An Action of Thrombin on Platelets in Accelerating Clotting

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While there is evidence for the thromboplastic activity of platelets plus a plasma factor during the initiation of clotting, the part played by platelets once coagulation has begun is not well defined. Quick has postulated that platelets are labilized by thrombin and that thereby a platelet factor is released in increasing quantities. This theory is based on the observations in a case of congenital afibrinogenemia studied by Pinniger and Prufty. These investigators noted that as native plasma of the patient was allowed to stand in vitro, thrombin appeared, and that although no fibrin clot formed, the platelets gradually agglutinated and disintegrated. Thrombin has also been postulated as an activator of so-called plasma Ac-globulin, and several investigators have described acceleration of prothrombin conversion by a factor which is active in serum. The purpose of the present investigation was to ascertain, if possible, the basis of the effect of thrombin in conjunction with platelets on the acceleration of clotting.

Methods

Samples of venous blood were mixed with Wintrobe’s double oxalate or collected in 1/10 volume of 0.1 M sodium citrate, or in 1/100 volume of 1.0 M sodium citrate. Platelet-rich plasma was separated by centrifugation at 150 g for 5 minutes. Platelets were then packed by spinning the supernatant plasma at 18,000 g for 30 minutes. This platelet button was gently broken up, and the platelets were washed at least four times in plain or buffered 0.85 per cent saline. Imidazole buffer was used to maintain pH 7.25. Red cells and platelet clumps were removed by repeated differential centrifugations. The remaining platelets were finally suspended in buffered or unbuffered saline and stored at 6 C. until used. Platelet-poor plasma was prepared by centrifuging separated plasma at 18,000 g for 60 minutes and collecting the supernatant.

The preparation of human or dog thrombin was made by adsorbing plasma with 100 mg. of barium sulfate per ml., incubating for 10 minutes at 37 C., and recovering the barium precipitate by centrifugation. The precipitate, on which prothrombin was adsorbed, was washed with saline at room temperature at least five times, in order to remove the unadsorbed plasma components. The barium sulfate was then treated with 0.2 M sodium citrate in a volume equal to one third of the original plasma, to elute prothrombin. The eluate was dialyzed against two changes of 0.85 per cent sodium chloride, each at least three hundred times the volume of the eluate, in order to obtain a saline solution of the prothrombin. This preparation was then recalcified and the amount of thrombin determined by the ability of various dilutions to clot a standard bovine fibrinogen solution, prepared according to Ware, Guest, and Seegers.

Topical thrombin (Parke-Davis) was the usual source of bovine thrombin. Through the courtesy of Dr. Walter H. Seegers, a highly purified preparation of bovine thrombin from his laboratory was used in several of the experiments.

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Platelets were counted directly in a counting chamber according to the method of Pohle.\textsuperscript{13} The effect of platelet preparations on the clotting time of recalcified plasma was determined by measuring the clotting time of 0.40 ml. of plasma after addition of 0.05 ml. of platelet preparation and 0.05 ml. of 0.15 M calcium chloride.

Whole blood clotting time was done in glass according to the method of Pohle and Taylor.\textsuperscript{13} A similar technic was used with silicone coated apparatus.

Serum prothrombin time was determined by a modification of Quick’s one-stage procedure\textsuperscript{14} in which fresh human plasma freed of prothrombin by treatment with barium sulfate was used as a diluent. The serum to be tested was aged for 4 hours at 37°C in order to destroy traces of thrombin. In our laboratory, the normal for serum prothrombin under these conditions is less than 15 per cent.

Two-stage prothrombin determinations were performed according to the method of Ware and Seegers.\textsuperscript{15} One ml. of an appropriate dilution of defibrinated plasma was added to 3.0 ml. of the reaction mixture which contained acacia, calcium, rabbit brain thromboplastin (Difco) diluted 1:10, and imidazole buffer at pH 7.25. Bovine serum was added to the reaction mix as a source of Ac-globulin unless otherwise indicated. At stated intervals, 0.4 ml. of this buffered mixture was added to 0.1 ml. of standard fibrinogen solution and the clotting time observed. In some experiments platelets or other preparations were substituted for thromboplastin in the reaction mix in order to determine their thromboplastic activity.

Platelet suspensions were treated with bovine thrombin by incubation at room temperature for 30 minutes with an equal volume of bovine thrombin in buffered 0.85 per cent saline. With human thrombin a different procedure was used, since the conversion of prothrombin to thrombin in the prothrombin solution was slow and incomplete. Platelets were therefore incubated with this reagent at room temperature for several hours and the amount of thrombin present was determined at the end of the incubation period. The platelets were then washed at least four times with buffered saline in order to remove free thrombin. As a control, in all experiments, equal aliquots of platelets were carried through the same manipulations without the addition of thrombin. These two types of platelet preparation were designated treated and untreated, respectively.

