect upon factor IX or factor X (Rapaport, Schiffman, Patch & Ames 1963), we believe these data furnish very strong evidence that factor VIII *requires* activation by a trace of thrombin before it can interact effectively with factor IX in serum.

The minuteness of the amount of thrombin needed to activate the factor VIII in our incubation mixtures — 0.005 NIH units per ml — deserves emphasis for two reasons. First, it puts in perspective the question: how can factor VIII require activation by thrombin when factor VIII is needed for the effective generation of thrombin? We must look for an initial mechanism or mechanisms — different from the sequence of reactions subsequently responsible for the rapid and massive generation of thrombin — which convert only the first tiny fraction (possibly only 1/10,000th) of the prothrombin in plasma to thrombin. Second, it warns investigators just how significant 'insignificant' amounts (or, unless very sensitive techniques are used, 'unmeasurable' amounts) of thrombin may be in intrinsic clotting mixtures.

The currently popular 'cascade' (Macfarlane 1964) and 'waterfall' (Davie & Ratnoff 1964) theories of clotting state that as each factor becomes activated, it, in turn, activates a factor further along in the reaction sequence. Intrinsic clotting is pictured as a sequence of ever forward-moving reactions between successive pairs of clotting factors in which the job of one is to activate the other.

Like rushing water, such a sequence of reactions seems difficult to subject to the fine control necessary for normal hemostasis. The potential for a 'runaway reaction' seems ever present. One can readily appreciate the added safety of a step in this sequence in which two factors, factors IX and VIII, each activated by a different mechanism, must exist simultaneously in an activated state before they can interact with each other. Moreover, one can admire the self-contained efficacy of a control mechanism in which traces of thrombin activate factor VIII, whereas the larger amounts of thrombin, forming as a consequence of the activation, destroy factor VIII.

It is important to recall that traces of thrombin also activate factor V (Ware, Murphy & Seegers 1947). Only small amounts of thrombin are required to initiate this activation (Hjort 1957). A further parallel with factor VIII exists in that larger amounts of thrombin destroy factor V in human blood. Although it has yet to be shown that factor V *must* be activated by thrombin before it can participate effectively in clotting, it is tempting to believe that this will prove to be true. Since factor V participates in extrinsic as well as intrinsic clotting, this would mean that a trace of thrombin, however formed, releases an important brake upon both the clotting systems.

The evidence that factor VIII undergoes a 'double activation', first by thrombin and then by factor IX, means that a system of symbols will have to be devised to designate (1) native factor VIII, (2) thrombin-activated factor VIII, and (3) thrombin-activated factor VIII after it has been further activated by factor IX. One possibility would

be to use the subscript $_t$ for the thrombin-acti-vated state. Thus VIII would be the native state, $VIII_t$ the thrombin-activated state and $VIII_a$ (as already proposed by Macfarlane, Biggs, Ash & Denson 1964) would be the final state of activation produced by the inter-action with factor IX. Clearly, this or a related system of nomenclature is essential if confusion is to be avoided.

We believe that the data presented herein prove that factor VIII must be activated by thrombin before adsorbed plasma and serum can interact normally in the generation of intrinsic prothrombinase in *in vitro* incuba-tion mixtures. Since traces of thrombin also markedly shorten partial thromboplastin times (Rapaport, Schiffman, Patch & Ames 1963) we believe that activation of factor VIII by thrombin is also a prerequisite for effective intrinsic prothrombinase synthesis in this *in vitro* clotting system. The foregoing discussion has implied that a similar activa-tion of factor VIII is required before fac-tor VIII can react with factor IX in hemo-stasis *in vivo*. This generalization, of course, remains to be proven. Moreover, other mechanisms of activation, besides activation by thrombin, could also operate *in vivo,* e.g. activation by other proteolytic enzymes in blood, by an agent liberated from platelets, or by a factor such as collagen which the blood contacts during tissue injury. Further work to evaluate such possibilities is obvi-ously needed.

SUMMARY

The combined use of hirudin in incubation mixtures and of a factor VIII anticoagulant in clotting mixtures has enabled us to show that factor VIII *must* be activated by minute amounts of thrombin before it can interact effectively with serum in intrinsic prothrom-binase generation. The implications of this observation have been discussed.

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