4. Magnusson, C. E., Stevens, H. C., *Am. J. Physiol.*, 1911, v29, 124.
5. Barlow, H. B., Kohn, H. I., Walsh, E. G., *ibid.*, 1946, v148, 372.

Received July 16, 1959. P.S.E.B.M., 1959, v102.

Cation Specificity of Thrombocyte Agglutinating Activity (TAg) of Canine Plasma.* (25210)

R. G. MASON,† E. C. LEROY† AND K. M. BRINKHOUS
Dept. of Pathology, University of North Carolina, Chapel Hill

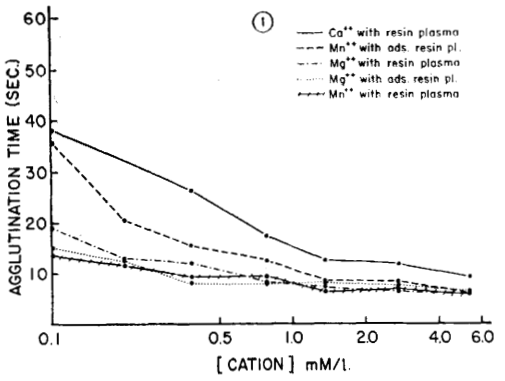
Canine plasma possesses the ability to agglutinate homologous and autologous platelets rapidly in the presence of Mg^{++} or Mn^{++}(1). This property of plasma has been termed thrombocyte agglutinating activity (TAg). TAg is contained in crude globulin fractions of plasma, but not in the albumin fractions. It is non-dialyzable and heat-labile. TAg appears to be separate from the plasma procoagulants, fibrinogen and antihemophilic factor, as well as from prothrombin and related $BaSO_4$-adsorbable factors. In this study TAg was tested for activity with 15 separate cations. The active ions were studied further to determine the relative amounts needed for prompt agglutination of platelets with TAg.

Methods. Details of the macroscopic platelet agglutination test for TAg have been described(1). The test is done in 2 stages. In the first or incubation stage, canine plasma is incubated for 30 min. with a cation chloride solution. In the second or agglutination stage, a standard suspension of washed canine platelets is added to the incubation mixture and time and degree of agglutination determined. Three types of canine plasma were used. *Resin plasma* was prepared by use of Dowex 50 resin columns(1). *Oxalate plasma* has been described(2). *EDTA plasma* was prepared by mixing 9 parts of whole dog blood with one part of stock EDTA solution(1), previously diluted one to 3.27 with normal saline. Plasma dialysis against 0.154 M NaCl was carried out with Visking casing (18 hr continuous agitation, 4°C). *Adsorbed plasmas* (resin, oxalate, or EDTA) were prepared by adding to each ml plasma 100 mg $BaSO_4$ (Merck) (contact time 30 min, 4°C) and repeating the procedure once. In the prothrombin time test with adsorbed plasmas no clot formed in 10 min. The chloride salts of various cations were used; all salts were Baker A.R., except $FeCl_3$ (Mallincrodt), $PbCl_2$ (Fisher), and $BaCl_2$ (Merck). Solutions were standardized by chloride analyses, and diluted to 0.108 M. For weaker solutions, serial 2-fold dilutions were made with 0.154 M NaCl. Cation concentration in the experiments is expressed as mM cation *added* per liter of plasma incubation mixture. *Platelet suspensions*(1) were prepared from EDTA canine plasma and contained $4 \cdot 10^5$ platelets/cmm. In several experiments, platelet preparation was modified by resuspending the platelets in solutions containing 0.5% bovine albumin (Armour). With this modification the platelet pellet could be resuspended rapidly, and platelet reactivity was unchanged.

Results. TAg and $BaSO_4$ adsorption of plasma. A series of tests were performed to determine the effect of $BaSO_4$ adsorption of plasma on platelet agglutination by TAg. Fig. 1 shows the results obtained with resin plasma before and after adsorption. With either Mg^{++} or Mn^{++}, rapid agglutination occurred, regardless of plasma adsorption or cation concentration used. Only with adsorbed plasma at the lower Mn^{++} concentrations were agglutination times less rapid than with non-adsorbed plasma. With Ca^{++}, on the other hand, no agglutination occurred with adsorbed plasma; with non-adsorbed plasma, clotting occurred in the incubation phase and the resultant serum caused moderately rapid agglutination.

