STUDIES ON THROMBIN-INDUCED PLATELET AGGLUTINATION*

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The importance of morphologic alteration, clumping, and subsequent fusion of blood platelets in hemostasis and thrombosis is well known. The mechanisms underlying these platelet changes, often referred to as agglutination and viscous metamorphosis, are poorly understood. Since the early work of Wright and Minot (1), many studies have suggested that thrombin may be involved. Widely divergent conclusions, however, have been reached regarding the role of thrombin in bringing about these platelet changes. These conclusions have ranged from the proposal that thrombin acts directly to cause platelet agglutination, to denial that it is concerned in a vital way with the process (2). In some studies, serum containing thrombin caused platelet agglutination (3–8). In other studies, thrombin preparations rather than serum have been employed. While a few of the data suggest that thrombin may act directly to agglutinate platelets (9–11), most investigators have found that thrombin by itself is inactive (3, 12–18). Other data have indicated that inadequately washed platelets were agglutinated by thrombin, but that no clumping occurred if the platelets were first thoroughly washed (17, 18). Washed platelets suspended in serum (5), plasma (3, 18), or any of several buffers (3) have also been reported to be clumped by the addition of thrombin. Sharp (19), however, could not confirm such direct action of thrombin, and he has questioned if thrombin is concerned with the earlier stages of platelet agglutination.

Many studies have indicated that one or more cofactors are needed for the agglutination of platelets by thrombin. We have previously reported (20) that a combination of thrombin and calcium is highly active in producing agglutination of carefully washed platelets, no other cofactors being necessary. Other workers have observed that thrombin together with calcium will not agglutinate washed platelets (16) unless certain additional cofactors are present (3, 4, 10, 13–15). This list of cofactors includes glucose and other monosaccharides as

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well as their phosphoric esters (10, 13), certain anions (10), and albumin (3, 14). From the conflicting data available it is difficult to assess the importance of thrombin, either alone or in conjunction with other factors, in platelet agglutination.

In this paper the results of a systematic study of the agglutination of washed platelets by thrombin are reported. Use was made of a recently devised macroscopic platelet agglutination test (12), in which the end point is sharp and platelet agglutination is apparent in a matter of seconds, rather than in minutes or hours as is the case with most other test systems. Previous work carried out in this laboratory (21–23) with another thrombocyte agglutinating system, designated TAg, which is distinct from the thrombin agglutinating system, showed that species of origin of both plasma and platelets, as well as type of cation, was frequently critical in determining whether or not agglutination occurred. In this study, particular attention was given to the influence of these same variables and of certain physical conditions, as ionic strength, pH, and temperature, on the agglutination reaction.

Materials and Methods

Thrombin Preparations.—Human and canine thrombins were prepared by citrate activation of prothrombin according to the method of Seegers (24). Bovine thrombin was obtained from a commercially available biothrombin (topical thrombin, Parke, Davis & Co., Detroit) and was used as such ("crude" thrombin) or was twice precipitated with equal volumes of pre-chilled (−20°C) acetone as a preliminary purification step. For further purification of thrombin from each species, the preparations were dialyzed for 6 hours against 12 × 1 liter of 0.05 M phosphate buffer, pH 7.0, and then chromatographed on a 1.1 × 14.0 cm amberlite IRC-50 resin column (Rohm & Haas Co., Philadelphia) (in equilibrium with 0.05 M phosphate buffer, pH 7.0). A modification of Rasmussen's technique (25) was employed. All effluent fractions were analyzed for thrombin activity by the method of Seegers and Smith (26). Protein concentrations of the most highly active fractions were estimated by ultraviolet light absorption at 280 mμ and 320 mμ in a Beckman DU spectrophotometer. Purity of the preparations ranged from 5,000 to 10,000 Iowa units per mg protein N. Before use in the platelet agglutination tests each preparation was dialyzed for 6 hours against 12 × 1 liter of 0.154 M NaCl in order to remove phosphate buffer.

Fibrinogen Preparation.—Fibrinogen was prepared from $BaSO_4$-adsorbed canine plasma by thrice precipitating with one-fourth saturated $(NH_4)_2SO_4$ (27).

Cation Chloride Solutions.—The cation chloride solutions, except for NaCl, were standardized by chloride analyses and diluted to the desired concentration. For concentrations less than 0.108 M, serial dilutions of 0.108 M solutions were made with saline (0.154 M NaCl), unless otherwise indicated. All reagents were Baker AR (J. T. Baker and Co., Phillipsburg, New Jersey) except $NiCl_2$, which was Mallinckrodt AR (Mallinckrodt Chemical Works, St. Louis).

