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An Enzymatic Method to Determine Damage in Human Platelets.* (32168)

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Platelet concentrates (PC) are frequently prepared for metabolic and clinical studies by centrifugation of platelet-rich plasma (PRP). This step, and subsequent resuspension of the platelets in about 10 ml of plasma, often causes platelet clumping which may be associated with platelet damage. Acid phosphatase is released from platelets during clotting(1,2), and measurement of released enzymes may possibly provide a quantitative means of assessing platelet damage during preparation of PC. The objectives of this investigation were to find a simple *in vitro* method of detecting platelet damage, and use it to determine whether the degree of damage was affected by the nature of the anticoagulant and, if so, under what conditions it could be minimized.

Methods. Platelet-rich plasma was obtained by centrifuging one unit (about 500 ml) of whole blood containing anticoagulant acid-citrate-dextrose* (PRP-A) or citrate-phosphate-dextrose(3) (PRP-C) in a triple plastic bag (Fenwal Company) at 10°C at 2500 × *g* for 100 seconds, and was expressed into the satellite bag. Experiments were performed on pools of 2 to 4 units of PRP obtained from blood of the same group and type, redivided into equal parts (volume approximately 200 ml). When necessary, acid or adenosine was added to each bag, mixed well, and the pH measured at 10°C. In experiments which included both additives, acid was introduced first, mixed and followed by adenosine. For the preparation of PC, PRP was then centrifuged at 5100 × *g* for 10 minutes at 10°C. The platelets were resuspended in 10-15 ml of the supernatant plasma to obtain PC(4). One ml of the suspension was centrifuged in a plastic tube at 0°C at 35,000 × *g* for 10 minutes. Activity of the enzymes nucleoside diphosphokinase (NDPK), 3-phosphoglycerate kinase (PGK), and enolase was determined in the supernatant so that the amount of enzyme released to the plasma in the PC could be calculated.

Total enzyme content of PRP was determined in lysed platelets. For lysis, 10 ml of PRP was centrifuged at 0°C at 35,000 × *g* for 10 minutes, the plasma was removed and the platelets lysed with 1.35 ml of distilled water. Buffer (0.15 ml of 1.0 M Tris-acetate, pH 7.5) was added, and the lysate was centrifuged at 35,000 × *g* for 10 minutes at 0°C. Enzyme activity was measured in aliquots of the supernatant. We assayed for enzymes which can be measured directly or indirectly by oxidation or reduction of DPN or TPN, and found detectable amounts of the following in the platelet lysates: NDPK, PGK, enolase, lactic dehydrogenase, pyruvate kinase, adenosine triphosphatase, glutathione reductase, hexokinase, glucose-6-

* NIH Formula A.

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phosphate dehydrogenase, and hexose isomerase. Of these, NDPK, PGK and enolase were present in large amounts but were not detectable in fresh platelet-poor plasma.

Enzyme activity measurements were carried out at 30°C by following the decrease in absorbency at 340mμ in a Beckman DU Spectrophotometer equipped with a Gilford converter and a Honeywell recorder. NDPK was measured according to the method of Mourad and Parks(5) except that 4×10^{-4} M thymidine-5′-diphosphate was substituted for deoxyguanosine-5′-diphosphate. The assay for PGK was similar to that employed by Bücher(6). One ml of reaction mixture contained: 2.25 μmoles ATP; 3.5 μmoles 3-phosphoglycerate; 40 μg glyceraldehyde-3-phosphate dehydrogenase; 0.1 μmole DPNH; 25 μmoles $MgCl_2$; 10 μmoles KCl, and 0.15 mmole Tris-acetate buffer, pH 7.5. Enolase was determined by a modification of the assay for phosphoglycerate(7), using the coupled pyruvate kinase-lactate dehydrogenase reaction to measure the formation of phospho(enol) pyruvate from 2-phosphoglycerate. One ml of reaction mixture contained: 3.0 μmoles 2-phosphoglycerate; 1.0 μmole ADP; 2.0 μg pyruvate kinase; 10 μg lactic dehydrogenase; 0.1 μmole DPNH; 25 μmoles $MgCl_2$; 10 μmoles KCl; 0.15 mmole Tris-acetate buffer, pH 7.5. The reaction was usually started by adding appropriate aliquots of plasma or of lysed platelet supernatant containing enzymes.

One unit of enzyme activity is the amount of enzyme which converts 1.0 μmole of substrate per minute at 30°C.

The 3 enzymes are also present in red blood cells (RBC), but the concentration per unit volume is only one-tenth as high as in platelets. In these samples, the RBC in PC ranged from 1-5% of the total cell count. Since a platelet has only one-eighth the volume of a red cell(8), the contribution of RBC enzymes to the lysed platelets should not exceed 4%.

