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Macroscopic Studies of Platelet Agglutination; Nature of Thrombocyte Agglutinating Activity of Plasma.* (24050)

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The spontaneous agglutination of platelets is poorly understood. From the earliest work(1) to the present, studies have been based primarily on microscopic observations. This report describes a test by which time and degree of agglutination are determined macroscopically. Optimum conditions for rapid agglutination of platelets are outlined.

Materials and methods. Preparation of sodium citrate, sodium oxalate, citrate-saline and saturated ammonium sulfate solutions has been described(2). Solutions of $CaCl_2$, $MgCl_2$, and $MnCl_2$ (Baker A.R.) were standardized by chloride analysis and diluted to 0.108 M. Buffered isotonic *EDTA* solution was prepared from the di- and trisodium salts of ethylenediamine tetraacetic acid (Geigy). Na_2EDTA and Na_3EDTA concentrations were 0.04086 M and 0.04734 M respectively. pH was 7.38. The solution was isosmotic with 0.156 M NaCl as determined by freezing point depression. Plasmas and platelet preparations were made from normal or hemophilic canine blood. Methods for preparing citrate plasma, oxalate plasma, fibrinogen(2), and plasmas adsorbed with $BaSO_4$ or fuller's earth (f.e.)(3) have been described. For preparation of *resin plasma*, 7 parts of blood were drawn into a syringe containing 1 part moist resin (Na cycle Dowex 50 cation exchange resin, 20-50 mesh). The blood was then passed twice through a column containing 2½ parts resin, at a flow rate of 6 ml/min. After centrifugation (2500 *g*, 20 min), the plasma was passed through a fresh resin column at the same rate. The albumin and globulin fractions were prepared from oxalate plasma using ammonium sulfate and were dialyzed with Visking casing at 4°C until free of sulfate. *Prothrombin* was a citrate eluate of $BaSO_4$ adsorbate of oxalate plasma, dialyzed against citrate-saline for 18 hrs, 4°C, diluted with saline to 50 Iowa units/ml. *Thrombin* was prepared by a modified citrate activation procedure(4). *AHF* was prepared from f.e.-adsorbed plasma by a modified phosphate-citrate technic(5). The test for fibrinogen has been described(3). *Standard platelet suspension*: Silicone-treated glassware was used. Blood, drawn into an equal volume of EDTA solution previously diluted 1:10 with saline (0.9% NaCl), was centrifuged at 4°C 8-10 min, 425 *g*, and the platelet-rich supernate recentrifuged for 5 min, 2075 *g*. The supernatant plasma (EDTA plasma) was decanted. The platelet pellet was suspended by gentle agitation at 20°C in EDTA solution which had been diluted 1:100 with saline. After centrifugation, 2 resuspensions or "washings" were made in citrate-saline. The final suspension, after centrifugation to remove rbc's (7 min, 90 *g*), was counted with the phase microscope and diluted to the desired concentration, usually 4 x 10^5 platelets/cmm. *Citrate platelet suspension*: The standard procedure was modified by using citrate and citrate-saline instead of the 2 dilute EDTA solutions. *Resin platelet suspension*: The initial plasma-resin column (v.s.) was flushed with 50 ml saline. The platelet-rich wash was

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TABLE I. Effect of Platelet, Cation, and Plasma Variations on Platelet Agglutination.

Exp. No.	Platelet prep.	Plasma or derivative	Ion	Agglutination time (sec.)
		Platelets and ions		
1	Citrate	Resin	Mg	8
2	Resin	"	"	13
3	Standard	"	"	8
4	" , fresh	"	Mn	8
5	" , 28°, 4 hr	"	"	15
6	" , 28°, 20 "	"	"	19
7	" , 2°, 20 "	"	"	9
8	"	Oxalate	Mg	8
9	" , −20°, 1 hr	"	"	no aggl.
10	" , 37°, 5 min.	"	"	10
11	" , 50°, 5 "	"	"	27
12	" , 60°, 5 "	"	"	no aggl.
13	"	Resin*	Ca	"
		Plasma and plasma fractions		
14	Standard	Citrate	Mg	7
15	"	EDTA	"	16
16	"	Citrate†	"	8
17	"	$BaSO_4$-ads.†	"	8
18	"	Resin†	"	10
19	"	EDTA‡	"	13
20	"	Globulin	Mn	12
21	"	Albumin	"	no aggl.
22	"	Fibrinogen	"	8
23	"	Resin, 54°	"	40
24	"	Cit. f.c. ads.	Mg	7
25	"	$BaSO_4$-ads.	"	9
26	"	Prothrombin	"	no aggl.
27	"	Thrombin	NaCl	"
28	"	"	Mg	120
29	"	AHF	"	18
		Hemophilic plasma and platelets		
30	Standard	Hemophilic	Mg	10
31	"	Normal	"	10
32	" , hemophilic	"	"	10
33	" , "	Hemophilic	"	10

* Clotting time 4 min.