Platelet Suspensions.—The platelet preparation has been described previously (12). The suspensions contained 400,000 platelets/mm^3 and were made from human or

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canine blood collected with EDTA (ethylenediamine-tetraacetic acid) as anticoagulant.

Platelet Agglutination Test.—A modification of the macroscopic test for platelet agglutination, reported previously from this laboratory (12), was used. One part of divalent cation chloride solution was added to four parts of thrombin solution, which had been previously diluted to the desired concentration with saline; 0.2 ml of this mixture was added immediately to an equal volume of platelet suspension. This final mixture contained thrombin in a concentration of 2 Iowa units per ml unless thrombin concentration was the variable in the experiment. The moment of beginning of platelet clumping was recorded as the agglutination time. The degree of agglutination at 2 minutes after mixing was recorded on a scale of 0 to 4 plus, based on the size of platelet clumps (12), with 0 indicating no agglutination. Within 30 seconds after completion of the 2 minute macroscopic observation, negative and questionable mixtures were examined by phase contrast microscopy. The test mixtures were again examined at 30 minutes. The agglutination tests were carried out at 28° ± 1°C, unless temperature was the experimental variable.

TABLE I

Action of Thrombin on Canine Platelets and Effect of Ca^{++} and Mg^{++}

Test mixture		Agglutination	
Thrombin*	Cation‡	Time	Degree
units/ml		*sec.*	
2	None	None in 1800	—
None	None	None in 1800	—
2	Ca^{++}	9.0	4+
None	Ca^{++}	None in 1800	—
2	Mg^{++}	10.2	4+
None	Mg^{++}	None in 1800	—

* Thrombin concentration in final reaction mixture.

‡ Cation concentration, 2.7 mM/liter, final reaction mixture.

RESULTS

Platelet-Agglutinating Activity of Thrombin.—The action of canine thrombin as an agglutinating agent for platelets was tested in both the presence and the absence of the divalent cations, calcium and magnesium. The results of a representative experiment are given in Table I. Thrombin in the absence of added cation did not cause the platelets to agglutinate within the 30 minute period of observation. Examination of the platelets at this time with the phase microscope (430 × magnification) showed them to be discrete and intact, and they could not be distinguished from the control tests in which platelets were suspended in saline solution. Thrombin with either calcium or magnesium

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caused prompt agglutination of platelets, usually within 8 to 12 seconds. Preliminary incubation of thrombin and cation for varying periods of up to 30 minutes affected neither the agglutination time nor the degree of agglutination. The platelet aggregates were large and irregular in contour. The outlines of individual platelets could be clearly discerned in the aggregates if examined soon after clumping, but after 30 minutes the outlines could no longer be identified in the aggregates which were now amorphous.

Effect of Species of Origin of Platelets and of Thrombin on the Platelet Agglutination Reaction.—A series of experiments was performed to determine if the species source of platelets or of thrombin would influence platelet agglutination.

TABLE II

Agglutination of Canine and Human Platelets by Human, Canine, and Bovine Thrombin Preparations

Thrombin,* species	Platelets, species	Agglutination‡		
		With Ca^{++} §	With Mg^{++} §	No Ca^{++} or Mg^{++}
		sec.	*sec.*	*sec.*
None	Canine	None in 1800	None in 1800	None in 1800
Canine	"	9.0	10.2	" " 1800
Bovine	"	11.5	12.5	" " 1800
Human	"	14.0	17.2	" " 1800
None	Human	None in 1800	None in 1800	" " 1800
Canine	"	11.5	21.5	" " 1800
Bovine	"	19.2	41.0 (2+)	" " 1800
Human	"	11.0	37.5	" " 1800

* Thrombin concentration, 2 units/ml, final reaction mixture.

‡ Degree of agglutination of all positive reactions was 4+, unless noted otherwise.

§ Cation concentration, 2.7 mM/liter, final reaction mixture.

The results of one group of experiments are shown in Table II. Human and canine platelets were tested with thrombins of human, canine, and bovine origin, with and without calcium and magnesium. As in the experiments with homologous canine reagents (Table I), the thrombins were inactive unless one of the divalent cations was present. In the presence of either cation, the platelets were agglutinated by thrombin of each species. Bovine thrombin was more active with canine platelets than with human platelets. Also, human platelets were agglutinated more slowly with magnesium than with calcium. Agglutination times as a rule were most rapid with homologous reagents.

