1.0

1.1

1.25

4.5
5.0
5.6
6.3

2.8

3.2

3.6

4.0

1.4

2.5

2.2

2.0

1.8

1.6

MICROCOPY RESOLUTION TEST CHART
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SIZE DEPENDENT PLATELET SUBPOPULATIONS: RELATIONSHIP OF PLATELET VOLUME

TO ULTRASTRUCTURE, ENZYMATIC ACTIVITY, AND FUNCTION

by

C. B. THOMPSON, K. A. EATON, S. M. PRINCIOTTA, C. A. RUSHIN,

AND

C. R. VALERI

NAVAL BLOOD RESEARCH LABORATORY
BOSTON UNIVERSITY SCHOOL OF MEDICINE
615 ALBANY STREET
BOSTON, MA 02118

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A method for the separation of platelets on the basis of their size has been developed using counterflow centrifugation. Platelets were separated, free of plasma proteins and other cells, into 7 subpopulations. The smallest-sized platelets, designated as Fraction 1, had a mean platelet volume (MPV) of $3.94 \pm .60$ $u^3$ (SD). Each successive fraction had a progressively larger MPV. The MPV for the largest-sized platelets, designated Fraction 7, was $8.19 \pm .64$ $u^3$. The MPV for the original platelets prior to fractionation was $6.57 \pm .61$ $u^3$.

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The mean density of Fraction 1 platelets was $1.067 \pm .002$ gm/$cm^3$, while Fraction 7 had a mean density of $1.072 \pm .001$ gm/$cm^3$. Transmission electron microscopy demonstrated that Fraction 1 had $4.3 \pm 0.9$ dense bodies per platelet, and Fraction 7 had $12.6 \pm 2.4$ dense bodies per platelet. Platelet LDH activity showed that the Fraction 1 platelets had $4.77 \pm 0.92$ IU per $10^{10}$ platelets; Fraction 7 platelets had $14.9 \pm 1.23$ IU per $10^{10}$ platelets. The LDH activity in the platelets before separation into subpopulations was $9.47 \pm 1.45$ IU per $10^{10}$ platelets.

Platelet function was measured by ADP-induced aggregation, serotonin uptake, and thrombin-induced release. Progressively more rapid and more complete aggregation was observed as the platelet size increased over the 7 fractions. Serotonin uptake was 4.2 times greater in the Fraction 7 platelets than in the Fraction 1 platelets. Quantitative release of serotonin following thrombin stimulation was significantly greater in the larger-sized platelets than in the smaller-sized platelets.

The observed differences in platelet aggregation, dense body content, LDH activity, and serotonin uptake and release suggest that large platelets may be functionally more important than smaller platelets.

# SUMMARY

*cu microns*

A method for the separation of platelets on the basis of their size has been developed using counterflow centrifugation. Platelets were separated, free of plasma proteins and other cells, into 7 subpopulations. The smallest-sized platelets, designated as Fraction 1, had a mean platelet volume (MPV) of 3.94 $\pm$ .60 $u^3$ (SD). Each successive fraction had a progressively larger MPV. The MPV for the largest-sized platelets, designated Fraction 7, was 8.19 $\pm$ .64 $u^3$. The MPV for the original platelets prior to fractionation was 6.57 $\pm$ .61 $u^3$. The mean density of Fraction 1 platelets was 1.067 $\pm$ .002 $gm/cm^3$, while Fraction 7 had a mean density of 1.072 $\pm$ .001 $gm/cm^3$. Transmission electron microscopy demonstrated that Fraction 1 had 4.3 $\pm$ 0.9 dense bodies per platelet, and Fraction 7 had 12.6 $\pm$ 2.4 dense bodies per platelet. Platelet LDH activity showed that the Fraction 1 platelets had 4.77 $\pm$ 0.92 IU per $10^{10}$ platelets; Fraction 7 platelets had 14.9 $\pm$ 1.23 IU per $10^{10}$ platelets. The LDH activity in the platelets before separation into subpopulations was 9.47 $\pm$ 1.45 IU per $10^{10}$ platelets.

Platelet function was measured by ADP-induced aggregation, serotonin uptake, and thrombin-induced release. Progressively more rapid and more complete aggregation was observed as the platelet size increased over the 7 fractions. Serotonin uptake was 4.2 times greater in the Fraction 7 platelets than in the Fraction 1 platelets. Quantitative release of serotonin following thrombin stimulation was significantly greater in the larger-sized platelets than in the smaller-sized platelets. — $\rangle$ *p. 3*

The observed differences in platelet aggregation, dense body content, LDH activity, and serotonin uptake and release suggest that large platelets may be functionally more important than smaller platelets.

# INTRODUCTION

Platelet heterogeneity has been noted for many years (Olef, 1936). Recent works of several investigators (Karpatkin, 1969a; Karpatkin, 1969b; Karpatkin and Charmatz, 1970; Charmatz and Karpatkin, 1974; Corash et al., 1977; Corash et al., 1978) have suggested that young platelets are denser, larger, and more functional than old ones when platelets were separated into subpopulations using density gradient techniques. The validity of these data has been questioned by Penington and associates (1976), who observed that platelets degranulate during density gradient separation and the measurements of platelet density, size, and function may reflect only the degranulation process. In addition, several reports (Mezzano and Aster, 1979; Boneu et al., 1973; Minter and Ingram, 1971; Busch and Olson, 1973; Paulus, 1975; Penington et al., 1976) have shown a lack of correlation between the density of platelets and their in vivo survival.

