

PLATELETS AS A SOURCE OF SERUM ACID NITROPHENYLPHOSPHATASE *

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(Submitted for publication August 1, 1958; accepted September 25, 1958)

Recent interest in serum enzyme activity in pathological conditions has led to renewed curiosity concerning the source of the enzymes present in serum under normal conditions. Since blood platelets contain acid phosphatase (1-8), it seemed possible that they might liberate this enzyme during blood coagulation. This possibility was investigated by comparing the acid phosphatase activity of serum prepared from platelet-rich and platelet-poor plasmas. Preliminary studies suggested that the platelets contribute virtually all of the activity of normal serum against β -glycerophosphate at acid pH (9). The present investigation was carried out using *p*-nitrophenylphosphate as substrate.

METHODS

Acid phosphatase activity was measured using *p*-nitrophenylphosphate¹ as substrate (10-12). This method gave highly reproducible values; for example, nine out of 10 determinations on a sample of fresh serum were 0.39 μ M per ml. per hour and the tenth was 0.41.

The subjects were either healthy young adults or, in a few instances, patients with metastatic carcinoma of the prostate.² To avoid the use of anticoagulants, native platelet-rich and platelet-poor plasmas were prepared by centrifugation of blood drawn in chilled siliconed syringes and cooled in an ice bath in siliconed tubes. Most of the red and white cells were sedimented by centrifuging for five to 10 minutes at about 1,000 rpm. Two ml. of the supernatant platelet-rich plasma was set aside in an ice bath and a platelet count taken. The remainder of the native blood and platelet-rich plasma was centrifuged in a multispeed attachment at about 10,000 rpm for 10 minutes to obtain platelet-poor plasma. About 1 ml. of each plasma was then placed in each of two glass tubes. One pair of plasmas was incubated at 37° C. and a second pair was placed at room temperature for one hour. The platelet-rich samples always clotted, but the platelet-poor samples often contained only traces of fibrin. Since spontaneous retraction did not usually occur, the clots were

removed with an applicator stick at the end of incubation, and acid phosphatase activity was measured. These samples are designated as "serum from platelet-rich plasma" and "serum from platelet-poor plasma."

The time course of acid phosphatase liberation was studied by placing 0.4 ml. of native platelet-rich plasma in each of a series of new glass tubes (rinsed with distilled water and oven-dried) at 37° C. and, at intervals, removing the platelets. In the samples which had not yet clotted, this was accomplished by brief high-speed centrifugation and the fibrin which formed subsequently in the platelet-poor plasma was removed with an applicator stick. In the samples which had clotted, the clot and entrapped platelets were removed together.

To determine whether anticoagulants inhibited enzyme activity, one-fifth volume of 0.1 M sodium oxalate, 0.11 M sodium citrate, or 0.027 M (1 per cent) disodium ethylenediaminetetraacetate in saline (EDTA) was added to serum separated from native platelet-rich plasma or from whole blood. To study the effect of the anticoagulants on the color of nitrophenol, saline was used instead of serum. The activity of whole platelets was tested by comparing the acid phosphatase activity of platelet-rich and platelet-poor plasmas separated from blood in which clotting was prevented by the addition of one-ninth volume of anticoagulant. Other studies were made on washed platelets, obtained by differential centrifugation of blood collected in EDTA and washed three times with isotonic saline at 4° C. in a multispeed attachment. A platelet count of about 500,000 per cu. mm. on the saline platelet suspension was appropriate for the measurement of nitrophenylphosphatase activity when 0.2 ml. of suspension was used. All platelet counts were done with a phase microscope (13).

RESULTS

In contrast to the results with β -glycerophosphate (9), substantial activity was found in the serum from native platelet-poor plasma (Table I). Identical values were obtained whether the platelet-poor plasma was allowed to incubate at 37° C. or at room temperature, indicating that the enzyme in this serum was not very heat labile. As found with glycerophosphate, serum prepared from native platelet-rich plasma had higher activity than that prepared from platelet-poor plasma. The liberation of platelet acid phosphatase was

* Supported by grants from the American Heart Association and the United States Army (DA-49-007-MD-673).

¹ Sigma Chemical Co., St. Louis, Mo.

² We are grateful to Dr. Waleed Maloof for selecting these patients.