Optimal Concentrations of Calcium and Magnesium for Platelet Agglutination by Thrombin.—Further examination was made of the relative effectiveness of calcium and magnesium in the thrombin-induced platelet agglutination reaction. Both canine and human platelets, along with each of the three thrombins from different species, were tested with varying concentrations of the two ions.

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Canine platelets were used in one group of experiments (Fig. 1). It will be noted that the agglutination times were determined more by the concentration

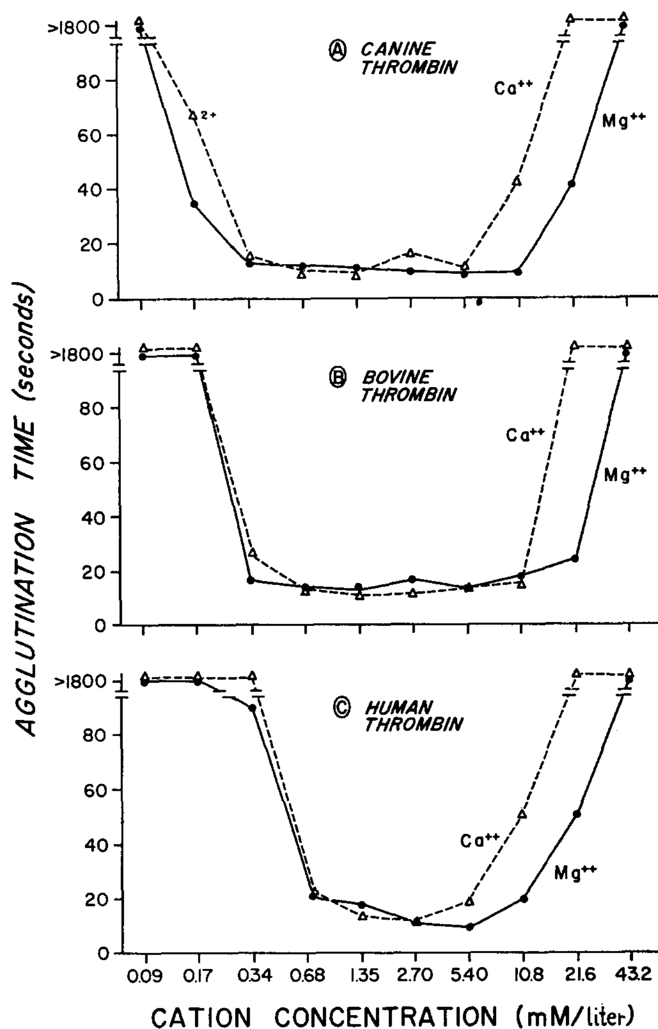

FIG. 1. Comparison of agglutination times of canine platelets with varying concentrations of calcium and magnesium, and with canine (*A*), bovine (*B*), or human (*C*) thrombins. Degree of agglutination was 4+, unless indicated otherwise; if no agglutination occurred within 1800 seconds, the test was reported as negative.

than by the type of cation. The two curves with calcium and magnesium in each of the three experiments shown in Fig. 1 are nearly superimposable, except at the higher concentrations of the cations. At these higher levels of cation, calcium appears to be slightly more inhibitory than magnesium. The range of

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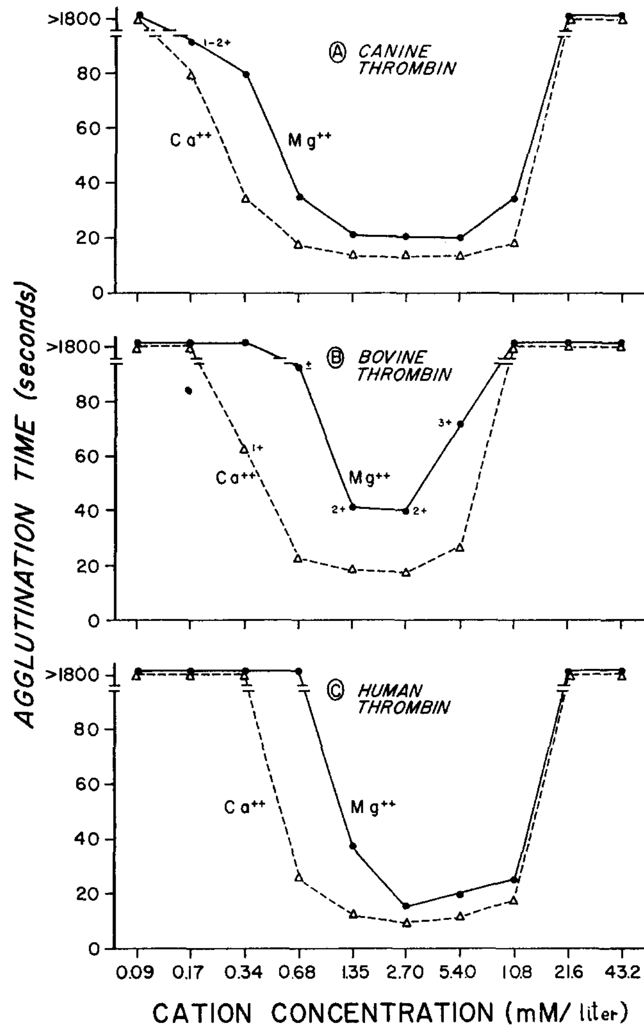