Results and discussion. Total units of each enzyme in 200 ml pooled PRP ranged from 80-110 for NDPK, 90-120 for PGK, and 9-12 for enolase, regardless of whether the anticoagulant was ACD or CPD. About 1 of 20 samples gave values outside this range. More enzyme was released during preparation of PC from CPD blood (pH 7.3) than from ACD blood (pH 6.9), the average release from 10 experiments being 19% as compared with 12%. The results of a typical experiment are shown in Table I. Platelet clumping was observed with both anticoagulants. Reduction of the pH of PRP-C from 7.3 to 6.8, 6.7 and 6.65 with 0.1 N HCl, 0.1 N lactic acid, or extra ACD solution caused a corresponding decrease in the averaged values for the amount of the three enzymes released in PC (Fig. 1). Similar results were obtained for PRP-A. Like enzyme release, platelet clumping was more marked at pH 7.3 than at pH 6.65.

TABLE I. Enzyme Release to the Plasma in Platelet Concentrates Prepared from ACD and CPD Blood.

	ACD plasma (pH 6.9)		CPD plasma (pH 7.3)	
Enzymes	Units*	%†	Units*	%†
NDPK	18	17	24.5	22
3-PGK	18.8	17	22.3	23
Enolase	1.84	16	2.5	17
% Averages		17		21

* Enzyme units released from platelets to the supernatant plasma in PC.

† Release as percentage of total amount of enzymes in lysed platelets.

The relationship between enzyme release and pH was altered by adding adenosine (Fig. 1). While 10^{-5} M adenosine reduced enzyme release at pH 6.9 to the value obtained at pH 6.65 in the absence of nucleoside, and prevented platelet clumping, 10^{-4} M adenosine was required to produce similar effects at pH 7.3.

Platelet clumping induced in PRP by exogenous ADP is optimal at pH 8 and inhibited either by lowering the pH(9) or by the addition of adenosine(10). Our results show that a lower pH or addition of adenosine diminishes both enzyme release and platelet clumping during the preparation of PC. This suggests that the macroscopic clumping in platelet concentrates is dependent on platelet ADP, and that enzyme release depends on physical damage to the platelet membrane incurred when platelets are processed into concentrates.

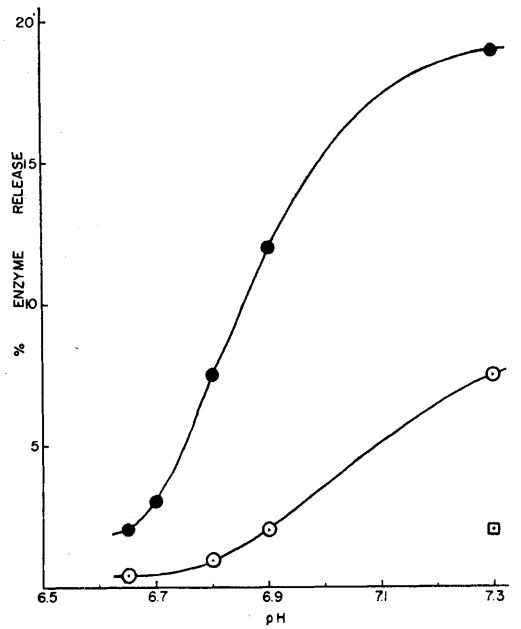

FIG. 1. Effect of pH and adenosine on enzyme release from platelets. Points represent average release in per cent of 3 enzymes. Each point represents 2-4 experiments. ● , no adenosine; ⊙ , 10^{-6}M adenosine; square with dot in it, + 10^{-4}M adenosine.

Summary. 1. Nucleoside diphosphokinase, 3-phosphoglycerate kinase and enolase are released from human platelets during the preparation of platelet concentrates. 2. Enzyme release is reduced by lowering the pH or by addition of adenosine. 3. Enzyme release is greater when the platelets in the concentrates are clumped.

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Measurement of Low Rates of Oxygen Consumption With a Horizontal Capillary-Differential Syringe Manometer.* (32169)

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The simplicity and accuracy of measurement obtainable with differential respirometers, especially where low rates of gas consumption are concerned, are decided advantages when this type of instrument is compared with conventional open limb manometers. Differential manometry is especially useful in systems where it is necessary to distinguish oxygen consumption by cells from a high rate of oxygen uptake by the suspending medium (sperm cells in seminal plasma, for example). Other applications and advantages of the differential techniques have been well described by Umbreit(1). This report describes a small differential syringe manometer with a 30 mm horizontal capillary which offers important advantages over

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