TABLE I
Acid nitrophenylphosphatase activity of serum obtained from blood treated in various ways

Subject,* sex	Native platelet-poor plasma		Whole blood 23°	Native platelet-rich plasma		Platelet contribution† 37°
	37°	23°		37°	23°	
	$\mu\text{M/ml./hr.}$		$\mu\text{M/ml./hr.}$	$\mu\text{M/ml./hr.}$		$\mu\text{M}/10^9 \text{ platelets/hr.}$
1A ♀		0.05			0.27	
2A ♀	0.23	0.19	0.34	0.96	0.45	2.37
3A ♂	0.42	0.47	0.65	0.69	0.47	1.00
4A ♂	0.46	0.51	0.55	0.82	0.59	0.91
5A ♀	0.48	0.44	0.50	0.88	0.58	0.55
2B ♀	0.42	0.36		0.91	0.55	1.02
Patients with prostate carcinoma						
6A ♂	0.65		1.50	0.76		
6B ♂	0.65	1.46	1.63	0.74	1.75	
7A ♂	0.79	2.48	2.65	1.71	2.57	

* Each number designates a different subject. Each letter indicates a different blood sample.

† $\frac{\mu\text{M/ml./hr. in serum from platelet-rich plasma} - \mu\text{M/ml./hr. in serum from platelet-poor plasma}}{\text{Platelet count in platelet-rich plasma in } 10^9/\text{ml.}}$

consistently greater in the samples allowed to clot at 37° C. than in those clotted at room temperature. The amount of phosphatase contributed by the platelets was calculated by subtracting the activity of serum from platelet-poor plasma from the activity of serum from platelet-rich plasma clotted at 37° C. and dividing the

difference by the platelet count in 10^9 per ml. It ranged from 0.55 to 2.37 μM nitrophenol per 10^9 platelets per hour.

The time course of phosphatase liberation is shown in Figure 1. Since retraction did not usually occur, the values on the curve were determined on serum expressed from the clot with

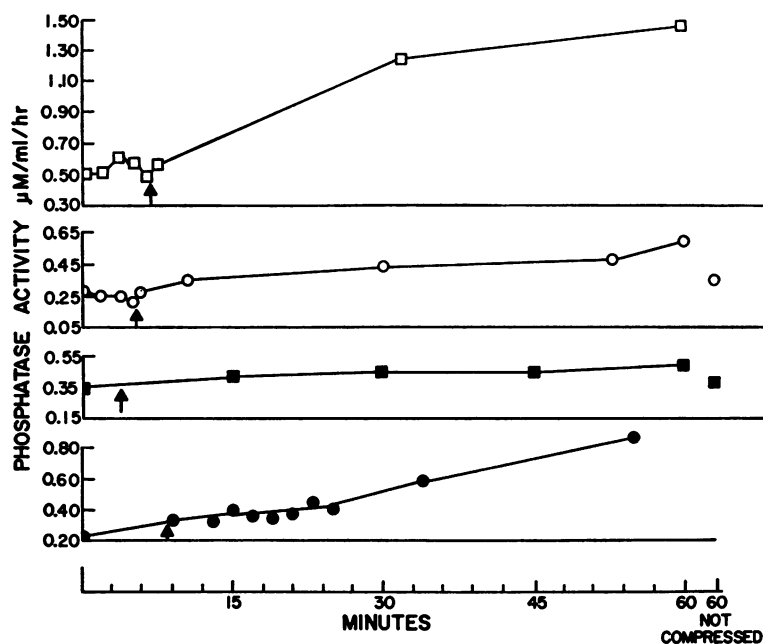


FIG. 1. ACID NITROPHENYLPHOSPHATASE ACTIVITY OF SERUM SEPARATED AT VARIOUS INTERVALS FROM NATIVE PLATELET-RICH PLASMA OF FOUR NORMAL SUBJECTS

Time of clotting is indicated by the arrow.