Results

Acceleration of the Clotting of Whole Blood by Thrombin-Treated Platelets

The clotting of hemophilic blood in glass and in silicone was accelerated strikingly by thrombin-treated platelets and to a less extent by control platelets in equal concentrations (table 1). Similarly, the clotting time of normal blood in silicone was shortened distinctly more by treated platelets than by untreated platelets.

Shortening of the Recalcification Time of Plasma by Thrombin-Treated Platelets

When platelets treated with bovine thrombin were added to normal or to hemophilic plasma, either platelet rich or platelet poor, the recalcification time in glass and in silicone was considerably shortened; addition of untreated platelets produced less acceleration.

Increased Prothrombin Consumption in the Presence of Treated Platelets

Traces of thrombin adsorbed on platelets could produce acceleration of whole blood clotting or shortening of recalcification time by causing the conversion of fibrinogen to fibrin in these systems. To exclude this possibility, observations were made only on the conversion of prothrombin to thrombin by studying prothrombin consumption in the presence of thrombin-treated platelets.
Table 1.—The Effect of Thrombin-Treated and of Untreated Platelets on Clotting Time and Prothrombin Consumption of Hemophilic Blood

<table>
<thead>
<tr>
<th>Preparations*</th>
<th>Clotting time (min.)</th>
<th>Serum prothrombin (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-stage method</td>
<td>1-stage method</td>
</tr>
<tr>
<td>Treated platelets 200,000/cu. mm. in buffered saline</td>
<td>1</td>
<td>94</td>
</tr>
<tr>
<td>Untreated platelets 210,000/cu. mm. in buffered saline</td>
<td>13</td>
<td>133</td>
</tr>
<tr>
<td>Buffered saline</td>
<td>85</td>
<td>164</td>
</tr>
<tr>
<td>Control</td>
<td>210</td>
<td>362</td>
</tr>
</tbody>
</table>

* Two ml. of whole hemophilic blood were added to 0.5 ml. of the designated preparations and observations of silicone clotting time and prothrombin consumption were made on duplicate samples of each.

The influence of treated platelets was observed on the prothrombin consumption of hemophilic blood, which commonly is diminished because of defective thromboplastic activity. Aliquots of whole blood were added to platelet preparations in silicone tubes. Four hours after coagulation, serum prothrombin was measured. In table 1 are presented the effects of the presence of treated and of untreated platelets on prothrombin consumption in such blood. Duplicate samples were tested. Residual prothrombin in the serum was found to be decreased in all samples in which thrombin-treated platelets were present. This decrease was more striking when prothrombin was measured by the two-stage method, which minimizes the effect of serum accelerators. This experiment, repeated several times with similar results, demonstrates an acceleration of clotting related to increased utilization of prothrombin independent of possible effects of contaminating thrombin on fibrinogen.

Increased Rate of Thrombin Formation in the Presence of Treated Platelets

Two-stage prothrombin technics were used in order to observe the rate and the degree of thrombin production resulting from the substitution of either a suspension of treated platelets or dilute thrombin solutions for thromboplastin in the reaction mixture. When fresh normal plasma was used as a source of prothrombin, it was found that platelets treated with bovine thrombin (either the Parke-Davis or Dr. Seegers' preparation), when substituted for thromboplastin in the reaction mixture, were active, while untreated platelets had little effect (fig. 1). With fresh hemophilic plasma, the same results were evident (fig. 2).

Platelets treated with bovine thrombin were also effective in bringing about thrombin production in stored hemophilic plasma. Without bovine serum as a source of Ac-globulin, however, thrombin production was slow and incomplete. The curve was similar to that obtained when thromboplastin was present in the reaction mix, without Ac-globulin in the diluent. Only minimal thrombin production was attained in this experiment when untreated platelets were
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Fig. 1.—Effect of substitution of thrombin-treated and of untreated platelets for thromboplastin in two-stage prothrombin determination. Defibrinated fresh platelet-free normal plasma in buffered saline was used as a source of prothrombin.

In curve A, untreated platelets at a concentration of 1,000,000/cu. mm., and in curve B, treated platelets at a concentration of 1,100,000/cu. mm., were substituted for thromboplastin. Curve C is the control curve, in which standard thromboplastin was used.

substituted for thromboplastin; the shortest time observed was 105 seconds, achieved after incubation for 25 minutes. In the presence of bovine serum as a source of A-globulin, the prothrombin curves were similar to those obtained with fresh plasma. Bovine serum was ineffective in prothrombin conversion in the absence of thromboplastin or treated platelets.