† Exp. 16, 17, 18: dialyzed *vs* cit.-saline for 4, 18, and 24 hr respectively.

‡ Dialyzed *vs* ox.-sal. for 24 hr.

Degree of agglutination was ++++ in all positive experiments except 6 (+++), 11 (++), and 28 (+).

centrifuged and the platelets twice resuspended in saline.

Procedure. Macroscopic platelet agglutination test was performed as follows: One ml plasma or plasma derivative was mixed with 0.25 ml cation solution (Ca, Mg, or Mn). After incubation for 30-40 min at 28°C, a 0.2 ml aliquot was aded to 0.2 ml platelet suspension and a stop watch started. The tube was tapped gently and the moment of appearance of platelet clumps was determined (agglutination time). Two minutes later the clumps were graded macroscopically on a scale of +, ++, +++, ++++. Microscopic examination of platelet aggregates revealed about 5 platelets per + aggregate, 10-25 per ++, 30-100 per +++, and over 100 per ++++ aggregate. Unagglutinated tests were observed at 2,10 and 30-60 min before being recorded as negative. The agglutination test was *modified* in some experiments by using 0.0108 M $MnCl_2$, in others by incubating 10 volumes of plasma or plasma dilution with 1 volume of 0.027 M $MgCl_2$ for 10 min. In these experiments the standard cation solutions were diluted with saline.

Results. I. Platelet Agglutination. Three components were essential for rapid agglutination—platelets, divalent cations, and plasma or plasma derivatives. Representa-