TABLE II

Acid nitrophenylphosphatase activity of platelet-rich and platelet-poor plasma from citrated, EDTA and oxalated blood

Subject	Time after venepuncture	Temperature	Citrated			EDTA			Oxalated		
			Platelet-rich plasma	Platelet-poor plasma*	Platelet-contribution	Platelet-rich plasma	Platelet-poor plasma*	Platelet-contribution	Platelet-rich plasma	Platelet-poor plasma*	Platelet-contribution
	hrs.	° C.	$\mu\text{M}/\text{ml.}/\text{hr.}$	$\mu\text{M}/\text{ml.}/\text{hr.}$	$\mu\text{M}/10^9/\text{hr.}$	$\mu\text{M}/\text{ml.}/\text{hr.}$	$\mu\text{M}/\text{ml.}/\text{hr.}$	$\mu\text{M}/10^9/\text{hr.}$	$\mu\text{M}/\text{ml.}/\text{hr.}$	$\mu\text{M}/\text{ml.}/\text{hr.}$	$\mu\text{M}/10^9/\text{hr.}$
1A	1.5	?	1.12	0.22	1.10						
2A	1.5	?	0.77	0.31	1.26	0.67	0.33	0.89	1.11	0.29	2.22
3A	1.25	4	0.57	0.38	0.34	2.63	0.43	3.24	0.73	0.45	0.44
	3.5†	4				0.66	(0.43)	0.34	0.59	(0.45)	0.23
4A	1	?	0.80	0.43	1.06	1.37	(0.43)	1.83			
2B	10 min.	24	0.74	(0.39)	0.91	1.19	(0.39)	2.05			
	6†	4	0.47‡	(0.39)	0.15‡	0.45‡	(0.39)	0.15‡			
2C	10 min.	4	0.45	0.34	0.20	0.50	0.32	0.46			
	10 min.	24				0.45	0.26	0.49			

* Values in parentheses represent activity of serum from platelet-poor plasma.

† Time after first determination.

‡ Values after freezing once were 2.42, 5.36, 3.41 and 7.82 $\mu\text{M}/\text{ml.}/\text{hr.}$, respectively.

an applicator stick. No liberation of phosphatase was evident immediately after clotting had occurred (indicated by the arrow) but the serum activity increased progressively thereafter. In samples in which the clot was freed from the wall of the tube, allowed to retract, and removed without compression, serum phosphatase activity even at one hour showed little increase over the value obtained when the platelets were removed prior to clotting.

The activity of ordinary serum, separated by centrifugation from whole blood allowed to remain at room temperature for one hour, was

usually only slightly higher (4 to 32 per cent) than that of serum from platelet-poor plasma (Table I). Activity was slightly or not at all increased by shaking the blood with glass beads during clotting.

The nitrophenylphosphatase activities of platelet-poor plasma from citrated, EDTA or oxalated plasma (Table II) showed no significant differences from each other or from serum prepared from native platelet-poor plasma from the same subject (Table I). Addition of citrate or EDTA to serum had no effect on acid nitrophenylphosphatase activity in two experiments;

TABLE III

Acid nitrophenylphosphatase activity ($\mu\text{M}/10^9$ platelets/hr.) of washed platelets treated in various ways

Subject	0.9% NaCl, unfrozen	0.9% NaCl, frozen	0.45% NaCl, frozen		Platelet-poor plasma,* 37° C.	Viscous metamorphosis	
			Super-natant	Sedi-ment		Super-natant	Sedi-ment
1A	3.18		0.42	4.50		0.28	2.29
2A	1.86		2.59	6.00		0.10	1.50
3A	1.52		1.06	5.40	1.30	0.06	0.86
4A	6.54		3.90	8.40	1.00†	0.46‡	1.98‡
5A	1.53	9.31	1.58	5.86			
2B	5.12	14.40	2.48	7.63			
2C	2.48	9.58		7.50§			1.47§
2D	3.66	12.90					
Patients with prostate carcinoma							
6B	5.09	10.30	1.98	6.18		0.28	2.28
7A	5.63		5.38	9.88			
7B		3.22					

* Activity of serum from platelet-poor plasma subtracted from that of serum obtained after adding platelets, then divided by number of platelets.

† 1.00 when incubated at room temperature.

‡ Values were 0.40 and 2.26, respectively, when incubated at 37° C. rather than at room temperature.

§ Activity of entire sample, not separated into supernatant and sediment.

oxalate decreased the activity 18 per cent in a normal male subject but had no effect in a female subject. None of the anticoagulants affected the color developed by nitrophenol itself. The activity of platelet-rich plasma was always higher than that of platelet-poor plasma (Table II) but it varied in an unpredictable manner. For example, in Samples 3A and 2B, EDTA platelet-rich plasma had much more activity than citrated platelet-rich plasma, but this high activity disappeared after three to six hours at 4° C. However, in another experiment, 2C, on one of the same donors, this difference between citrated and EDTA samples was not evident even when the determinations were made very promptly on chilled blood. Because of this variability, the calculated activity per 10⁹ platelets in platelet-rich plasma was sometimes lower and sometimes higher than it was in serum from native platelet-rich plasma.