* This investigation was supported in part by grant from Nat. Heart Inst., N.I.H., P.H.S.

† Post-Sophomore Research Fellow, N.I.H., P.H.S.



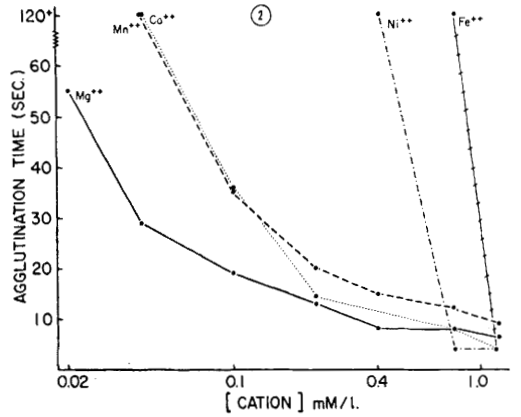


FIG. 1. Effect of $BaSO_4$ adsorption of plasma on TAg activity.

FIG. 2. Effect of varying cation concentration on TAg activity.

In subsequent tests adsorbed plasmas were used, thus avoiding clotting.

Cations and TAg. Resin, oxalate, EDTA, and dialyzed EDTA plasmas were each tested with 15 divalent or trivalent cations for TAg activity (Table I). With resin plasma, only 5 ions gave positive results: Mg^{++}, Mn^{++}, Fe^{++}, Co^{++}, and Ni^{++}. With plasmas prepared with the chelating agents, oxalate and EDTA, somewhat different results were obtained. With oxalate plasma, the same group of cations as well as Ca^{++} caused agglutination. With EDTA plasma, only Mg^{++}, Mn^{++}, and Ca^{++} were active. However, after EDTA was removed by dialysis, the plasma reacted with the same 5 ions as did resin plasma (last column, Table I), but not with Ca^{++}. With both oxalate and dialyzed EDTA plasmas, agglutination times were longer than with resin plasma.

Quantitative cation studies. The effect of varying cation concentration on TAg activity of resin plasma was next studied. Results obtained with ions possessing specific activity are shown in Fig. 2. Fe^{++} is required in relatively high concentration, while Mg^{++} is still active in comparatively low concentration. Ni^{++}, Co^{++} or Mn^{++} were somewhat less active than Mg^{++}. The other 10 ions were inactive in this dilution series.

Discussion. In this study of the effect of cations on TAg activity of plasma, a number of problems were encountered in obtaining conditions which would produce constant and reproducible results. Since $BaSO_4$ adsorption of plasma influenced TAg activity little, if at all (Fig. 1), the use of adsorbed plasma in the agglutination test was advantageous as it eliminated interference from fibrin formation in tests with Ca^{++} and Sr^{++}. Resin-treated plasma appeared to be more reliable than oxalate or EDTA plasma (Table I). In the presence of either oxalate or EDTA, false positive results were obtained with Ca^{++}. In the presence of EDTA, false negative results were obtained with Fe^{++}, Co^{++}, and Ni^{++}. After removal of the EDTA chelate by dialysis, the plasma gave qualitatively the same reactions with the different ions as did resin plasma. A number of factors appear to be responsible for the divergent results obtained with plasmas containing chelating agents. Included are plasma concentration of the added chelating agent, solubility of the chelate complex, and the stability constant or relative affinity of cation for chelating agent(3). These as well as other factors influence the availability of specific cations for platelet agglutination with TAg. Certain of the cations (see footnote, Table I) in high enough concentration caused both non-specific clumping of platelets in the absence of TAg and formation of a metal proteinate precipitate in plasma.

Of the cations which caused platelet agglutination with TAg, 4 of the 5—Mn^{++}, Fe^{++}, Co^{++}, and Ni^{++}—belong to the first transitional series of elements and have the atomic numbers, 25-28. The fifth ion, magnesium, is not a member of this series. Nevertheless, it was the most active of the ions tested (Fig. 2)—a concentration of only 1/2 - 1/40 as much as

TABLE I. Test of 15 Cations for Activity with TAg in Adsorbed Canine Plasma.