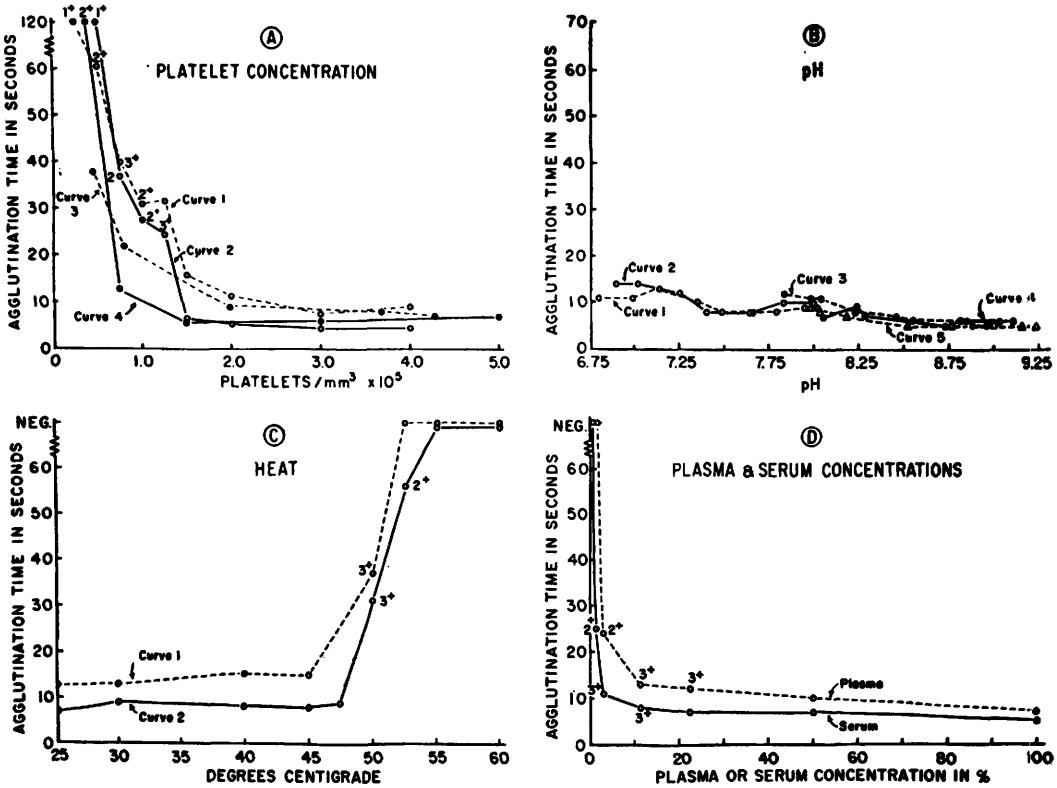

FIG. 1. Effect of platelet conc., pH, heating of plasma, and plasma conc. on platelet agglutination. Resin plasma was used throughout. Unless otherwise indicated, agglutination reactions were ++++. (A) *Platelet concentration.* Final conc. in agglutination test is indicated on abscissa. Curve 1: Test done with Mg. Curve 2: Test done with Mn. Curves 3 and 4: Modified agglutination test with Mg. (B) *pH.* pH was determined with a glass electrode immediately after agglutination. Modified test with Mg was used. Curve 1: 0.3 ml 0.2 M imidazole buffer of varying pH (3) was added to final agglutination tubes. Curve 2: Plasma was diluted with equal vol of imidazole prior to incubation. Curve 3: pH of plasma varied by adding 0.15 N NaOH prior to incubation. Curve 4: Plasma diluted with equal vol 0.2 M glycine buffer adjusted with N/5 NaOH; half-strength Mg. Curve 5: 0.2 ml glycine buffer was added to final agglutination tubes. (C) *Heat.* Plasma heated at indicated temperature for 10 min. Curve 1: With Mg. Curve 2: With Mn. (D) *Plasma and serum concentrations.* Dilutions were made with normal saline. Plasma or serum conc. on abscissa refers to their strength prior to use in test. Mg was used.

tive experiments in Table I and Fig. 1 illustrate the effect of variations in these 3 components. No agglutination was observed when either cation or plasma alone were tested.

Platelets. Various *anticoagulants* were used to prepare platelet suspensions. The results are shown in Exp. 1-3, Table I. In further experiments EDTA platelets gave the most reproducible results and became our standard. The platelets in these preparations were uniform biconvex discs when examined in the hanging drop with the phase microscope (970 x). *Washing* the platelets as many as 10 times did not alter their agglutinability. One preparation was divided into 6 lots, washed 1, 2, 3, 5, 7, and 10 times respectively, and tested with Mg and $BaSO_4$-treated plasma. Each lot reacted equally in the test. The influence of platelet *concentration* is shown in Fig. 1A. A final concentration of 2 x 10^5/cmm or higher gave best results, while agglutination times were longer and platelet clumps smaller with lower concentrations. *Storage* of platelet preparations at 28°C for 20 hr caused progressive reduction in their agglutinability (Exp. 4-6). If the suspensions were maintained at 2°C for as long as 20 hr and warmed to 28°C immedi-

ately before use, the platelets showed little change in reactivity (Exp. 7). Platelets frozen and thawed lost their agglutinability (cp. Exp. 8 and 9), as did those heated to 60°C (cp. Exp. 8 and 10-12).

Comparison of Ca, Mg, and Mn ions. In many experiments with plasma, either resin or $BaSO_4$-adsorbed, platelet agglutination was delayed or absent in the presence of Ca, but was rapid with Mg or Mn (cp. Exp. 13 and 3-4). The fact that Ca caused clotting, while Mg and Mn did not, suggests disassociation of platelet agglutination and coagulation.

pH. The effect of varying pH on platelet agglutination is shown in Fig. 1B. Between pH values of 6.8 to 9.25, agglutination times were in the narrow range of 5-14 sec. In our other studies, the pH was 7.6-7.9.

Plasma and serum. Prompt agglutination occurred with all plasmas tested, regardless of the anticoagulant used in plasma preparation (Exp. 3, 8, 14, 15). EDTA plasma gave the longest agglutination times. Plasma stored at −20°C retained its agglutinating activity. In one experiment, resin plasma which had been frozen for 3 months still had its original agglutinating power. Serum was found to be as active as plasma (Fig. 1D). The foregoing experiments suggest that a potent thrombocyte agglutinating factor(s) is present in plasma and serum.

II. Nature of Thrombocyte Agglutinating Factor (TAg) of Plasma. Additional experiments were undertaken to characterize this factor further. Fig. 1C shows that TAg was inactivated by heating at 52.5°-55°C. After dialysis the plasma factor was still present (Exp. 16-19). Plasma and serum retained high agglutinating capacities until diluted to concentrations below about 6-12% (Fig. 1D). On fractionation of plasma, TAg activity was found in the globulin but not in the albumin fraction (Exp. 20, 21).

In a final group of experiments, the possible identity of TAg with certain coagulation proteins was investigated. *Fibrinogen* preparations uniformly revealed high agglutinating activity (Exp. 22). On the other hand, serum, with no detectable fibrinogen, was equally active (Fig. 1D). Attempts were made to separate fibrinogen from TAg in plasma. Resin plasma heated to 54°C for 90 sec no longer clotted with thrombin, yet agglutination still occurred (Exp. 23). Citrate plasma from which fibrinogen had been removed by adsorption with fuller's earth(3) retained full agglutinating activity (Exp. 24). These experiments appear to separate TAg from fibrinogen.

