The Activation of Factor VIII by Thrombin

By A. H. Ozge-Anwar, G. E. Connell and J. F. Mustard

There is now substantial evidence that thrombin may play a part in coagulation of blood at stages other than the conversion of fibrinogen to fibrin. Therriault et al.1 and Penick2 found suggestive evidence that thrombin might interact specifically with factor VIII and, more recently, Rapaport et al.3 have described a systematic investigation of this possibility. Rapaport et al.3 have shown that a minute amount of thrombin can bring about an apparent increase of five- to 40-fold in the activity of factor VIII during short periods of incubation of thrombin with plasma.

In the present work, activation by thrombin of factor VIII in partly purified preparations has been demonstrated. The activation was attributed to the interaction of factor VIII and thrombin by, first, comparing the effect of thrombin on factor VIII preparations and on similar preparations from factor VIII-deficient plasma, and, secondly, by demonstrating that activation was inhibited by diisopropylfluorophosphate (DFP), a relatively specific inhibitor of thrombin. The methods which were employed also permitted examination of the effects of thrombin on factor VIII in isolation from most other clotting factors.

Materials and Methods

Factor VIII Assay

The method of Breckenridge and Ratnoff4 was used in factor VIII assays and in enzymic activation studies. This method is basically a partial thromboplastin time with kaolin activation. The following reagents were employed.

Standard Plasma: Blood from volunteer Red Cross donors was collected from the antecubital vein through plastic tubing into plastic tubes containing 3.8 per cent trisodium citrate. The volume of anticoagulant was one-tenth of the final volume of blood. The blood was collected while flowing freely, and the tubes were subjected to continuous mixing. The blood was chilled in crushed ice and centrifuged in the cold immediately for 20 minutes at 5000 r.p.m. (3020 g.). The cellular layer and the lower one-fourth of the plasma were discarded. The remainder of the plasma was recentrifuged under the same conditions. Plasma from 5 to 6 donors was pooled, dispensed in 1 ml. portions in silicone-coated* glass tubes, frozen rapidly and stored in a liquid nitrogen.

From the Departments of Biochemistry and Medicine, University of Toronto.

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Ayse H. Ozge-Anwar, M.D.: Research Fellow of the Ontario Heart Foundation; Graduate Student in the Departments of Biochemistry and of Medicine, University of Toronto, Toronto, Canada. George E. Connell, Ph.D.: Associate Professor of Biochemistry, University of Toronto, Toronto, Canada. J. Fraser Mustard, M.D., Ph.D.: Assistant Professor of Pathology, Associate, Department of Medicine; M.R.C. Associate, University of Toronto, Toronto, Canada.

*Obtained from Canadian General Electric, Sc. 87.
refrigerator. The factor VIII activity did not change during three to four months of storage.

**Deficient Plasma:** This was obtained by the same procedure from a single patient with severe factor VIII deficiency. Thromboplastin generation was always found to be defective in plasma prepared at different times over a period of 2 years although slight fluctuations in the degree of deficiency were observed.

**Veronal Buffered Isotonic Saline (VBIS) pH 7.35:** This contained 9.0 Gm. NaCl, 200 ml. 0.10 M. sodium diethyl barbiturate, and 144 ml. 0.10 M HCl per liter of solution. This solution was used as a diluent in the assay.

**Kaolin:** This was obtained from the Fisher Scientific Company. (Acid Washed-American Standard, Cat. No. K-5).

**Lecithin (Centrolex-O):** This was obtained from Central Soya, Chemicry Division, Chicago, Illinois. A solution, 0.1 per cent with respect to Centrolex, in physiologic saline was prepared by emulsifying the lecithin granules in a grinder. The final suspension was divided into 5 ml. aliquots, in glass test tubes, covered with parafilm and stored at −20 C. For use in the assay procedure 3 mg. of kaolin was added to each milligram of Centrolex suspension.

**CaCl₂:** A 0.025 M. CaCl₂ solution was used.

Plastic pipettes (Falcon Plastic Company, Los Angeles, California) were used for transfer operations in enzyme studies. Except for standard plasma, plastic tubes (Falcon Plastic Company) were used for storage of materials. Pyrex glass test tubes 10 x 75 mm. were used for clotting tests.

**Preparation of Factor VIII:** The procedure of Blombäck⁵ was followed for the preparation of fraction I-O from normal plasma. Typical preparations were found to be 30- to 40-fold higher in specific activity (per mg. nitrogen) than standard plasma. The preparation was dissolved in 0.055 M sodium citrate, dispensed in 1 ml. portions, frozen rapidly, and stored in a liquid nitrogen refrigerator.