Irrespective of this contradictory data, the clinical relevance of platelet density determinations is limited. The methods for its measurement are cumbersome and difficult to standardize. However, recent advances in electrical cell sizing have made the mean platelet volume, a parameter analogous to the red cell MCV, routinely available in most clinical laboratories (Rowan et al., 1979). Most of the data on the significance of the mean platelet volume has been inferential because a method for size separation of platelets has never been developed.

We report here on a new method for the separation of platelets into 7 subpopulations on the basis of platelet volume using counterflow centrifugation, and on the relationship of platelet volume to the density, ultrastructure, enzymatic activity, and in vitro function of these 7 platelet fractions.

## METHODS AND MATERIALS

### Blood Collection and Platelet Isolation

The donors were 20 healthy volunteers, 12 males and 8 females, between the ages of 19 and 37. Twenty-five ml of blood were collected from each donor and transferred immediately to a 50 ml plastic tube (#25330, Corning Glass Works, Corning, N.Y.) containing 3.75 ml of anticoagulant citrate dextrose solution, USP, Formula A (ACD) (Travenol Laboratories, Deerfield, IL).

Platelet isolation was performed as follows: a 25 ml volume of blood collected in 3.75 ml of ACD was diluted with a 25 ml volume of a solution containing phosphate-buffered saline and albumin (PBS-A) (105.5 mM NaCl, 12.8 mM $Na_2HPO_4$, 2.8 mM $KH_2PO_4$, 1.3 mM $Na_2EDTA$, 15% V:V ACD, 0.5% W:V fatty acid free bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 3 M NaOH to titrate pH to 6.5, osmolarity 308 mOsm/L). The diluted blood was separated into 2 equal volumes and transferred to two 50 ml plastic tubes; the volume in each tube was centrifuged at 160 X g for 7 minutes (McBride, 1968). The platelet-rich supernatant was removed with a plastic pipette. The red cell concentrate was resuspended in 25 ml of PBS-A, after which each tube was centrifuged again and the supernatant solution removed from each tube. The platelet-rich supernatants were pooled and acidified to pH 6.5 with ACD. The acidified platelet suspension was separated into 2 equal volumes and transferred to two tubes and centrifuged at 700 X g for 15 minutes. The supernatant solution was removed. The platelet pellet was resuspended in 10 ml of PBS-A, and the platelet suspension was centrifuged at 700 X g

for 15 minutes. The supernatant solution was removed. Finally, the platelets were resuspended in 3.5-4.5 ml of PBS-A. The average recovery of platelets from whole blood by this method was 81%.

## Counterflow Centrifugation (Elutriation)

The platelets were separated into 7 subpopulations using a Beckman Centrifuge #J21B with an elutriator rotor (#JE6) and a Sanderson cell separation chamber (#335206). The rotor was disassembled and thoroughly rinsed daily. The chamber was stored overnight in dilute NaOH for cleaning and was coated once or twice a month with Prosil-28 (PCK Research Chemicals, Inc., Gainesville, FL). All separations were done in PBS-A at 20 C and 3500 rpm. The separation chamber was loaded directly through a 3-way stopcock using a plastic syringe.

The subpopulations were separated as follows: Between 3.5 and 4.5 ml of platelet suspension (0.8-1.2 X $10^9$ cells/ml) were loaded and the stopcock was flushed with 1 to 2 ml of buffer. The blood sample was allowed to flow into the chamber at a rate of 1 ml/minute for 4 minutes, after which the rate was increased to 2.5 ml/minute for 3 minutes, then to 3 ml/minute for 3 minutes, and then progressively to 3.5, 4.0, 5.0, 6.0, 7.0 and 8.0 ml/minute each for 2 minutes. The eluted sample at each of the last 7 flow rates was collected and labeled sequentially for a total of 7 subpopulations. The rotor and pump were stopped, and the separation chamber was disconnected. The cells in the chamber were collected into a plastic syringe through an 18-gauge needle. The platelets in each of the subpopulations were counted and sized.

## Platelet Counts and Sizing

Platelets in the whole blood and in the platelet suspension were counted by phase microscopy using an improved Neubauer hemacytometer, 0.1 mm deep (American Optical Co., Buffalo, NY) and a Phase-Star microscope (American Optical Co.) on 40X phase (Brecher and Cronkite, 1950). Duplicate counts were made by two observers. White blood cells and red blood cells present in the platelet subpopulations were counted by phase microscopy.

Before and after fractionation of the subpopulations, triplicate platelet counts were done electronically in a Coulter ZBI counter (Coulter Electronics, Hialeah, FL) using a 70 micron aperture and settings as follows: amplitude 1/2; aperture 1/2; gain 10; matching switch 20K; lower threshold 6; upper threshold 77.

Platelets were sized on a linear scale using a Coulter ZB counter with an H4 channelyzer attachment and a 50/60 aperture. Settings used were: amplification 1; aperture current 0.354; gain 10; matching switch 20K; lower threshold 5; upper threshold 100. Channelyzer settings were: 50 micron resolution, 1-6 exclusion threshold. At these settings latex particles of a diameter of 2.02 microns were counted in channel numbers 60-62, with a scale factor of 0.07 by the H4 program. Forty thousand-eighty thousand cells were sized in PBS (10 mM phosphate, 3 mM potassium, 305 milliosmolar, pH 7.4) at a concentration of about 100,000 cells/ml. In compiling sizing data, we used only those particles with volumes between 2 and 15 cubic microns at the above settings.

