Platelet Release Reaction During Clotting of Native Human Platelet-Rich Plasma[1] (36223)

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(Introduced by S. Sherry)

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Previous studies (1) on the platelet release reaction occurring during the interaction between washed platelets and thrombin have revealed a pattern by which all substances released (metabolically inactive ATP and ADP, serotonin and numerous acid hydrolases) are located in denses bodies and α-granules. All substances retained by the cells during this interaction (10 different enzymes), are located in cytoplasm, membranes, and mitochondria. These findings led to the suggestion (2) that the platelet release reaction is a specific process, consisting only in extrusion of material located in platelet granules. These results have been extended by the present investigation on the release of platelet constituents during the clotting of native platelet-rich plasma (PRP). The morphological appearance of the platelet-fibrin clot (3) suggests that greater alterations occur in this system than have been observed during the action of thrombin on washed platelets. Platelets have been shown to be the source of serum *p*-nitrophenyl phosphatase (4) but this enzyme, in contrast to the other lysosomal acid hydrolases, is retained by both washed platelets (1) and platelets in citrated platelet-rich plasma exposed to thrombin (5). Therefore special attention has been paid to the behavior of acid phosphatase in the present work.

Materials. Substrates for acid hydrolases and chemicals[2] for other enzyme determinations have been described previously (1, 6).

Preparation of native and citrate PRP. All glassware used was siliconized (Siliclad, Clay Adams); whereas plastic ware was not. From each healthy donor two 18 ml aliquots of blood were drawn by free flow (venipuncture) with a blood collection set[3] into separate 50 ml plastic tubes, one containing 2 ml of 0.11 *M* sodium citrate (for preparation of citrate PRP), the other containing 2 ml of 0.15 *M* NaCl (for preparation of native PRP). The tubes were kept in ice during blood collection and until they were centrifuged at 4° for 10 min at $G_{max} = 225g$. The citrate and native PRP so obtained were transferred to different tubes and kept on ice until start of release experiment. Part of the citrate PRP was centrifuged at 4° for 20 min at $G_{max} = 1800g$ to obtain platelet-poor plasma (PPP).

Release experiments. Two milliliter portions of native PRP, citrate PRP, and PPP were incubated at 37° in plastic tubes. The native PRP clotted within 3–5 min; after which, all samples were incubated for a recorded period of time. The tubes were then chilled; and the clot was removed from the native PRP samples. The serum so obtained was centrifuged at 4° for 20 min at $G_{max} = 1800g$ to remove any residual platelets. Sam-

[1] A preliminary report on this work was presented at the 54th Annu. Meet. Fed. Amer. Soc. Exp. Biol., Atlantic City, 1970, Abstr. No. 2124 [Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29, No. 2 (1970)]. Financial support for this work was provided through the Heart Association of Southeastern Pennsylvania, the General Research Support Grant 5S-01-FR-05417-08 awarded to Temple University School of Medicine, and General Clinical Research Center Grant No. MO1-RR-349, U.S. Public Health Grants HE 13175-01, HE 14217-01.

[2] Obtained from Sigma Chemical Co., St. Louis, MO.

[3] Plexitron R 241H Blood Collection Set, Travenol Labs., Inc., Morton Grove, IL.

TABLE I. Levels of Enzymes and Substances in PRP, PPP, and Serum Percentage Release During Clotting.

Native PRP, citrate PRP, and PPP were incubated for 60 min at 37° after clotting of the native PRP. The clot was removed and the serum, PRP, and PPP were assayed for enzymes and substances; enzyme activities (μmoles/min/liter); substances (μmoles/liter). Platelet numbers varied between 3.3 and 4.5 $\times$ 10^8 cells/ml.