The fraction I-O was examined for the presence of prothrombin, factor V, factor IX, and factor XI by the following methods. The one-stage prothrombin time which gives an overall impression of abnormality in prothrombin and factors V, VII and X was carried out according to the technic described by Quick.⁶ In this assay 0.1 ml. of a 1/10 dilution of fraction I-O + 0.2 ml. Simplastin* (rabbit brain thromboplastin + CaCl₂) were mixed and the clotting time recorded at 37 C. A similar test was also carried out in the presence of Al(OH₃)₃-absorbed citrated human plasma as an additional source of fibrinogen and factor V. This reaction mixture consisted of 0.1 ml. of a 1/10 dilution of fraction I-O + 0.1 ml. double Al(OH₃)₃-absorbed citrated human plasma + 0.2 ml. Simplastin. In both instances the clotting times were found to be longer than 5 minutes.

The factor V assay was carried out according to the technic outlined by Owren.⁷ No factor V activity was found.

Factor IX activity was assayed using the thromboplastin generation test of Biggs and Douglas.⁸ The effect of fraction I-O in correcting a factor IX deficient system was assayed. The generation mixture consisted of 0.2 ml. of a 1/10 diluted double Al(OH₃)₃-absorbed citrated human plasma + 0.2 ml. of a 1/10 dilution of fraction I-O + 0.4 ml. of a 1/20 dilution of factor IX deficient serum + 0.4 ml. of 0.1 per cent soybean lecithin + 0.4 ml. of 0.025 M. CaCl₂. No factor IX activity could be demonstrated.

Factor XI activity was assayed using artificially depleted factor XI deficient substrate according to the technic of Waaler.⁹ There was no evidence of factor XI activity in this assay.

The fraction I-O preparations were not tested specifically for factors VII and X but they failed to correct a prolonged prothrombin time of a patient who had been receiving dicoumarol therapy for several months.

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*Obtained from Warner-Chilcott Div., Morris Plains, N. J., U. S. A.
†Obtained from British Drug Houses.
Table 1.—Factor VIII Assay on Fraction I-O from Normal and Factor VIII-Deficient Plasma

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Clotting Times in Seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undilated</td>
</tr>
<tr>
<td>Plasma (undiluted)</td>
<td>79.6</td>
</tr>
<tr>
<td>Fraction I-O (normal)</td>
<td>77.6</td>
</tr>
<tr>
<td>Fraction I-O (Factor VIII-deficient)</td>
<td>166.9*</td>
</tr>
</tbody>
</table>

For these experiments the Fractions I-O were dissolved in a volume which was equivalent to that of the plasma from which the fraction was prepared. Thus the tests reported under the heading “undiluted” were performed on preparations which were diluted ten-fold relative to ordinary preparations. In the tests reported under the heading VBIS, veronal-buffered isotonic saline replaced the test material.

*Mean of ten determinations.
†Mean of two determinations.

A fraction I-O preparation using factor VIII-deficient plasma was carried out in exactly the same way. The results of factor VIII assays on typical normal and deficient preparations at selected concentrations are presented in table 1. These assays and others at greater dilution showed that the relation between clotting time and \( \log_{10} \) of dilution was linear for normal fraction I-O down to a dilution of 1/800. The line was almost parallel to the corresponding line for standard plasma. The values for undiluted plasma and I-O preparations were not on the linear portion of the graph of clotting time vs. \( \log_{10} \) of dilution. Factor VIII assays on the deficient I-O preparation were not significantly different from the VBIS controls in 10 experiments.

Special Reagents

Bovine Thrombin: This was obtained from Parke Davis and Company (Thrombin-Topical) and treated as described by Rapaport et al. The preparation showed four major zones in starch gel electrophoresis and other minor components. It has been our experience that this thrombin in the concentration used in this study has no correcting effect on factor IX-deficient serum in the thromboplastin generation test. It is assumed therefore that the factor IX content of the preparation was very low.

Trypsin: Crystalline bovine trypsin was a gift of Canada Packers Limited. This preparation gave a single zone on starch gel electrophoresis.

Soybean Trypsin Inhibitor (SI-5488): This was obtained from the Worthington Biochemical Corporation.

Diisopropylfluorophosphate (DFP): This was kindly provided by Merck, Sharp and Dohme Limited. A 0.10 M. stock solution of DFP in isopropyl alcohol was stored at —20 C. This stock solution was diluted a further five-fold with VBIS to give a working solution of 0.020 M.