FIG. 2. Comparison of agglutination times of human platelets with varying concentrations of calcium and magnesium, and with canine (*A*), bovine (*B*), or human (*C*) thrombins. Degree of agglutination was 4+, unless indicated otherwise; if no agglutination occurred within 1800 seconds, the test was reported as negative.

cation concentration causing prompt agglutination was more limited with human thrombin than with canine or bovine thrombin.

Human platelets were used in the other group of experiments of this series (Fig. 2). With each of the three types of thrombin, calcium caused more rapid agglutination of platelets than did magnesium. Calcium was also active at lower concentration than was magnesium. Unlike the results with canine platelets, the two ions appear to be equally inhibitory at the higher levels tested.

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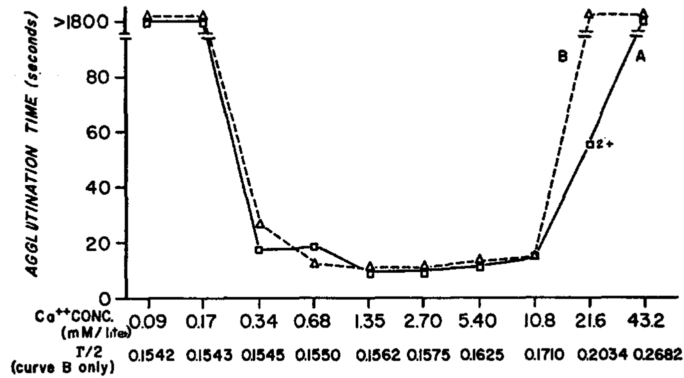

FIG. 3. Influence of ionic strength on the agglutination of canine platelets by bovine thrombin with varying concentrations of calcium. In curve *A*, calcium concentration varied, but $\Gamma/2$ was maintained at 0.154 by adjusting NaCl concentration. In curve *B*, calcium concentration varied; $\Gamma/2$ also varied as indicated on abscissa. Degree of agglutination was 4+, unless indicated otherwise; if no agglutination occurred within 1800 seconds, the test was reported as negative.

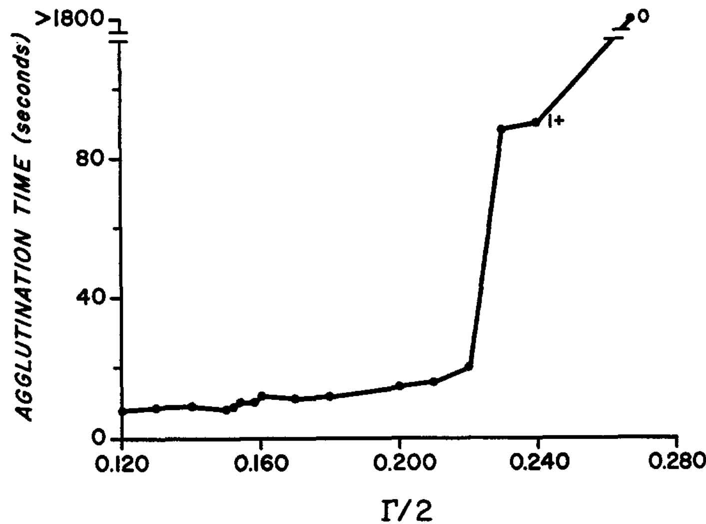

FIG. 4. Influence of ionic strength on the agglutination of canine platelets by bovine thrombin and an optimal and constant concentration of calcium (2.7 mM/liter). Ionic strength was varied by adjusting the NaCl concentration. Duplicate experiments were done, and the average agglutination times plotted. Degree of agglutination was 4+, unless indicated otherwise.