Even after 30 minute incubation of platelet-rich plasma in the buffered substrate, the platelets appeared morphologically intact, with processes but no balloons or vesicles. Thus, unless some of the platelet enzyme had leaked out of the platelets despite their morphologic integrity, unruptured, unwashed platelets can hydrolyze nitrophenylphosphate. When platelet-rich plasma was frozen and thawed once, the activity was higher than that of an unfrozen sample (Table II, footnote).

Acid nitrophenylphosphatase activity was determined on washed saline-suspended platelets treated in various ways. The results are shown in Table III. Suspensions of washed unfrozen platelets had higher acid phosphatase activity than suspensions of platelets which had not been separated or washed (*i.e.*, platelets in platelet-rich plasma), and an increase of several-fold was observed after freezing and thawing the saline suspension once. The platelets were separated into supernatant and sediment by centrifuging for five minutes at 2,000 rpm after they had been frozen and thawed in 0.45 per cent saline. The sediment was resuspended in the original volume of saline. The activity of the supernatant and sediment was somewhat less than that of platelets frozen in isotonic saline, whether it was determined by adding the values for supernatant and sediment or by measuring the activity

without separating the two fractions (Experiment 2D). The activity of the supernatant was between 9 and 32 per cent of the total activity and was approximately equal to the activity contributed by the platelets to serum from platelet-rich plasma.

Release of acid phosphatase activity from washed platelets during clotting was studied by adding them to native platelet-poor plasma which was allowed to incubate for 60 minutes at 37° C. before removing the clot and entrapped platelets. The activity liberated was almost identical with that liberated from unwashed platelets during the clotting of native platelet-rich plasma (*cf.* Table I). In other words, washing the platelets did not affect the amount of phosphatase liberated during clotting at 37° C. However, washing did facilitate phosphatase liberation at room temperature; when washed platelets were used, the same amount of phosphatase was liberated whether clotting took place at room temperature or at 37° C., whereas a much smaller amount was liberated from native platelet-rich plasma when it was clotted at room temperature rather than at 37° C.

Studies were also carried out on platelets which had undergone viscous metamorphosis (14) (Table III). To five volumes of platelets suspended in saline were added three volumes of tris-maleate buffer (pH 7.4) and two volumes of human thrombin (about 5 units per ml.) (15). After a few minutes at room temperature with occasional agitation, the platelets clumped, and soon thereafter, the platelets in the clumps fused together. After 60 minutes, the samples were separated into supernatant and sediment and the sediment was resuspended in saline. The activity of the supernatant was much less than either the activity of the supernatant obtained after freezing the platelets in hypotonic saline or the activity contributed to the serum by the platelets during clotting. The activity of the sediment was much less than that of frozen platelets, and often less than that of unfrozen washed platelets, perhaps because the platelets in the resuspended sediment were clumped.

Two patients with metastatic carcinoma of the prostate gland were studied who had elevated acid phosphatase values as determined on ordinary serum (Table I). The levels in serum

from platelet-poor plasma maintained at room temperature were above the maximum level which we observed in normal individuals ($0.51 \mu\text{M}$ per ml. per hour). Thus, the prostate enzyme is not bound to platelets but is presumably in the plasma. The values for serum from platelet-poor plasma incubated at 37°C . were lower than those on serum from the same plasma incubated at room temperature, demonstrating the well-known heat lability of the prostate enzyme in serum (16, 17). Values for serum from platelet-rich plasma incubated at 23°C . were only slightly higher than those for serum from platelet-poor plasma, since liberation of the enzyme from the platelets is minimal at this temperature. Liberation of the platelet activity at 37°C . could not be quantified because of the destruction of the prostate enzyme at this temperature. The activity of acid phosphatase in washed whole platelets from two patients with elevated prostatic serum phosphatase levels was not abnormal (Table III).

Studies with inhibitors were carried out on frozen saline-suspended platelets of normal Subject 2 and Patient 6B with carcinoma of the prostate gland. L-Tartrate (18) produced 14 and 7 per cent inhibition, and formaldehyde (18) caused 81 and 76 per cent inhibition, respectively. Magnesium (0.01 M) inhibited the normal platelets 14 per cent.