Enzyme Activation Studies: The basic experimental design for enzyme activation studies is illustrated in figure 1. A preparation of fraction I-O (diluted one to ten with VBIS) containing factor VIII is incubated with thrombin and subsequently with DFP, while an identical factor VIII-deficient preparation is treated with equivalent volumes of VBIS. The two preparations are then mixed and a portion is tested by the standard factor VIII assay method. In a reciprocal experiment, the deficient preparation is treated with thrombin and DFP, while the factor VIII preparation is treated with equivalent volumes of VBIS. All experiments of this type were conducted with coded samples so that the nature of the experimental materials was unknown by the person who determined the clotting times.
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Results

Preliminary Experiments Using Enzymes and Inhibitors

The general scheme for the experiments was to incubate thrombin with preparations of factor VIII, to inactivate the thrombin with DFP, and finally to compare the treated factor VIII with untreated controls. In order to execute these experiments, it was desirable to determine conditions under which the thrombin used in preincubation could be inactivated completely by DFP, without permitting the DFP to affect the assay system.

A series of preliminary experiments were performed in which DFP, thrombin, and a mixture of the two were incubated at various concentrations. At intervals, portions of these solutions were withdrawn and mixed with a preparation of factor VIII (fraction I-O, diluted 1/10). After further incubation (20 seconds), the factor VIII preparation was assayed in the usual man-
Table 2.—Effect of DFP on Thrombin Activity

<table>
<thead>
<tr>
<th>Duration of Preincubation of Mixtures (Mm.)</th>
<th>0.4 ml. VBIS</th>
<th>0.05 ml. Thr.</th>
<th>0.4 ml. VBIS</th>
<th>0.05 ml. VBIS</th>
<th>0.05 ml. DFP (M/50)</th>
<th>0.06 ml. VBIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1'</td>
<td>70.2</td>
<td>66.0</td>
<td>88.7</td>
<td>85.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3'</td>
<td>73.3</td>
<td>61.8</td>
<td>95.8</td>
<td>85.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9'</td>
<td>78.0</td>
<td>66.6</td>
<td>88.1</td>
<td>86.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15'</td>
<td>88.2</td>
<td>64.0</td>
<td>89.1</td>
<td>87.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The mixtures described in the column headings were added to equal volumes of diluted fraction I-O at the times indicated. The fraction I-O had been diluted with VBIS 1:12.5. 0.10 ml. of the final mixture was used for factor VIII assay.

A shorter clotting time than in control experiments was taken to indicate residual thrombin activity in the preincubated solution at the time of mixing with fraction I-O. Results of a typical experiment are presented in table 2. The second column shows that thrombin is progressively inactivated by M/500 DFP at +4 C., and that the inactivation is complete after 15 minutes. The experiments in the third column show that if DFP is omitted, the clotting times remain shorter than controls by approximately 20 seconds during the course of the experiment. This is a measure of the degree of activation of fraction I-O by thrombin under these conditions. The fourth column shows that the presence of DFP alone does not affect the clotting times appreciably. The final concentration of DFP in the assay system was M/5000 under these conditions.

These experiments show that thrombin in the concentration used can be completely inactivated by incubation with M/500 DFP for 15 minutes at 4 C. It proved to be possible to shorten the time of treatment to 5 minutes by raising the temperature to 37 C. This procedure did not affect the results in any other way, and it was adopted as the normal procedure for inactivating thrombin in later experiments.

Activation of Fraction I-O

In table 3, the effects of thrombin on a normal and factor VIII-deficient preparation are compared using the test system described in figure 1. The mean clotting time in experiments in which normal I-O was treated with thrombin (column 1) was shorter by 24 seconds than the mean for untreated controls (column 3). The mean for experiments in which deficient I-O was treated with thrombin (column 2) was not significantly different from the mean for the control experiments.

Although thrombin appeared to activate factor VIII, it was possible that the effect was less than maximal because of simultaneous destruction of factor VIII. In order to test this possibility a new set of experiments were carried out in which normal fraction I-O (untreated with thrombin) was combined with the treated I-O prior to assay.