There did not appear, in these and other experiments, to be any consistent differences in the relative effectiveness of the different thrombins, except for the decreased activity in the case of bovine thrombin and human platelets (see Table II). In all of the experiments, the most rapid agglutination times

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were obtained with 2.7 mM/liter of either calcium or magnesium regardless of the source of platelets or of thrombin.

Influence of Ionic Strength on Platelet Agglutination.—In the experiments above (Figs. 1 and 2) the ionic strength varied directly with the divalent cation concentration. Two groups of experiments were performed to determine the influence of ionic strength on the time and degree of platelet agglutination. In one group the calcium concentration was varied as before, but the ionic

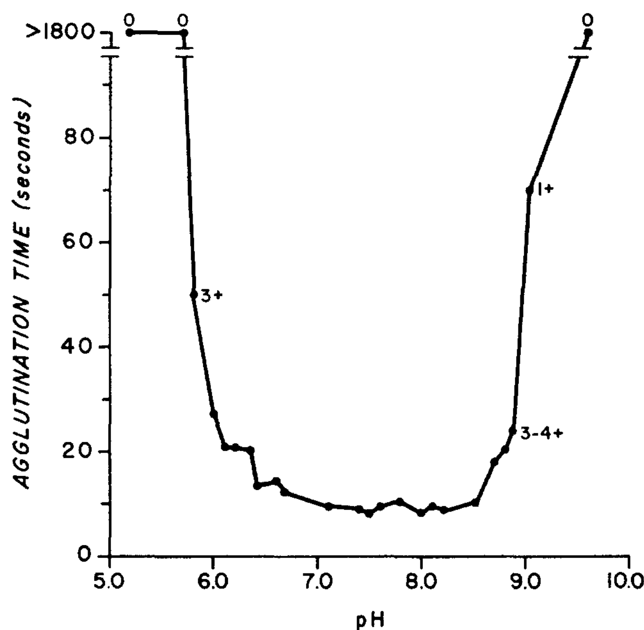

FIG. 5. Effect of pH on agglutination of canine platelets by purified bovine thrombin and calcium (2.7 mM/liter). Imidazole buffer was used in the pH range of 5.7–7.4, and tris(hydroxymethyl)aminomethane ("tris") buffer in the range of 7.5–8.9. The concentration of each buffer in the final reaction mixture was 8.5 mM/liter. pH was determined with a glass electrode (Beckman Zeromatic pH meter). Degree of agglutination was 4+, unless otherwise indicated.

strength was kept constant. In the other group of experiments, the reverse arrangement was adopted in which the calcium concentration was kept constant and optimal, while the ionic strength was varied.

The results of an experiment in which ionic strength was the constant ($\Gamma/2 = 0.154$) are shown in Fig. 3, curve *A*. The results of the comparable experiment, in which both the ionic strength and calcium concentration were varied (Fig. 1 *B*), are included in Fig. 3 as curve *B*. Little or no difference in results of the two experiments was noted except at a high level of calcium, 21.6 mM/liter; here agglutination occurred only at the lower ionic strength.

The results of a typical experiment in which ionic strength was the variable

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are shown in Fig. 4. In the range of ionic strength from 0.12 to 0.21 the agglutination times were rapid, 8 to 16 seconds. Agglutination was most rapid with lower ionic strength. With higher ionic strength (0.220 to 0.268), the reaction was progressively inhibited, even though the calcium ion concentration remained constant. All of these data suggest that inhibition can be due to both high ionic strength and specific action of a high concentration of the divalent cation.