DISCUSSION

Preliminary studies (9) indicated that virtually no activity against β -glycerophosphate was found in serum prepared from platelet-poor plasma, whereas values up to 0.49 Bodansky units (mg. P per 100 ml. per hour) were found in serum from platelet-rich plasma. These data suggested that the platelets are the major source of normal serum acid glycerophosphatase activity. When *p*-nitrophenylphosphate was used, serum from native platelet-poor plasma had some activity, indicating that the platelets were not the only source of serum activity against this substrate. However, greater activity was always observed in serum from platelet-rich plasma, especially after incubation for one hour at 37°C . In such serum, between 39 and 76 per cent of the activity was derived from the platelets. In ordinary serum, separated from whole blood centrifuged after remaining for one hour at room

temperature, less than 32 per cent of the activity was attributable to the platelets.

Acid glycerophosphatase activity has been previously described in rabbit (1), horse (2), calf (3) and human (4, 7) platelets. Platelet acid phosphatase was unaltered during menstruation (4), but reduced in subjects with hepatic cirrhosis (5) or receiving dicumarol (6). Our results and those of Morita and Asada (8) indicate that human platelets are able to hydrolyze nitrophenylphosphate as well as glycerophosphate. The range of values for both frozen and unfrozen platelets of normal subjects is quite wide.

Platelet acid nitrophenylphosphatase is inhibited to a considerable degree by formaldehyde, but very little by L-tartrate or magnesium. In these respects, it behaves like the enzyme in normal serum (18, 19). Others have shown that L-tartrate inhibits acid phosphatase from prostate, liver and spleen, but not the normal serum enzyme (18). Formaldehyde partially inhibits the enzyme in serum and in several tissues, but completely inhibits the enzymes in red blood cells (18). Magnesium has little effect on acid phosphatase from most sources but may activate one of the enzymes in the red cells (19).

Investigation of factors controlling liberation of platelet acid phosphatase and perhaps other platelet enzymes (9) may shed light upon platelet function. Less than 25 per cent of the total platelet nitrophenylphosphatase activity is liberated into the serum when native platelet-rich plasma clots at 37°C . and the clot is compressed one hour later. Over two-thirds of the platelet activity against nitrophenylphosphate is insoluble and remains in the sediment after freezing the platelets in a hypotonic medium. The activity liberated during clotting is equal to between 23 and 94 per cent of the activity of the soluble platelet fraction. Liberation of enzyme from platelets occurs progressively during the hour following clotting. Both serotonin (20, 21) and potassium (22) are also liberated from the platelets during clotting, but their release occurs more rapidly than that of acid phosphatase. Although thrombin liberates all of the serotonin from washed platelets suspended in buffer (21), less phosphatase activity is liberated under these conditions than by clotting or freezing. This suggests that

viscous metamorphosis produced in this manner does not fully reproduce the platelet changes which occur after clotting of platelet-rich plasma.

Even after thorough washing, human platelets contain serotonin (21), factor V (accelerator globulin) (23) and fibrinogen (24). There is good evidence that the former two substances are taken up from the environment and bound to the platelets (23, 25), and it seems probable that platelet fibrinogen is acquired in a similar manner. Hence, one wonders whether platelet acid phosphatase has originated elsewhere and is simply carried by platelets. Since acid phosphatase can be demonstrated histologically in the cytoplasm of megakaryocytes (26), it seems unlikely that the circulating platelets take up the enzyme.

In patients with metastatic carcinoma of the prostate gland and elevated serum acid phosphatase, acid nitrophenylphosphatase activity in serum from platelet-poor plasma was above the normal level. Thus, the enzyme was presumably present in the circulating plasma. There is no evidence that the prostate enzyme becomes bound to the platelets.

SUMMARY

A significant proportion of serum enzyme activity against *p*-nitrophenylphosphate at acid pH originates from platelets and is liberated during clotting. Suspensions of intact washed platelets in saline and of unwashed platelets in platelet-rich plasma have acid nitrophenylphosphatase activity. Greater activity is observed after freezing and thawing these suspensions. Some of the factors influencing platelet liberation of acid phosphatase have been investigated. In patients with elevated serum acid phosphatase activity attributable to metastatic carcinoma of the prostate, the abnormal enzyme is presumably in the circulating plasma, since the activity of serum prepared from platelet-poor plasma is elevated, and since platelet acid phosphatase activity is normal.

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