The results (table 4) in the first column show that the clotting times in
Table 3.—Thrombin Activation of Fraction I-O

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Normal I-O treated with thrombin and DFP + Deficient I-O treated with VBIS</th>
<th>Normal I-O treated with VBIS + Deficient I-O treated with thrombin and DFP</th>
<th>Normal I-O treated with VBIS + Deficient I-O treated with VBIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57.8</td>
<td>70.0</td>
<td>68.0</td>
</tr>
<tr>
<td>2</td>
<td>52.2</td>
<td>60.4</td>
<td>65.8</td>
</tr>
<tr>
<td>3</td>
<td>52.0</td>
<td>78.2</td>
<td>88.5</td>
</tr>
<tr>
<td>4</td>
<td>54.0</td>
<td>77.5</td>
<td>81.2</td>
</tr>
<tr>
<td>5</td>
<td>52.0</td>
<td>73.3</td>
<td>75.6</td>
</tr>
<tr>
<td>6</td>
<td>55.3</td>
<td>83.3</td>
<td>88.2</td>
</tr>
<tr>
<td>7</td>
<td>56.6</td>
<td>79.0</td>
<td>85.6</td>
</tr>
</tbody>
</table>

Mean ± S.E. 54.3 ± 2.3 74.5 ± 7.5 78.7 ± 9.5

(Relative to column 3)

p < 0.005 < 0.15

The values reported in this table are clotting times in the standard factor VIII assay procedure using the mixtures indicated in column headings as test materials. The plan of the experiments is summarized in figure 1.

Table 4.—Thrombin Activation in the Presence and Absence of Additional Fraction I-O

<table>
<thead>
<tr>
<th>No. of experiments</th>
<th>Normal I-O treated with thrombin and DFP + Normal I-O treated with VBIS + Deficient I-O treated with VBIS</th>
<th>Normal I-O treated with VBIS + Deficient I-O treated with thrombin and DFP + Deficient I-O treated with VBIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>53.3 ± 4.9</td>
<td>54.3 ± 2.3</td>
</tr>
<tr>
<td>7</td>
<td>55.6 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

The values reported in this table are clotting times in the standard factor VIII assay procedure using the mixtures indicated in column headings as test materials. The plan of the experiments is summarized in figure 1.

*The same experiments as column (1) of table 3.

experiments in which the treated I-O was supplemented with additional normal I-O were not shorter than those in the partly deficient system shown in the second column. The results in the third column show that essentially the same results can be achieved by using only a single portion of normal I-O treated with thrombin and DFP.

Dependence of Activation on the Concentration of Activated Fraction I-O

In order to investigate the relationship between the concentration of activated fraction I-O and the degree of activation, an experiment was designed similar to that illustrated in figure 1. Two identical portions of normal fraction I-O were prepared. One of these was treated with thrombin and DFP
in the usual manner whereas the other was incubated with VBIS. The thrombin-treated portion was immediately diluted, mixed with the untreated fraction I-O and subjected to the assay procedure in the usual manner. The clotting times observed in a series of such experiments were plotted against the logarithm of the relative concentration of the treated fraction I-O in figure 2. A straight line has been fitted to the data by the method of least squares. These results indicate that the concentration of the activated fraction I-O is a rate limiting factor under the conditions of these experiments.

The Effect of Trypsin on Factor VIII

A similar series of experiments were performed in which thrombin was replaced by trypsin, and soybean trypsin inhibitor was used in place of DFP. The results of these experiments are presented in table 5.

No evidence for the activation of factor VIII by trypsin was obtained. On the contrary, the clotting times were very much prolonged in those experiments in which trypsin was incubated with normal fraction I-O. The prolonged times probably reflect the destruction of factor VIII in the preparation by proteolytic digestion. The data in table 6 show that effects of trypsin can be corrected by addition of fresh undigested fraction I-O to the system. In other experiments, the trypsin was diluted to the point where its destructive action on normal fraction I-O had virtually vanished. No activation could be detected even at this concentration.

Discussion

The experiments described above show that a constituent of commercial
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Table 5.—Trypsin Activation of Fraction I-O

<table>
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<tbody>
<tr>
<td>1</td>
<td>160.2</td>
<td>89.3</td>
<td>85.6</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>151.0</td>
<td>71.5</td>
<td>96.0</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>151.6</td>
<td>96.2</td>
<td>88.2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td>169.0</td>
<td>88.3</td>
<td>93.0</td>
<td></td>
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</tr>
<tr>
<td>5</td>
<td>127.5</td>
<td>60.6</td>
<td>88.2</td>
<td></td>
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<tr>
<td>6</td>
<td>172.5</td>
<td>89.5</td>
<td>88.2</td>
<td></td>
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</tr>
<tr>
<td>Mean ± S.E.</td>
<td>155.3 ± 16.2</td>
<td>82.6 ± 13.5</td>
<td>89.0 ± 5.6</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>t</td>
<td>8.668</td>
<td>0.988</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>&lt; 0.0005</td>
<td>&lt; 0.2</td>
<td>&lt; 0.2</td>
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</tr>
</tbody>
</table>

The values reported in this table are clotting times in the standard factor VIII assay procedure using the mixtures indicated in the column headings as test materials. The plan of the experiments is summarized in figure 1 except that trypsin was used in place of thrombin. Trypsin was freshly prepared in VBIS at a concentration of 0.005 per cent. The trypsin inhibitor was prepared at a concentration which was sufficient to inactivate the trypsin completely.