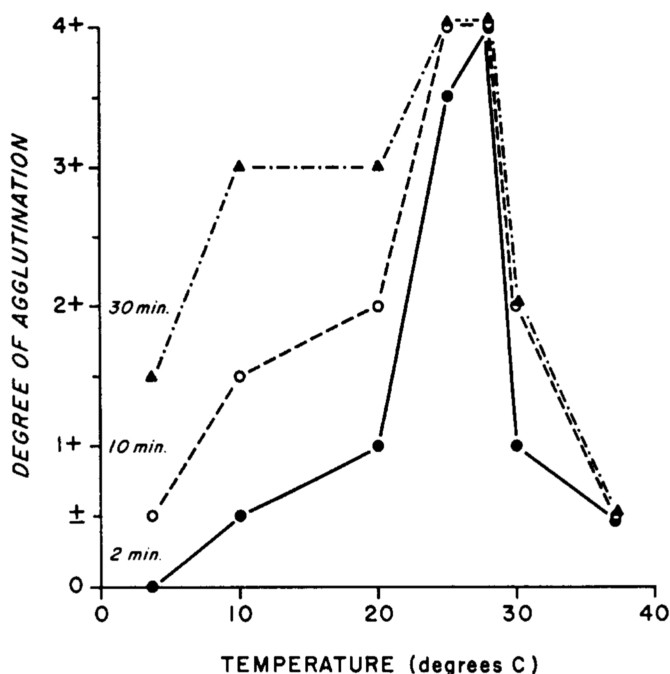

FIG. 6. Influence of temperature on degree of agglutination of canine platelets. Purified bovine thrombin and calcium (2.7 mM/liter) were used. The reagents were first equilibrated at the appropriate temperature, usually in a water bath. Triplicate test mixtures were maintained at each temperature investigated, and one of the mixtures was examined at 2, 10, or 30 minutes to determine degree of agglutination.

Effect of pH and Temperature on the Thrombin-Induced Platelet Agglutination Reaction.—The effect of pH was tested over a range of 5.0–9.6 (Fig. 5). Buffered systems, with imidazole or tris buffers, and unbuffered systems, with added 0.1 N HCl or 0.1 N NaOH, were employed to vary the pH. In neither buffered nor unbuffered systems did agglutination occur at any pH unless calcium and thrombin were added. Over a relatively wide range of pH, 6.4–8.6, agglutination was prompt. Agglutination was slow or did not occur at pH values below 6.0 or above 9.0.

The influence of temperature on the platelet agglutination reaction was tested

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in the range of 4–37°C (Fig. 6). At 28°C, maximal agglutination had occurred at 2 minutes. At lower temperatures, the reaction proceeded at a slower rate. At 37°C, only scattered clumps of 2 to 4 platelets were noted, with many of the platelets remaining unagglutinated. Platelet suspensions, alone or with either thrombin or cation, maintained at the various temperatures served as controls; no agglutination or other observable changes were noted.

Thrombin Concentration, Platelet Agglutination Time, and Fibrinogen Clotting

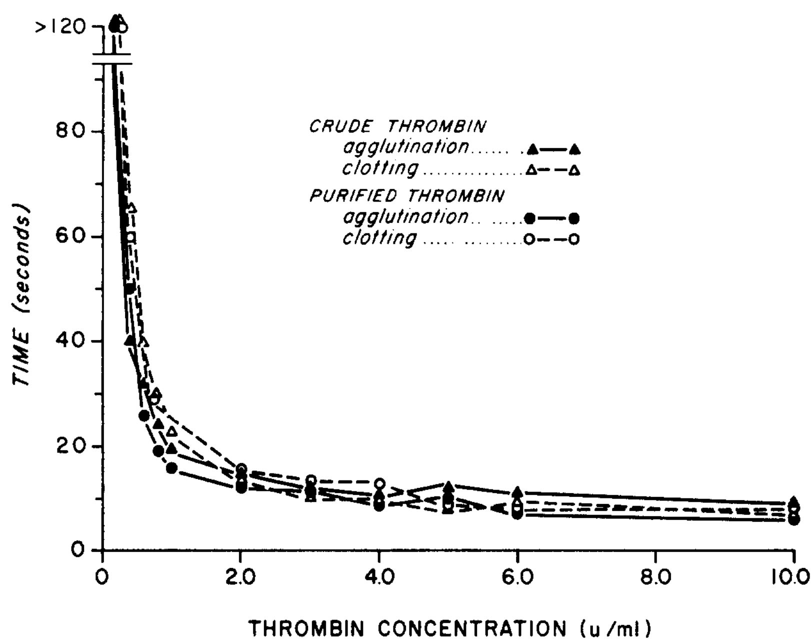

FIG. 7. Comparison of the effect of varying concentrations of crude and purified bovine thrombin on the platelet agglutination time and the fibrinogen clotting time. Potencies of thrombins were: 1180 units/mg protein N for crude thrombin, 8340 units/mg protein N for purified thrombin. Calcium concentration in each test was 2.7 mM/liter.