The relation of TAg to *prothrombin* and *thrombin* was then studied. Agglutinating activity was present in $BaSO_4$-adsorbed plasma in which no prothrombin could be detected with either the 1-stage or the 2-stage tests (Exp. 25). Conversely, a prothrombin fraction of plasma had no TAg activity (Exp. 26). Thrombin which clotted fibrinogen in 2-3 sec caused no platelet agglutination (Exp. 27). However, the addition of cation caused variable agglutination (Exp. 28). Serum prepared by recalcification of resin plasma had no agglutinating activity (Exp. 13). This serum contained thrombin, as it clotted equal parts of fibrinogen in 49 sec. It thus appears that platelet agglutination by TAg is not dependent upon either prothrombin or thrombin.

An AHF fraction prepared from fibrinogen-free plasma showed moderate agglutinating activity (Exp. 29). On the other hand, canine hemophilic oxalate plasma, devoid of AHF, was as active as normal oxalate plasma (Exp. 30, 31). Thus TAg appears to be dissociated from AHF. In other studies suspensions of hemophilic platelets were agglutinated rapidly by both normal and hemophilic plasmas (Exp. 32, 33).

Discussion. Direct macroscopic observation of platelet agglutination provides a method by which rate and degree of agglutination can be readily determined. At the beginning of this study, agglutination times were in the range of 1-5 min. However, as optimum conditions for agglutination were determined, the times were shortened to 5-15 sec. Preparation of platelets was an important technical procedure in this study. The need for meticulous attention to such details as venepuncture and rapid mixing of the blood with anticoagulant cannot be overemphasized. Reports on use of EDTA for human platelet preparations suggest that altered forms regularly occur(6). In our standard preparations

the platelets were discrete with regular outline and without spicules or similar alterations. The EDTA used was isosmotic with saline. The isolated platelets showed no inherent agglutinability, but were rapidly agglutinated by plasma-cation mixtures. This property of platelets could be easily destroyed by either freeze-thaw or heating procedures.

The finding that Ca is not essential for platelet agglutination was surprising in view of the many reports of its important role in this phenomenon [Lüscher(7)]. It was consistently observed that more rapid agglutination and larger platelet aggregates occurred with Mg or Mn than with Ca. Concentrations of Mg and Mn were isosmotic with saline. While rapid agglutination occurred under these conditions, the ions were greatly in excess of physiological concentrations. The pH for this reaction did not appear critical within the limits of 7-9.

In the study of the platelet agglutinating activity of plasma and serum, the same results were obtained with both autologous and homologous systems. The agglutinating factor appears to be a protein. It may be closely related to fibrinogen and AHF, judging from its presence in purified preparations of these clotting factors. However, other findings indicate that platelet agglutination is separate from the fibrin clotting process. In the systems where agglutination was rapid, as with Mn, no coagulation occurred. Further, systems free of fibrinogen, AHF, prothrombin and other $BaSO_4$-adsorbable coagulants were very active. Also, purified prothrombin had no TAg activity. Further study is needed to determine the basis of the agglutinating activity of the thrombin fractions. The presence of high agglutinating activity in canine hemophilic plasma confirms similar findings in human hemophilia(8). In view of the Roskam-Bounameaux hypothesis(9) that isolated platelets agglutinate because of procoagulants contained in plasma "adsorbed" to their surface, it was interesting that repeated platelet washings failed to alter their agglutinability. We have designated plasma activity as thrombocyte agglutinating factor(s) or TAg. Consideration was given first to the term viscous metamorphosis factor (VMF), but this was abandoned since VM has been so often related to the procoagulant plasma proteins. Also, this term has acquired many special connotations, including clot retraction(10), since its use by Eberth and Schimmelbusch(11).

Summary and conclusions. 1. Optimal conditions for platelet agglutination were determined in the dog by a macroscopic test. Most rapid agglutination was observed with Mg^{++} or Mn^{++}, undiluted plasma, and a platelet concentration above 200,000/cmm. Frozen or heated platelets were not agglutinable. 2. The thrombocyte agglutinating (TAg) factor found in plasma was thermolabile and nondialyzable. Under optimal conditions it caused gross platelet clumping in 5-15 seconds. This factor was active in the absence of fibrin coagulation, Ca^{++}, fibrinogen, AHF, prothrombin and related coagulant factors, suggesting the TAg may act independently of the coagulation process.

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