## Removal of Protein

The plasma protein removed during the counterflow centrifugation

procedure was evaluated by adding ^{125}I-labeled albumin to the blood prior to processing and measuring the ^{125}I in each of the isolated platelet fractions.

## Platelet LDH Activity

A volume containing $2 \times 10^8$ platelets from each subpopulation was placed in a 12 ml plastic tube (#2059, Falcon B-D, Oxnard, CA), centrifuged at 2000 X g for 30 minutes, and the supernatant removed and discarded. Each platelet pellet was resuspended in a 2 ml volume of distilled water and sonicated at 0 C using 25 watts for two 30-second pulses (Lab-Line Ultratip Labsonic System #9100, Tip #MT-1, Lab-Line Instruments, Melrose Park, IL). Each fraction was then centrifuged to remove particulate material, and supernatant LDH was assayed using a Multistat III Analyzer (Instrumentation Laboratories, Lexington, MA) by measuring at 25 C the conversion of NAD to NADH at 340 nm in the presence of lactate (Wacker et al., 1956). All measurements were performed in duplicate and corrected for the absorbance of the reagent.

## Platelet Density Studies

Platelet density was studied using a modification of the albumin density gradient described by Charmatz and Karpatkin (1974). Five hundred grams of bovine serum albumin (BSA), Fraction V (Sigma Chemical Co., St. Louis, MO) were added to 1 liter of distilled water containing 2 g of dissolved NaCl. The mixture was stirred under vacuum until the BSA was dissolved, and the solution with a pH of 6.8, 305 mOsm/kg $H_2O$, and a density of $1.0940 \pm 0.0018$ was stored at -80 C in 50 ml sterile centrifuge tubes (Corning Glass Works, Corning, NY) until it was used.

High density BSA (density 1.085, 87.5% albumin solution, 12.5% PBS-A) and low density BSA (density 1.044, 40% albumin solution, 60% PBS-A) solutions were used to make continuous linear gradients using an LKB 11300 Ultragrad gradient mixer (LKB Products, Bromma, Sweden) and an AutoAnalyzer proportioning pump (Technicon Instruments, Woburn, MA). Nine ml gradients were layered in plastic tubes (#2059) onto 1.2 ml cushions of high density albumin at 0.6 ml/minute (AutoAnalyzer pump tubes (#116-0532P09), Technicon) for 15 minutes.

Three ml of platelet suspension (5 X $10^7$ cells/ml in PBS-A) were layered on gradients and centrifuged at 1500 X g for 60 minutes. The centrifuge was stopped without braking. The gradients were carefully removed in 1 ml fractions by puncturing the bottom of the tubes with a 19-gauge needle and pumping out the contents at 1 ml/minute using an AutoAnalyzer proportioning pump (Technicon) and a 7000 Ultravac Fraction Collector (LKB Products). Electronic platelet counts were made on each fraction using the Coulter counter. The density of the fractions was measured using a DMA 45 digital density meter (Mettler-Paar, Heightstown, NJ).

## Electron Microscopy of Platelets

Autologous plasma was used for resuspension of the platelets in the original sample and the platelets in each of the 7 subpopulations. Two hundred mesh copper grids (Athene type, Polysciences, Warrington, PA) coated on the dull side with formvar in ethylene dichloride, 0.25% (Ernest F. Fullam, Inc., Schenectady, NY) and carbon were used to prepare platelet whole mounts for dense-body quantitation by electron microscopy (Costa et al., 1974). Ten microliters of the specimen were placed on

the coated side of of the grid. The grid was then blotted with #4 filter paper (Whatman, Ltd., England) to leave only a thin film. The grids were coded and the dense bodies were quantitated at 5000 X using a transmission electron microscope (JEM 100B, Jelco, Inc., Bedford, MA). Dense-body counts were performed on 100 platelets in each smear by a technician who was not aware of the significance of the coding system.

## Platelet Aggregation Studies

Platelet aggregation was measured photometrically by a modification of the method described by Born (1962). Platelet subpopulations were obtained as above. Additional blood was anticoagulated with sodium citrate (B-D, Rutherford, NJ) and centrifuged at 1800 X g for 10 minutes to obtain platelet-poor plasma (PPP). The pH of the plasma was adjusted to 6.5 with 3N HCl. Platelets were removed from PBS-A as previously described and the autologous PPP was used to resuspend the platelets in the original sample and in each of the 7 subpopulations to a platelet count of 2 X $10^5$/mm$^3$. To account for differences in optical density, each sample was adjusted to a baseline that set the optical density of the platelets in plasma at 10% light transmission. For all samples platelet-poor plasma was set at 90% light transmission.

Platelet response to 1, 10 and 25 micromolar concentrations of ADP (Calbiochem-Behring Corp., La Jolla, CA) was measured at 37 C with a Model 300 Chrono-Log platelet aggregometer (Chrono-Log Corp., Broomall, PA) using a teflon-coated stir bar at 1200 rpm. Measurements were made of the initial rate of aggregation and the maximum percent change in light trans-mission of the aggregation pattern. All samples were kept at room temperature

and then equilibrated at 37 C for 3 minutes prior to testing. The final pH of the platelet suspension was adjusted to pH 7.4 by adding 3N NaOH.