Time.—A comparative study of the effect of thrombin concentration on the rate of platelet agglutination and fibrinogen clotting was undertaken. A number of experiments were performed in which agglutination and clotting tests were done simultaneously with varying thrombin concentrations. Homologous thrombin and platelets, either canine or human, as well as heterologous reagents, as described in Figs. 1 and 2, were used with either calcium or magnesium. Similar results were obtained in all experiments, regardless of source of platelets, type of cation, or source or purity of thrombin. The greater the thrombin concentration, the shorter the agglutination time. The results of two experi-

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ments, one with crude thrombin and one with purified thrombin, are shown in Fig. 7. The values for platelet agglutination time and fibrinogen clotting time were nearly the same at each thrombin concentration tested in the range of 2 to 10 units of thrombin per ml. At lower concentrations of thrombin, the agglutination times were somewhat shorter than the clotting times. Since no fibrinoplastic agent as acacia was used, clotting times with unit thrombin concentration exceeded the 15 second value obtained with the Iowa assay procedures (26). At the same concentrations of thrombin, as determined by fibrinogen clotting, the crude and purified preparations were equally active in the agglutination reaction.

Other Cations and Platelet Agglutination.—Several cations, either divalent or trivalent, aside from calcium and magnesium, were tested for their effectiveness with thrombin in causing platelet agglutination. Preliminary screening experiments were performed with canine platelets and human thrombin. The cations tested and their concentrations (21) were as follows: Sr^{++} and Ba^{++}, 10.8 mM/liter; Mn^{++}, 1.08 mM/liter; Co^{++}, Cu^{++}, Ni^{++}, and Cd^{++}, 0.34 mM/liter; La^{+++}, 0.10 mM/liter; and Zn^{++}, Pb^{++}, and Hg^{++}, 0.05 mM/liter. Of this group of ions, only divalent manganese and cadmium gave positive results. Since divalent cobalt and nickel are active in the TAg reaction (21) and strontium is active in the clotting reaction, these three cations along with manganese and cadmium were tested over a range of concentrations, similar to the experiments in Figs. 1 and 2. Manganese and cadmium were regularly active with all combinations of thrombin and platelets. Cobalt was inactive except for weak and inconsistent agglutination in the case of canine platelets and bovine thrombin. Nickel and strontium were negative in all tests. Canine platelets reacted more rapidly than human platelets in these experiments. The shortest agglutination times were obtained at concentrations of 1.35 to 2.7 mM/liter with manganese (9 seconds), and 0.34 mM/liter with cadmium (16 seconds).

DISCUSSION

The experiments reported here indicate that thrombin has potent thrombocyte agglutinating properties, provided that certain specific divalent cations are present. Thrombin without the cations is inactive, confirming previous reports from this laboratory (12, 20) as well as from many others (3, 13–18). As judged by light and phase microscopy, the agglutinated platelets retain their individual identity in the aggregates for a short time, but after several minutes the aggregates become amorphous (viscous metamorphosis). No cofactor aside from the cation is needed for this action of thrombin. The finding that crude thrombin, with relatively low thrombic activity per milligram protein nitrogen, possessed approximately the same agglutinating ability, unit for unit, as highly purified preparations (Fig. 7), suggests that thrombin itself rather than some impurity is responsible for the agglutination reaction (3).

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Rapid agglutination of platelets occurred in these experiments at physiologic values for the different parameters investigated, temperature excepted, even though purified reagents and isolated platelets were used. The level of thrombin activity in whole blood serum may reach a concentration of 2 units/ml (28), the concentration of thrombin used in most of our studies. Both calcium and magnesium were active in the ranges of their normal serum levels, 2.3 to 3.0 mM/liter and 0.7 to 1.5 mM/liter, respectively. Agglutination was also prompt at physiologic values of both ionic strength and pH. In fact, rapid agglutination occurred at values well outside the physiologic range for calcium, magnesium, ionic strength, and pH. The limited ability of thrombin to agglutinate washed platelets at 37°C remains an enigma (Fig. 6), particularly since, at this temperature, agglutination in platelet-rich plasma regularly occurs as thrombin is generated (29). Regardless of the temperature, it was found in practice that the platelets need to be prepared fresh for each experiment and used promptly.

The cation requirements for platelet agglutination by thrombin differ in several respects from those of two naturally occurring platelet-agglutinating factors, TAg and TAg′, studied earlier (12, 21–23). The first of these factors, TAg, is present in $BaSO_4$-adsorbed plasma which contains no detectable prothrombin or thrombin (12). The second thrombocyte-agglutinating factor, tentatively designated TAg′, is adsorbed onto $BaSO_4$. A comparison of the cations which are active with TAg, TAg′, or thrombin in agglutination of canine platelets follows:

	Mg^{++}	Ca^{++}	Mn^{++}	Fe^{++}	Co^{++}	Ni^{++}	Cd^{++}
TAg	+	−	+	+	+	+	−
TAg′	−	+	−	−	−	−	−
Thrombin	+	+	+	−	−	−	+

No one cation is active in all three systems. In the TAg′ system, thrombin is regularly formed in the incubation mixtures prior to addition of platelets. This finding suggests that thrombin may be the agent responsible for agglutination. If so, calcium would appear to play a double role, one in the thrombin activation reaction, the other in the agglutination reaction. Magnesium, manganese, and cadmium would be substitutive only in the second of these reactions.