When platelets were treated with human thrombin prepared by eluting barium sulfate, their activity in conversion of prothrombin was similar to that of platelets treated with bovine thrombin. Since the method of treating platelets with reconstituted eluate differed from that used with bovine thrombin, these results could not be quantitatively compared. Occasionally it was noted that platelets treated with eluate were less active than platelets treated with bovine thrombin.

In order to determine whether calcium was necessary for this action on platelets, oxalate was used as a decalcifying agent. It was found that thrombin was equally effective in increasing the thromboplastic property of platelets when calcium was absent.

Platelets which had been heated to 60 C. before treatment with thrombin
FIG. 2.—Effect of substitution of thrombin-treated and of untreated platelets for thromboplastin in the two-stage prothrombin determination. Fresh hemophilic plasma was used as a source of prothrombin.

In curve A, untreated platelets at a concentration of 140,000/cu. mm., and in curve B, treated platelets at the same concentration were substituted for thromboplastin. In curve C, standard thromboplastin was used.

were as effective in prothrombin conversion as unheated platelets. Exposure to this temperature after treatment with thrombin, however, decreased the effectiveness of the platelets.

Thrombin solutions without platelets failed to affect thrombin production when substituted for thromboplastin. Since the concentration of the material in the preparations contributing to the activity of the platelets may have been different at their surface and in the surrounding medium, observations were made on serial concentrations of bovine thrombin. Their ineffectiveness may be seen in figure 3, in which the prothrombin source was defibrinated fresh hemophilic plasma. The slight conversion apparent when more dilute preparations of thrombin were substituted for thromboplastin is not significantly different from that seen when neither thrombin nor thromboplastin had been added.

Negative Effect of the Treatment of Red Cells with Thrombin

Similar experiments were carried out with red cells treated with thrombin. These red cells were found to cause no acceleration of prothrombin conversion.
Platelets which had been washed and suspended either in buffered or in unbuffered 0.85 per cent saline were incubated at 26°C for 1 hour with equal volumes of bovine thrombin at strengths varying up to 200 units/ml. of buffered saline. Comparison of platelet counts made before and after this procedure occasionally demonstrated a decrease in platelet concentration, but more often showed no significant change as a result of exposure to thrombin.

Because it was noted that platelets that had been exposed to thrombin were more difficult to resuspend during the washing process, an attempt was made to evaluate the change in their stickiness by counting platelets suspended in a thrombin solution before and after rotation or agitation in glass vessels at 26°C and at 37°C. These technics also failed to reveal any consistent difference between the control platelet samples and the samples treated with thrombin.
However, in some experiments, platelets treated with thrombin were found to clump and at times to disintegrate.

*Lack of Relationship Between Viscous Metamorphosis and Thrombin-Treated Platelets*

The phenomenon of clumping and disintegration of platelets during coagulation has been known for many years. The conditions requisite for this process were compared with those needed for the treatment of platelets with thrombin, in order to determine whether the two phenomena are related.

Wright and Minot described this process of agglutination, lysis, and disintegration of platelets and observed that for it to take place, certain factors were necessary. With the use of the phase microscope, we confirmed these observations of viscous metamorphosis of platelets and noted, as did the original investigators, that decalcification prevented the process from occurring. In contrast, decalcification did not interfere with the effect of thrombin on platelets with respect to acceleration of clotting.

Wright and Minot also described a factor in recalcified plasma necessary to produce viscous metamorphosis of platelets. They did not find thrombin prepared by Howell's technic to be the responsible material; the preparation used, however, was extremely weak in thrombic activity. In the present experiments with more potent preparations of human and bovine thrombin, this phenomenon was observed. The active factor in these preparations was found to be rather stable to heat, and some were kept at 60 °C. for 20 minutes without loss of activity. Thrombin-treated platelets, on the other hand, were more susceptible to the action of heat, and exposure to 60 °C. for 20 minutes markedly diminished their activity in prothrombin conversion.

The third component necessary for viscous metamorphosis was found to be an elusive property of platelets. Their ability to undergo this change was variable and often unpredictable. The property was heat labile and was destroyed by heating a suspension of platelets to 50 °C. for 10 minutes, or by keeping it at 37 °C. for more than an hour. Freezing and thawing of platelets abolished the property, as did their storage in the icebox for more than 48 hours. On the other hand, platelets could be made effective in prothrombin conversion by treatment with thrombin after they had been exposed to these conditions, or after they had been stored several weeks in the icebox. Other unknown factors must also play a part in viscous metamorphosis, since platelet suspensions prepared in seemingly identical fashion varied with respect to this property and some samples could not be made to clump and disintegrate. The factor allowing acquisition of special properties in prothrombin conversion after thrombin treatment was much more constant and stable. Whether viscous metamorphosis could be produced or not in the process of thrombin treatment, the resulting material was effective in prothrombin conversion. Thus the phenomenon of viscous metamorphosis did not appear to play a role in the action of thrombin on platelets in causing acceleration of prothrombin conversion.