## $^{14}C$ Serotonin Uptake and Release Assay

The platelets were isolated from 25 ml of ACD blood. A 10 ml volume of normal saline was added to the ACD blood, the mixture was centrifuged at 225 X g for 7 minutes, and the platelet-rich supernatant was removed with a plastic pipette. This process was repeated three more times, yielding approximately 30-35 ml of platelet-rich plasma. The platelet-rich supernatant was incubated with 2.6-2.8 X $10^{-6}$ M $^{14}C$-5-HT (hydroxytryptamine binoxalate, 51.5 mCi/mmole, New England Nuclear, Boston, MA) at 37 C for 60 minutes in a Dubnoff metabolic incubator agitated at 75 strokes/minute.

To determine serotonin uptake a 0.5 ml volume of platelet suspension was centrifuged at 7000 X g for 2 minutes in a Fisher Model 59 microfuge. The radioactivity in the supernatant was compared to that in the platelet suspension to determine $^{14}C$-5-HT uptake.

To separate the labeled platelets into subfractions, ACD was added to the labeled platelet suspension to achieve a final concentration of 10%, and the platelets were centrifuged at 2000 X g for 15 minutes in a Sorval 22 C centrifuge (duPont Instruments, Newtown, CT). The platelets were then resuspended in PBS-A prior to separation by counterflow centrifugation. Each of the 7 platelet subpopulations was concentrated by centrifugation at 2000 X g for 30 minutes to remove the PBS-A. The platelets were resuspended to a count of 5 X $10^5$/mm$^3$ in tris-saline-glucose buffer (TSG) (0.9% NaCl containing 0.5% glucose in 0.2 M Tris-HCl at a pH of 7.4). A 0.5 ml aliquot from each platelet fraction was placed in a 1 ml poly-

ethylene centrifuge tube. The $^{14}$C serotonin labeled platelets were stimulated by adding 0.02 ml aliquots of thrombin (Robblee et al., 1979). Final thrombin concentrations were 0.0088, 0.1489, 0.2885 and 0.3846 units per ml. Control samples were prepared using 0.02 ml of saline as the platelet stimulant.

Each of the samples was incubated for 5 minutes and then centrifuged at 7000 X g for 2 minutes in a Fisher Model 59 microfuge. A 0.100 ml aliquot of the supernatant was placed in a scintillation vial and mixed with Aquasol 2 (New England Nuclear, Boston, MA). Beta emissions were counted on a Mark III 6880 liquid scintillation system (Tracor Analytic, Elk Grove Village, IL). All counts were performed at least in triplicate and corrected for background activity.

## RESULTS

Fraction 1 contained the smallest platelets with a mean volume of 3.94 $\pm$ .60 u$^3$ (SD) (Table 1). Fractions 2 through 7 contained progressively larger platelets; the platelets in Fraction 7 were the largest, with an average volume of 8.19 $\pm$ .64 u$^3$. The average size of the original platelet suspension was 6.57 $\pm$ .61 u$^3$ (Table 1).   TABLE 1

The flow rate of the PBS-A solution through the separating chamber correlated significantly with the MPV of the platelet subpopulation. The correlation coefficient between flow rate and mean platelet volume was 0.982 $\pm$ 0.016 for the 15 experiments. We were able to accurately and reproducibly isolate the 7 fractions of platelets with increasing platelet volumes by controlling the rate of flow of the PBS-A solution through the separating chamber. The size distribution and MPV were not altered by the counterflow centrifugation procedure: before isolation of the subpopulations the MPV was 6.57 $\pm$ .61 u$^3$; the MPV was 6.47 $\pm$ .57 u$^3$ for the pooled subpopulations in 8 experiments (Figure 1). There were no   FIG. 1 platelet aggregates seen in any of the fractions.

The average recovery of platelets in the subpopulations was 96.4 $\pm$ 4.3%. The overall recovery of whole blood platelets in the 7 subpopulations was 78%. Approximately 99.97% of the iodinated albumin added to the blood sample was removed during the separation procedures prior to isolation of the 7 subpopulations. Most of the red cells and all of the white cells present in the platelet suspension before separation into sub-populations were subsequently found in the separation chamber (Figure 2).   FIG. 2 No white blood cell contamination was observed in any of the subpopulations and red blood cell contamination was less than 1 RBC per 1,000 platelets.

Platelets with a larger platelet volume had slightly higher platelet density (Table 2): In 5 experiments there was a statistically significant difference in mean platelet density between Fraction 1 (1.067 $\pm$ 0.002) and Fraction 7 (1.072 $\pm$ 0.001) (p<0.01) (Table 2).

Transmission electron microscopy showed that the Fraction 1 platelets with the smallest volume had a mean of 4.3 $\pm$ 0.9 dense bodies per platelet, and Fraction 7 platelets with the largest volume had a mean of 12.6 $\pm$ 2.4 dense bodies per platelet (Table 3). The platelets in the original platelet suspension had a mean of 8.7 $\pm$ 1.3 dense bodies per platelet. The number of dense bodies per cubic micron of platelet cytosol also was increased in the platelets with the largest volume (Table 3).

Table 4 reports LDH activity per platelet and per volume of platelet cytosol. The LDH activity was 4.77 $\pm$ 0.92 X $10^{10}$ IU per platelet for the platelets with the smallest volume (Fraction 1), 14.88 $\pm$ 1.23 X $10^{10}$ IU per platelet for the largest platelets (Fraction 7), and 9.47 $\pm$ 1.45 X $10^{10}$ IU per platelet for the original platelet suspension. The same patterns were observed when the LDH activity was reported per volume of platelet cytosol (Table 3).