Certain divalent cations are known to influence the action of thrombin on different substrates, particularly fibrinogen (30) and N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester (TAMe) (31). While the reactions are accelerated in the presence of these cations, the absolute need for them is less obvious than it is for the platelet agglutination reaction described here. A comparison between the "substrates"—fibrinogen, TAMe, and platelets—upon which throm-

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bin acts and the effective cations is seen below:

	Mg^{++}	Ca^{++}	Mn^{++}	Co^{++}	Ni^{++}	Sr^{++}	Cd^{++}
Fibrinogen (30)	+	+	+	+	+	+	
TAMe (31)	−	+	−	−	−	+	−
Platelets	+	+	+	−	−	−	+

The "cation profile" for each reaction is different, with only calcium being common to all of them.

The parallelism of the platelet agglutination time and the fibrinogen clotting time with varying concentrations of thrombin, shown in Fig. 7, is striking. The two curves are nearly equivalent. This finding suggests that the platelet agglutination time could serve as the basis for the assay of thrombin, just as the fibrinogen clotting time does. The decreasing agglutination time with decreasing ionic strength (Fig. 4) is reminiscent of the progressive shortening of the fibrinogen clotting time under similar circumstances (26).

The understanding of the basic mechanism of platelet agglutination with thrombin-cation mixtures may be aided by these studies. One simple explanation advanced by many authors (32–35) is that the platelet, even if washed many times, possesses a "plasmatic atmosphere" containing fibrinogen and various procoagulants. The agglutination of platelets by thrombin could then be due to polymerization of fibrin between adjacent platelets. In spite of the many similarities between agglutination and the fibrinogen clotting reaction, we doubt that fibrin polymerization is the chief mechanism determining agglutination in our experiments for several reasons: (*a*) The minimal amount of calcium needed for platelet agglutination is manyfold greater than that proposed for fibrin formation (36). (*b*) The "cation profile" for the two reactions is different. (*c*) Agglutination with TAg′ and presumably with thrombin is easily reversible for a limited time in the presence of a strong chelating agent (23). (*d*) Platelets in afibrinogenemia agglutinate in the presence of thrombin (18, 19). Perhaps the splitting of some substrate other than fibrinogen, located in or on the platelet membrane, may be responsible for the agglutination phenomenon. The cation might participate in the formation of a complex with enzyme or substrate or both. A possible role for the cation is the formation of a cationic "bridge" (37). The limited range of effective concentration of calcium and magnesium, and the inhibitory effect of high concentrations of these ions, aside from the effect of ionic strength, suggest such a "bridging" mechanism (38, 39). Bridging sites on the platelet surface could be made available by proteolytic activity of thrombin.

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SUMMARY

A one-stage macroscopic test for platelet agglutination was used to study the effect of thrombin and thrombin-cation mixtures on washed platelets. Conclusions regarding platelet agglutination are as follows:

(*a*) Canine, bovine, or human thrombin alone does not cause agglutination of canine or human platelets.

(*b*) Thrombin with calcium or magnesium causes rapid platelet agglutination. Both calcium and magnesium are active at physiologic concentrations. Divalent manganese or cadmium ions can be substituted for calcium or magnesium.

(*c*) The agglutination reaction is affected but little by the species of origin of thrombin or platelets, or by variations in ionic strength or pH over a broad range.

(*d*) Temperature at which the reaction is carried out is critical; optimal temperature for the test is 28°C.

(*e*) Agglutination is inhibited by high ionic strength, by pH values outside the range 6.4–8.6, and by temperatures outside the range 25–28°C. High concentrations of calcium have a specific inhibitory effect.

(*f*) Platelet agglutination time is as sensitive an index of thrombin concentration as is the fibrinogen clotting time.

A comparison is made between divalent cations which influence platelet agglutination induced by thrombin, TAg′, and TAg. A similar comparison is made of cations influencing the action of thrombin on the "substrates," fibrinogen, TAMe, and platelets.

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