Table 6.—Trypsin and Fraction I-O Destruction

<table>
<thead>
<tr>
<th>No. of experiments</th>
<th>Normal I-O treated with trypsin and inhibitor</th>
<th>Normal I-O treated with VBIS</th>
<th>Deficient I-O treated with VBIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td>80.8 ± 11.8</td>
<td>155.3 ± 16.2</td>
<td>80.5 ± 2.8</td>
</tr>
</tbody>
</table>

The values reported in this table are clotting times in the standard factor VIII assay procedure using the mixtures indicated in the column headings as test materials. The plan of the experiments is summarized in figure 1 except that trypsin was used in place of thrombin. Trypsin was freshly prepared in VBIS at a concentration of 0.005 per cent. The trypsin inhibitor was prepared at a concentration which was sufficient to inactivate the trypsin completely.

*The results in column (2) are the same as those in column (1) of table 5.

bovine thrombin preparations can change or "activate" a constituent of fraction I-O prepared from human plasma. The activator is probably thrombin itself, because the effect can be abolished by short incubation of the enzyme preparation with DFP. The activated substance is probably factor VIII because, (1) incubation of thrombin with fraction I-O from factor VIII-deficient plasma does not produce activation, (2) most other coagulation factors (prothrombin, factor V, IX and XI) are not present in the fraction I-O preparations.

The possibility exists that constituents of commercial bovine thrombin
preparations other than thrombin itself, may interact with factor VIII in a manner which would be sensitive to DFP. Although activated factor XI is sensitive to DFP inhibition, the presence or absence of this factor in thrombin preparations could not be demonstrated conclusively. Activated PTC (factor IX) is not believed to be a DFP-sensitive factor. Although the recent work of Macfarlane, Biggs, Ash and Denson indicates that the presence of factor IX is necessary for factor VIII activation, our material in the concentration used did not have detectable factor IX activity. With these reservations in mind the evidence in the present paper confirms by an alternative approach the findings of Rapaport, Schiffman, Patch and Ames. These conclusions are also compatible with the proposal of Macfarlane et al. that thrombin acts on factor VIII to make it more reactive with factor IX, although the role of factor IX was not investigated in the present work.

Attempts to demonstrate activation of factor VIII using trypsin in place of thrombin gave negative results. Factor VIII was rapidly destroyed on incubation with trypsin, and no evidence of a transient activation was obtained. Trypsin and thrombin have identical specificity with respect to small model substrates (e.g., acylated arginine or lysine esters) but they differ in their actions on proteins. For example, in the case of fibrinogen, thrombin splits only four peptide bonds of arginine whereas trypsin splits numerous peptide bonds of both arginine and lysine.

If the activation of factor VIII by thrombin depends on the cleavage of one or more arginine peptide bonds, it is likely that the same bond(s) are split by trypsin. The failure to detect activation by trypsin would suggest that other peptide bonds which are essential for factor VIII activity are cleaved at least as rapidly as the “activating” bond(s). Although trypsin accelerates prothrombin-thrombin conversion and possibly activates other factors (VII and X) under the present conditions trypsin cannot replace thrombin in the activation of factor VIII.

SUMMARY

The activation of human factor VIII by thrombin has been demonstrated by a new experimental approach. This method permitted investigation of the interaction of thrombin and factor VIII in the absence of most other clotting factors. The activation effect of thrombin is susceptible to inhibition by diisopropylfluorophosphate. Trypsin cannot replace thrombin in the activation reaction, and it destroys factor VIII activity rapidly.

SUMMARIO IN INTERLINGUA

Le activation de human factor VIII per thrombina esseva demonstrate per medio de un nove methodologia experimental. Isto permitte le investigation del interaction de thrombina con factor VIII in le absentia del majoritate del altere factores coagulatori. Le effecto activatori de thrombina es susceptible de esser inhibite per diisopropylfluorophosphato. Trypsina non pote rimplicar thrombina in le reaction activatori, e illo destrue rapidemente le activitate de factor VIII.
ACKNOWLEDGMENT

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REFERENCES

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