Cation	Atomic No.	[Cation] mM/l*	Adsorbed plasma tested: Resin Sec.	Resin Degree†	Oxalate Sec.	Oxalate Degree	EDTA Sec.	EDTA Degree	Dialyzed EDTA Sec.	Dialyzed EDTA Degree
Mg^{++}	12	21.6	5	4+	7	4+	7	4+	12	4+
		.78	10	4+	29	4+	Neg		‡	‡
Ca^{++}	20	21.6	Neg		16	2+	9	4+	Neg	
		.78	"		Neg		Neg		‡	‡
Mn^{++}	25	21.6	6	4+	7	4+	5	4+	49	3-4+
		.78	8	4+	10	4+	Neg		‡	‡
Fe^{++}	26	.78	10	4+	17	3+	"		17	4+
Fe^{+++}	26	.78	Neg		Neg		"		Neg	
Co^{++}	27	.78	5	4+	22	1+	"		15	4+
Ni^{++}	28	.78	6	4+	19	4+	"		31	4+
Cu^{++}	29	.78	Neg		Neg		"		Neg	
Zn^{++}	30	.096	"		"		"		"	
Sr^{++}	38	21.6	"		"		"		"	
Cd^{++}	48	.78	"		"		"		"	
Ba^{++}	56	21.6	"		"		"		"	
La^{+++}	57	.21	"		"		"		"	
Hg^{++}	80	.096	"		"		"		"	
Pb^{++}	82	.096	"		"		"		"	

* Greatest concentration of each cation was either 21.6 mM/l or that amount which did not cause a metal proteinate precipitate in the plasma during incubation stage of the test.

† Refers to size of platelet clumps graded macroscopically on scale of 1+ (about 5 platelets per aggregate) to 4+ (over 100 platelets/aggregate).

‡ Test not done.

Controls in which normal saline was substituted for plasma showed that the following cations, in ranges of concentration indicated, produced non-specific agglutination of washed platelets: Fe^{++}, 10.8-21.6 mM/l; Ni^{++}, Cu^{++}, and Pb^{++}, 5.36-21.6; Cd^{++} and La^{+++}, 2.72-21.6; Fe^{+++}, Zn^{++}, and Hg^{++}, 1.36-21.6. Controls with lesser concentrations and controls with the other cations did not cause non-specific agglutination.

that required for the other active ions was effective. Also, magnesium appears to be active in concentrations normally present in canine plasma(4). The other active ions, all "trace elements," appear to be required in concentrations higher than those normally present in plasma. It is of interest that the 2 ions, Ca^{++} and Sr^{++}, which are active in clotting were inactive with TAg in adsorbed resin plasma.

Summary. The effect of each of 15 divalent or trivalent cations on the thrombocyte agglutinating activity (TAg) of canine plasma was tested. Adsorption of plasma with $BaSO_4$ had little, if any, effect on TAg activity. In adsorbed resin-treated plasma TAg was active only in the presence of Mg^{++}, Mn^{++}, Fe^{++}, Co^{++}, or Ni^{++}, with Mg^{++} being effective in the lowest concentration. Seeming differences in cation specificity of TAg in plasmas containing the chelating agents, oxalate and EDTA, are discussed.

1. Brinkhous, K. M., LeRoy, E. C., Cornell, W. P., Brown, R. C., Hazelhurst, J. L., Vennart, G. P., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 379.

2. Wagner, R. H., Graham, J. B., Penick, G. D., Brinkhous, K. M., *The Coagulation of Blood,* Tocantins, L. M., Ed., Grune and Stratton, N. Y., 1955, p105.

3. Martell, A. E., Calvin, M., *Chemistry of the Metal Chelate Compounds,* Prentice-Hall, N. Y., 1952.

4. Carr, M. H., Schloerb, P. R., *J. Lab. & Clin. Med.,* 1959, v53, 646.

Received July 20, 1959. P.S.E.B.M., 1959, v102.