Figure 3 reports the rate and magnitude of aggregation for the original platelet suspension and the 7 platelet fractions exposed to 10 uM ADP. The initial rate of aggregation and the maximal percent light transmission were lower in platelets with smaller mean platelet volumes (Fractions 1, 2 and 3) than in the original platelet suspension, and were higher in the platelets with greater mean platelet volumes (Fractions 5, 6 and 7) (Figure 3). A concentration of 1 uM ADP produced only a change in

shape in each of the 7 fractions and in the original platelets; a concentration of 25 uM ADP produced full scale aggregation patterns in all 7 of the fractions and in the original platelet population.

In another phase of the study, platelets were labeled with $^{14}$C-serotonin before separation into 7 subpopulations. The serotonin uptake was greater for the larger platelets than for smaller platelets; this was true whether serotonin uptake was reported per platelet or per cubic micron of platelet cytosol (Table 5). The serotonin uptake of the original   <u>TABLE 5</u> platelet suspension was 7.62 $\pm$ 1.37 cpm/10$^4$ platelets; uptake of the smallest platelets was 3.60 $\pm$ 0.40 cpm/10$^4$ platelets and for the largest platelets 15.20 $\pm$ 1.24 cpm/10$^4$ platelets. No loss of platelet serotonin into the supernatant during separation was detected.

Treatment of $^{14}$C-labeled platelets with increasing concentrations of thrombin caused a greater serotonin release in the larger platelets than in the smaller platelets (Figure 4). Fraction 7, containing the largest   <u>FIG. 4</u> platelets, released 4.2 times more serotonin than did Fraction 2. When each of the 7 fractions was treated with saline instead of thrombin, the release of serotonin was similar for all fractions.

## DISCUSSION

The principle of counterflow centrifugation utilized a centrifugal force opposed by a flow force to separate particles. The velocity of flow necessary to remove a particle from the centrifugation chamber is proportional to:

$$\frac{r^2 \cdot g \cdot (P_p - P_m)}{k \cdot n}$$

where $r$ = particle radius, $g$ = centrifugal force, $P_p$ = density of the particle, $P_m$ = density of supporting medium, $k$ = particle shape, $n$ = viscosity of support medium. By selecting a support medium with a low density relative to the densities of the particles to be separated, the separation is influenced primarily by the size of the particle (Lindahl, 1948).

Counterflow centrifugation proved to be a highly reproducible method to separate platelets into 7 fractions by differences in platelet volume. In our studies the overall recovery of human platelets in the 7 subpopulations was 78%. This value compared favorably to the recovery values of Corash and associated (1977, 68%) and that of Penington and associated (1976, 79%) using density separation technics. The mean values of platelet volume, density, dense body content, enzymatic activity of LDH, platelet aggregation to ADP, and serotonin uptake were similar for the pool of 7 platelet subpopulations and the original platelet suspension, suggesting that the subpopulations were not damaged during separation. The large platelets had greater density, higher enzymatic activity of LDH, larger numbers of dense bodies per platelet and per cubic micron of platelet cytosol, greater uptake of serotonin, and greater release of serotonin

with increasing concentrations of thrombin.

Our data support the conclusions of Karpatkin and associates (1969a, 1969b, 1970) and of Corash and associates (1977, 1978) who used density gradients to separate platelets in fractions. Whether aging or hetero-geneity of platelet production accounts for the observed differences in the 7 platelet fractions is not clear. However, platelet heterogeneity does exist and may have clinical and therapeutic importance (O'Brien, 1974; Karpatkin, 1978; Bessman and Levin, 1979; Giles, 1981). Clinically, a bleeding diathesis is seen less frequently in patients with ITP and during bone marrow recovery following chemotherapy, in which cases the platelet counts are low and mean platelet volumes are high, than in patients with aplastic anemia who have low platelet counts and low mean platelet volumes (Garo et al., 1971; Harker and Slichter, 1972). Our observation that larger platelets have greater functional capabilities than smaller ones may explain in part the lower incidence of bleeding diathesis associated with low platelet counts and higher mean platelet volumes.

The counterflow centrifugation principle can potentially be utilized with several of the present apheresis instruments to separate the larger more functional platelets from the smaller ones. The selective isolation of large platelets may provide a more therapeutically effective platelet transfusion.

Counterflow centrifugation can be used to separate platelets into fractions by differences in platelet volume. These differences in platelet volume correlate with differences in density, dense body content, enzymatic activity of LDH, platelet aggregation to ADP, and serotonin uptake and release. Our data support the relevance of the mean platelet volume as a measure of platelet functional capability.