	No. of expts.	Levels (mean $\pm$ SD) PRP	PPP	Serum	Release[a] (%)
Lactate dehydrogenase	6	3830 $\pm$ 990	950 $\pm$ 190	1000 $\pm$ 140	1.9 $\pm$ 1.7
Pyruvate kinase	6	68 $\pm$ 49	31 $\pm$ 25	28 $\pm$ 19	—7.2 $\pm$ 12.2
Hexokinase	6	23 $\pm$ 6	13 $\pm$ 2	11 $\pm$ 6	—18.9 $\pm$ 29.9
Adenylate kinase	12	99 $\pm$ 33	5 $\pm$ 4	19 $\pm$ 6	17.1 $\pm$ 6.5
Acid phosphatase	6	141 $\pm$ 2	9 $\pm$ 3	15 $\pm$ 2	4.4 $\pm$ 2.0
β-Galactosidase	6	3.8 $\pm$ 0.9	0.7 $\pm$ 0.6	0.8 $\pm$ 0.5	5.8 $\pm$ 9.6
β-Glucuronidase	12	0.7 $\pm$ 0.2	0.3 $\pm$ 0.2	0.4 $\pm$ 0.2	36.1 $\pm$ 11.9
β-*N*-Acetylglucosaminidase	12	24 $\pm$ 0.5	14 $\pm$ 0.4	20 $\pm$ 0.4	60.2 $\pm$ 8.3
Adenine nucleotides (UV absorbing material)	12	26 $\pm$ 8	0 $\pm$ 0	18 $\pm$ 7[b]	75.2 $\pm$ 10.4
Serotonin	12	1.3 $\pm$ 0.9	0.1 $\pm$ 0.1	1.0 $\pm$ 0.1	80.1 $\pm$ 13.8

[a] Percentage release is calculated from the formula: $[(TA_{serum}-TA_{PPP})/(TA_{PRP}-TA_{PPP})] \times 100$. Where TA means total activity of an enzyme or total amounts of a substance. The values were worked out for each individual set of native PRP, citrate PRP, serum and PPP; the values given are the means ($\pm$ SD) of these individual percentages.

[b] Measured against plasma as blank.

ples of centrifuged sera were pooled and 10 μl of 1.1 *M* sodium citrate was added /ml of serum. All samples were frozen and thawed once before determination of enzymes and substances.

Determinations. Acid *p*-nitrophenyl phosphatase was measured according to Technical Bulletin No. 104, Sigma Chemical Co., with the inclusion of 0.16% Triton $\times$ 100 (final conc). Other enzymes were measured as previously described (6, 7).

Serotonin was measured by the method of Crawford and Rudd (8) using Aminco Bowman Spectro-Fluorometer with the following modifications: *n*-butanol was washed (in the stated order) with 0.1 *N* HCl and twice with H_2O before use; 2.0 ml of 3.4 *N* HCl was used in the last step of the procedure.

Adenine nucleotides. To 2.0 ml of 0.5 *M* $HClO_4$ was added 0.5 ml of the serum (incubated for 2 hr), PPP or PRP. The mixtures were centrifuged for 20 min at $G_{max} = 28$, 700*g* and the supernatants were read from 242 to 262 mμ at 2 mμ intervals against the PPP-extract. Due to the rapid breakdown of nucleotides in plasma (9), specific assays for released ADP and ATP were not used. Instead, total nucleotides were estimated by their optical density. For platelet-rich plasma, this was read at 260 mμ using ADP as a standard. For plasma and serum, in which adenine nucleotides were degraded to inosine and hypoxanthine (9), the extinction was read at 248 mμ using hypoxanthine as a standard.

Results. During clotting of native PRP the following enzymes were released into the serum in significant amounts: Adenylate kinase, β-glucuronidase, and β-*N*-acetylglucosaminidase (Table I). Serotonin and adenine nucleotides were also released, but lactic dehydrogenase and acid phosphatase were retained by the platelet. Pyruvate kinase and hexokinase were measured against relatively large plasma blanks and the negative differences between serum and plasma were attributed to an inactivation of up to 10% of these enzymes that occurred during 60-min incubation of serum. β-Galactosidase, unlike the other acid hydrolases, was rapidly inactivated in serum (more than 30%/hr. When measurements were made 10 min after

TABLE II. Comparison of Release Percentages in the Clotting PRP System and the Washed Platelet-Thrombin System.

The values from the clotting experiments are taken from Table I; whereas those for the washed platelet–thrombin system are computed from Tables II, III, and IV, of Ref. (1) and Table I of Ref. (26) (the results obtained in the presence of 2.5 m*M* Ca), using the formula in Table I of the present paper. The basis for comparison between the 60-min clotting experiments (40 units of thrombin/ml generated) and the 5-min experiments with washed platelets and 5 units of thrombin/ml are the following: With washed platelets, 100 units of thrombin give the same degree and pattern of release as 5 units/ml; and 60-min incubation does not give more release than that observed 2–3 min after thrombin addition. Incubation temperature (37°) and Ca concentrations in the two systems are the same.