**Discussion**

According to Ware and Seegers, besides causing fibrin formation, thrombin may accelerate conversion of prothrombin to thrombin by changing inactive
plasma accelerators of this process to active serum accelerators. Quick\(^1\) has shown that minute amounts of thrombin adsorbed on glass will accelerate coagulation in platelet-rich plasma, and Stefanini\(^2\) has demonstrated that greater amounts of thrombin cause increased prothrombin consumption only in the presence of an adequate number of platelets. The present experiments also demonstrate a means by which thrombin may accelerate blood coagulation: namely, by enhancing the thromboplastic activity of platelets.

The acceleration of clotting of hemophilic or of normal blood in the presence of thrombin-treated platelets appears to be due, in part, to increased speed of thrombin formation. From the two-stage prothrombin determinations there is evidence that this may be the result of a thromboplastic effect of thrombin-treated platelets. The increase in prothrombin consumption in the presence of thrombin-treated platelets is further indication of such activity. From our studies it is evident that possible formation of platelet accelerator, which is very similar to serum Ac-globulin,\(^3\) is not the complete mechanism because treated platelets, although effective in the absence of thromboplastin, were no more effective than thromboplastin in the presence of low concentrations of Ac-globulin. Moreover, the control platelets, which should have contained an equal amount of platelet Ac-globulin, were much less effective under the same conditions. Indeed, the possibility that the activity was due either to platelet Ac-globulin or to the production of serum Ac-globulin is even more remote in view of the ineffectiveness in prothrombin conversion of bovine serum, a potent source of Ac-globulin, in the absence of thromboplastin.

Thrombin, itself, was not responsible for this special property of treated platelets. Thrombin preparations alone in the reaction mixture of the two-stage system had no effect comparable to that of thrombin-treated platelets. Since the process of defibrination of the plasma for two-stage studies involved addition of the same thrombin used in treating platelets, it is unlikely that possible traces adsorbed could have had further effect. The treated platelets thus appeared to have an effect beyond that of Ac-globulin activity or of thrombin activity. Thus, while thrombin adsorbed on the platelets could have accelerated the recalcification time and whole blood clotting time, it could not have been responsible for the acceleration of prothrombin conversion noted in these experiments.

The thromboplastic activity of the platelets was clearly enhanced by exposure to the bovine thrombin preparations. While slight acceleration of the recalcification time by thrombin-treated red cells was evident, these red cells, unlike thrombin-treated platelets, were ineffective in causing prothrombin conversion. Thus, the reaction of thrombin on platelets does not appear to extend to red cells.

The effect of thrombin on platelets was not mediated through platelet lysis. The viscous metamorphosis of platelets appears to be a phenomenon separate from this action of thrombin on platelets. In preparations in which it was not possible to demonstrate viscous metamorphosis because of the presence of oxalate, the previous exposure of platelets to heat or the use of stored platelets, thrombin still caused the same potentiation of platelet activity. It seems, instead, that thrombin in some way prepares the platelets for more active participation in the process of prothrombin conversion.

Since none of the thrombin preparations was completely pure, a contaminating
material, perhaps related to the kinase described by Milstone,\textsuperscript{2} may have been the active fraction. However, it is present only in preparations in which thrombin can be demonstrated, and if not thrombin itself, must be some closely allied product of an early stage of coagulation.

**Conclusions**

1. Washed platelets, after exposure to thrombin preparations or to plasma in which thrombin has been formed, accelerate coagulation in the presence of calcium.
2. Lysis and disintegration of the platelets are not requisites of this action on platelets.
3. Treated platelets are effective as a substitute for thromboplastin and so used, increase the conversion of prothrombin to thrombin.

**Summario in Interlingua**

Le objectivo de iste studio esseva determinar, in tanto que possibile, le base del efecto de thrombina conjunctemente con plachettas super le acceleration del coagulamento. Le sequente observationes esseva facite:

1. Plachettas lavate que ha essit.e expoisite a preparationes de thrombina o a plasma in que thrombina se ha formate, produce accelerate coagulation in le presentia de calcium.
2. Le lyse e le disintegratioti del plachettas non es requirite pro iste action del thrombina super le plachettas.
3. Plachettas tractate es effective como substituto pro thromboplastinia. In iste uso illos augmenta le conversion de prothrombina a in thrombina.

**References**

\textsuperscript{5} Ware, A. G. and Seegers, W. H.: Serum Ac-globulin formation from plasma Ac-globulin; role in blood coagulation; partial purification; properties and quantitative determination. Am. J. Physiol. 152: 567, 1948.
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