## FIGURE 1

The volume distribution of the original platelet suspension prior to separation and of the pool of the subpopulations of platelets (Fractions 1-7) separated by counterflow centrifugation.
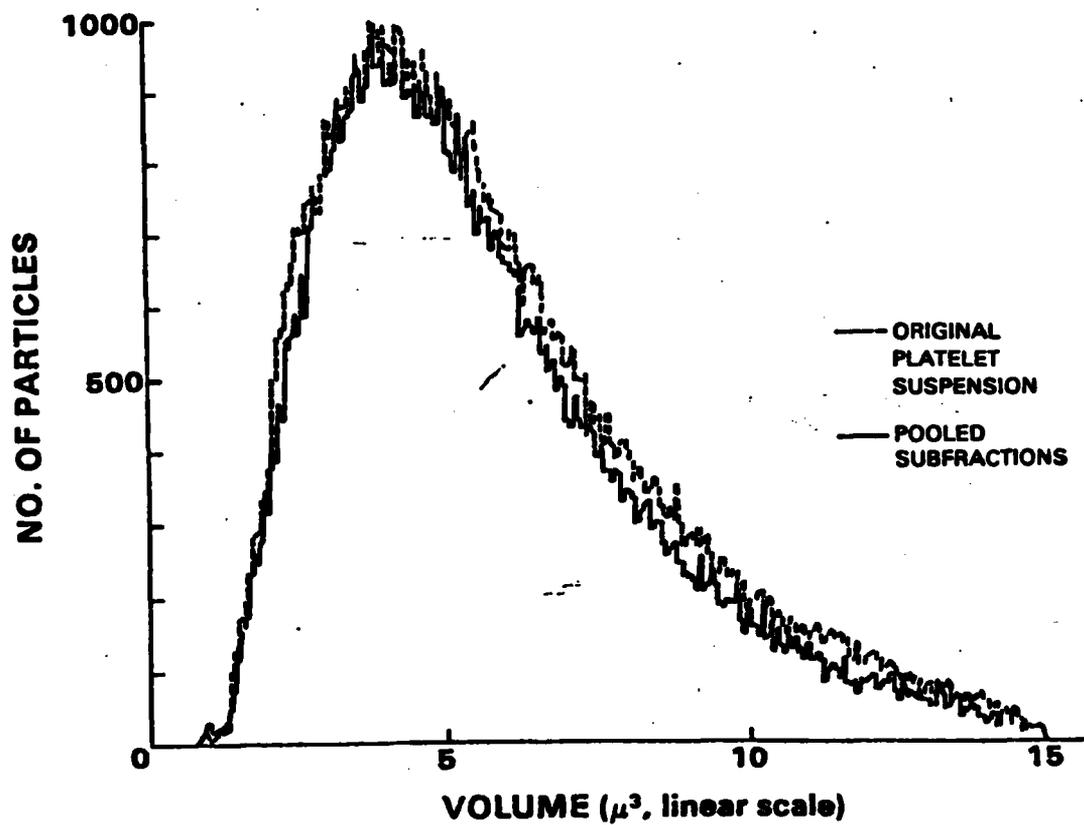
FIGURE 1

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# FIGURE 2

Volume distribution of the platelet suspension prior to separation and of the cells remaining in the elutriator chamber after removal of the platelets.
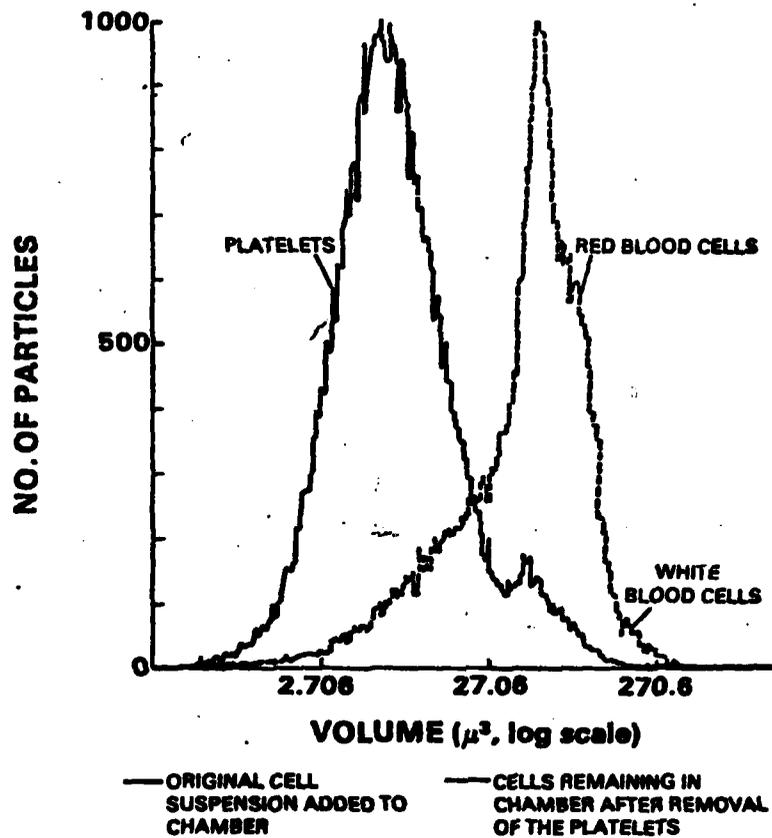
FIGURE 2