	Enzyme release (%) from markers for cytosol, membranes, and mitochondria			
	Lactate dehydrogenase	Pyruvate kinase	Hexokinase	Adenylate kinase
Clotting of PRP	1.9	—7.2	—18.9	17.1
Thrombin-washed platelets	1.7	—1.3	— 5.7	0.22

	Enzyme release (%) from markers for:					
	α-Granules				Dense bodies	
	Acid phosphatase	β-Galactosidase	β-Glucuronidase	β-*N*-acetyl-glucosam.	Adenine nucleo.	Serotonin
Clotting of PRP	4.4	15–35[a]	36.9	60.2	65.2[b]	75.2
Thrombin-washed platelets	2.9	44.0	38.6	45.3	64.1	72.0

[a] After clotting (10 min), 3 experiments.
[b] Corrected for 10% "release energy ATP" (see Results).

clotting, 15–35% of β-galactosidase was released (Table II). The release of adenine nucleotides and metabolites was measured as the difference between plasma, assumed to be free of adenine nucleotides, and PRP or serum. Plasma contained, however, considerable amounts of absorbing material (*i.e.*, uric acid, hypoxanthine). If "release energy ATP" being excreted as hypoxanthine during release amounts to 10% of platelet adenine nucleotides (10), 65.2% of platelet adenine nucleotides had been actually released during clotting. It should be noted that "percentage release" is defined in Table I as the amount of released material related to the amount of this material associated with the platelets of PRP. Though the serum contains 6% more acid phosphatase than did PPP, this increase represents only 4.1% of the total platelet acid phosphatase, which is too low to be regarded as part of the specific release reaction.

The time course of liberation of acid phosphatase from platelets after clotting of native PRP was slower than that for β-glucuronidase (Fig. 1). Centrifugation of serum (20,000*g* for 20 min) caused no appreciable reduction in its β-glucuronidase activity; whereas all of the acid phosphatase originating from platelets was removed. Hence, β-glucuronidase was released in a soluble form; whereas the acid phosphatase was particulate. High speed centrifugation also reduced the acid phosphatase activity of PPP and more of the activity was sedimented at 100,000*g* than at 20,000*g*. Similarly, the β-galactosidase activity of serum was reduced 26% by centrifuging for 20 min at 100,000*g*. In contrast, β-*N*-acetyl-glucosaminidase activity was not affected. When the sedimented material was resuspended, acid phosphatase but no β-galactosidase activity was found. Also, the total supernatant and sedimented

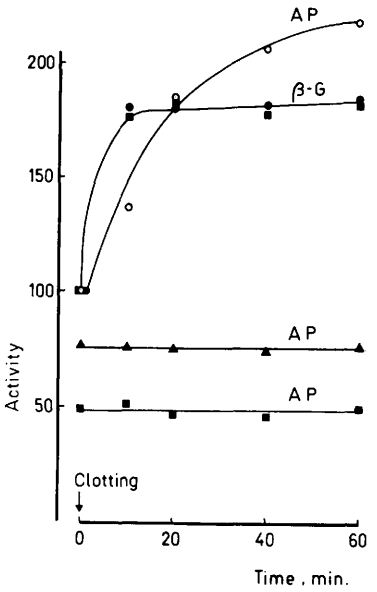

FIG. 1. Effect of incubation at 37° after clotting of native PRP on the activity of acid *p*-nitrophenylphosphatase (AP) and β-glucuronidase (β-G) found in serum centrifuged for 20 min at 2000*g* (○,●); for 20 min at 20,000*g* (▲); and for 90 min at 100,000*g* (■). The zero-time values represents activities found in citrate PPP from the same blood. The enzyme activities are expressed relative to that found in PPP centrifuged at 2000*g* (= 100).

acid phosphatase was found to be 71% of that in the original serum, indicating that both of these enzymes are inactivated to some extent during centrifugation.