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# FIGURE 3

The response to 10 uM ADP of the original platelet suspension and of each of the subpopulations of platelets (Fractions 1-7). The baseline was determined independently for each sample by setting the initial light transmittance of the sample to 10%. The figure is representative of 5 consecutive experiments.
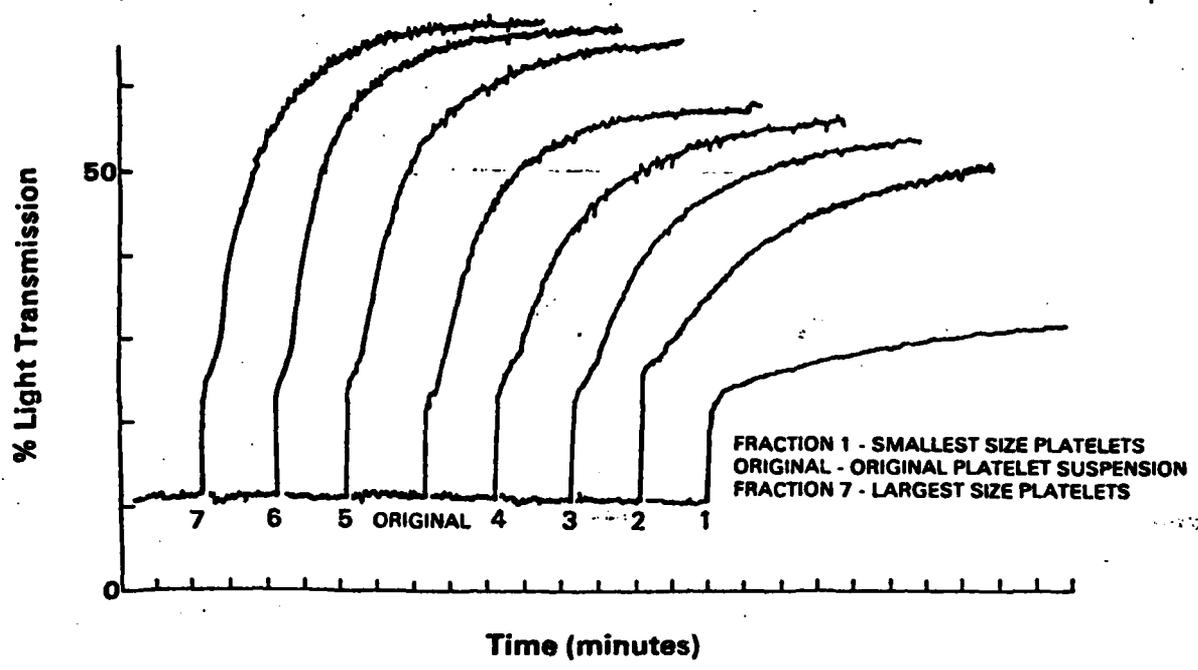
FIGURE 3

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## FIGURE 4

The release of serotonin (nanomoles/$10^9$ platelets) from the sub-populations of platelets (Fractions 2-7) treated with increasing concentrations of thrombin. An insufficient number of platelets was obtained in Fraction 1 to perform a dose response curve. (n=5)
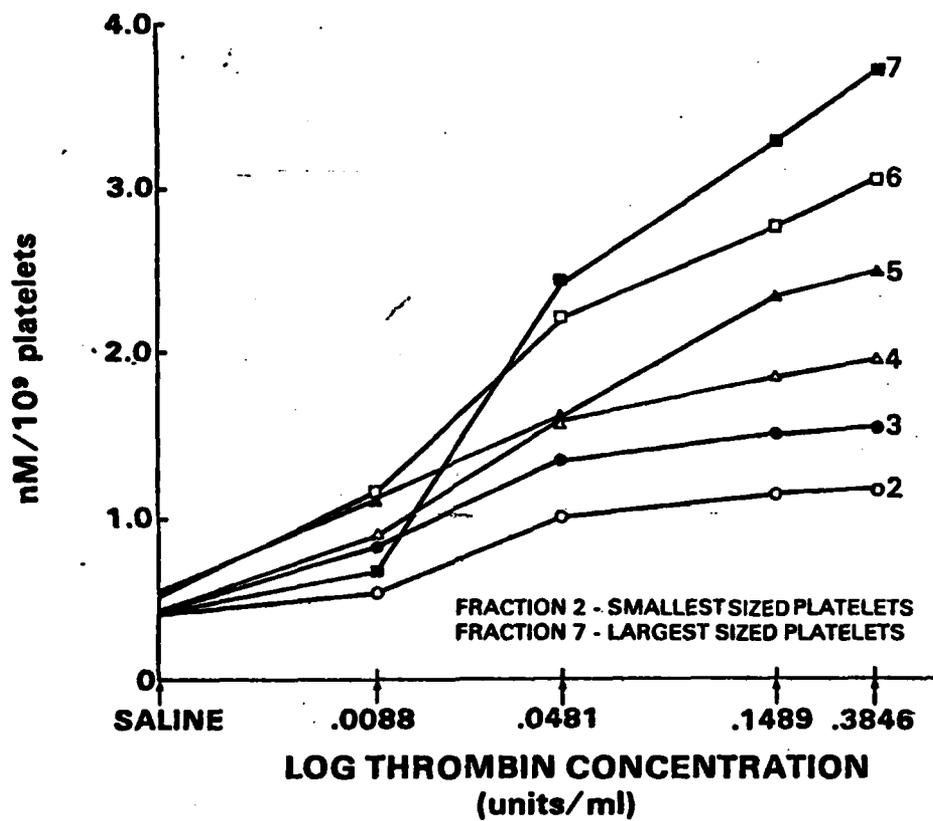
FIGURE 4