Effects of aspirin[4] on the release reaction occurring during clotting were investigated both *in vitro* (1.1 and 2.8 m*M* in PRP) and *in vivo* (0.65 g orally, 2 hr before venipuncture). For the *in vivo* experiments a control blood sample was taken from each of the 6 subjects before the aspirin was given. Oral aspirin had no demonstrable effect on the release of β-glucuronidase, adenylate kinase, adenine nucleotides, or serotonin, but caused a reduction of 11.9% (±10.1%, significant at $p < .05$) in the release of β-*N*-acetyl-glucosaminidase. *In vitro* additions of aspirin were without any detectable effect.

Discussion. During clotting of native platelet-rich plasma, approximately 9 units/ml of thrombin can be generated (11) in the presence of 2–2.5 m*M* calcium. In addition, the effects of clot retraction, polymerizing fibrin and plasma proteins might also provide a greater stimulus for platelet release reaction occurring *in vivo*. The morphological changes observed during clotting of PRP suggest widespread platelet lysis (3). The present work shows that only a small degree of lysis can have occurred, since certain platelet constituents appear extracellularly in a selective manner and in a soluble form. This selectivity is strikingly similar to that observed during the study of thrombin action on washed platelets (Table II), in that all nongranular platelet constituents released were those which had been shown to be associated with granules. These findings suggest that even avoiding use of anticoagulants, centrifugation, removal of platelets from plasma and exposure of the cells to artificial media, the previously described release pattern (1) is not altered. Furthermore, additional factors occurring in clotting of platelet-rich plasma, such as polymerizing fibrin, clot retraction, γ-globulin, which have been shown to have effects on platelets, do not seem to evoke other responses in the platelet than the specific platelet release reaction.

We have previously shown (1) that thrombin releases maximally 64.1% of platelet adenine nucleotides and 72% of the cell serotonin (Table II). During clotting, adenine nucleotides and serotonin were released to the same extent, indicating that when the material from dense granules is released, it is always maximal under normal circumstances, as has been pointed out for ADP and epinephrine-induced release (12). On the other hand, adenylate kinase was released to a greater extent during clotting than in the washed platelet–thrombin system. Significant release of adenylate kinase during clotting has been also demonstrated by others (13). The lack of release of adenylate kinase from washed platelets with thrombin might indicate that some of the platelet responsiveness was lost during platelet washing.

We have confirmed the findings of others

[4] Aspirin tablets (USP) or powder (USP) from Merck and Company, Inc., Rahway, NJ.

(4, 14) that platelets contribute significantly to the amount of acid *p*-nitrophenyl phosphatase in the serum. The rise in acid phosphatase during clotting appeared to be only slightly greater than that observed when citrate PRP was incubated for the same time period, a rise noted with all enzymes studied. Both acid phosphatase and β-galactosidase accumulate in serum during clotting with a time course distinctly different from that observed with the platelet constituents released as discussed above. Furthermore, the two acid hydrolases were liberated during clotting in a sedimentable form and showed some degree of inactivation during sedimentation in contrast to the released material which appeared in a soluble form. The particulate nature of serum acid phosphatase has been previously described by Polasek (15) who also noted that platelet factor 3 (PF3) is associated with these particles. Thus, acid phosphatase and PF3 are liberated from platelets by mechanisms other than by the platelet release reaction. Aspirin, a compound known to inhibit the release reaction induced by ADP, epinephrine, and low doses of thrombin (12, 16–18), had little effect on the release reaction induced by clotting, whether added *in vitro* or ingested prior to drawing the blood samples. This was probably due to the known ineffectiveness of aspirin towards the large amounts of thrombin (18) which are generated during the clotting process.

Summary. During clotting of PRP, 20 to 80% of the platelet serotonin, adenine nucleotides, β-*N*-acetyl-glucosaminidase, β-glucuronidase, and β-galactosidase were released to serum in a soluble form. Other enzymes, such as lactate dehydrogenase, pyruvate kinase, and hexokinase were retained. Small amounts of acid phosphatase were lost from the platelets in a particulate form. These results agreed well with those obtained previously on the action of thrombin on washed platelets. It is concluded that, during clotting, platelets do not lyse initially, but release only those substances stored in intracellular granules, a process previously described as the platelet release reaction.

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