**FRACTION 2 - SMALLEST SIZED PLATELETS**
**FRACTION 7 - LARGEST SIZED PLATELETS**

**LOG THROMBIN CONCENTRATION**
**(units/ml)**

FIGURE 4

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## TABLE 1

The Mean Platelet Volume and % of the Original Platelet Suspension Recovered in Each of the 7 Fractions. Average Recovery of the Original Suspension was 96.4 ± 4.3 %. (n=15, Mean ± SD)

| Platelet Fraction # | % Recovery of Original Platelets | Mean Platelet Volume (u$^3$) |
|---|---|---|
| 1 | 3.3 ± 2.1 | 3.94 ± 0.60 |
| 2 | 9.6 ± 4.4 | 4.36 ± 0.68 |
| 3 | 16.0 ± 3.8 | 5.08 ± 0.74 |
| 4 | 20.8 ± 2.3 | 5.93 ± 0.75 |
| 5 | 22.6 ± 3.5 | 6.93 ± 0.75 |
| 6 | 15.2 ± 5.8 | 7.64 ± 0.73 |
| 7 | 8.6 ± 2.5 | 8.19 ± 0.64 |
| Original Platelet Suspension: | ---------- | 6.57 ± 0.61 |

## TABLE 2

The Mean Density of the Platelets in the Original Platelet Suspension Prior to Separation and in Each of the Subpopulations of Platelets. (n=5, Mean ± SD)

| Platelet Fraction # | Mean Platelet Density (gm/cm$^3$) |
|---|---|
| 1 | 1.067 ± 0.002 |
| 2 | 1.070 ± 0.001 |
| 3 | 1.071 ± 0.002 |
| 4 | 1.071 ± 0.002 |
| 5 | 1.071 ± 0.002 |
| 6 | 1.072 ± 0.001 |
| 7 | 1.072 ± 0.001 |
| Original Platelet Suspension: | 1.070 ± 0.001 |

## TABLE 3

The Number of Platelet Dense Bodies in the Original Platelet Suspension and in Each of the Subpopulations of Platelets. (n=7, Mean $\pm$ SD)

| Platelet Fraction # | Mean Dense Bodies (DB/plt.) | Mean Dense Bodies* (DB/$u^3$ plt. cytosol) |
|---|---|---|
| 1 | 4.3 $\pm$ 0.9 | 1.11 $\pm$ 0.17 |
| 2 | 5.1 $\pm$ 1.9 | 1.18 $\pm$ 0.28 |
| 3 | 6.9 $\pm$ 1.1 | 1.33 $\pm$ 0.22 |
| 4 | 7.4 $\pm$ 1.3 | 1.22 $\pm$ 0.21 |
| 5 | 9.5 $\pm$ 2.1 | 1.36 $\pm$ 0.28 |
| 6 | 10.8 $\pm$ 1.7 | 1.47 $\pm$ 0.14 |
| 7 | 12.6 $\pm$ 2.4 | 1.61 $\pm$ 0.31 |
| Original Platelet Suspension: | 8.7 $\pm$ 1.3 | 1.41 $\pm$ 0.22 |

*In each experiment the mean dense bodies per platelet was divided by the mean platelet volume for each fraction to estimate the dense bodies per cubic micron platelet cytosol.

## TABLE 4

The LDH Enzymatic Activity in the Original Platelet Suspension and in Each
of the Subpopulations. (n=5, Mean ± SD)

| Platelet Fraction # | LDH $(IU/10^{10}$ plt.) | LDH $(IU/10^{10}$ plt. cytosol)[*] |
|---|---|---|
| 1 | 4.77 ± 0.92 | 1.13 ± 0.21 |
| 2 | 6.16 ± 0.97 | 1.37 ± 0.24 |
| 3 | 6.94 ± 0.91 | 1.34 ± 0.22 |
| 4 | 8.33 ± 0.82 | 1.39 ± 0.22 |
| 5 | 10.11 ± 0.56 | 1.42 ± 0.15 |
| 6 | 11.78 ± 0.61 | 1.52 ± 0.19 |
| 7 | 14.88 ± 1.23 | 1.76 ± 0.14 |
| Original Platelet Suspension: | 9.47 ± 1.45 | 1.47 ± 0.16 |

[*]In each experiment LDH per $10^{10}$ platelets was divided by the mean platelet volume for each fraction to estimate the LDH per $10^{10}$ micron cubes platelet cytosol.

## TABLE 5

The $^{14}$C-Serotonin Uptake by the Original Platelet Suspension and by Each of the Subpopulations of Platelets.   (n=5, Mean $\pm$ SD)

| Platelet Fraction # | $^{14}$C-Serotonin (cpm/10$^4$ platelets) | $^{14}$C-Serotonin (cpm/10$^4$ u$^3$ plt. cytosol)* |
|---|---|---|
| 1 | 3.60 $\pm$ 0.40 | 0.99 $\pm$ 0.08 |
| 2 | 4.51 $\pm$ 0.56 | 1.15 $\pm$ 0.15 |
| 3 | 6.18 $\pm$ 0.79 | 1.38 $\pm$ 0.16 |
| 4 | 7.71 $\pm$ 0.86 | 1.47 $\pm$ 0.17 |
| 5 | 10.19 $\pm$ 1.05 | 1.62 $\pm$ 0.18 |
| 6 | 12.49 $\pm$ 1.24 | 1.77 $\pm$ 0.20 |
| 7 | 15.20 $\pm$ 1.24 | 1.95 $\pm$ 0.21 |
| Original Platelet Suspension: | 7.62 $\pm$ 1.37 | ---------- |

*In each experiment $^{14}$C serotonin per 10$^4$ platelets was divided by the mean platelet volume for each fraction to estimate the $^{14}$C serotonin per 10$^4$ micron cubes platelet cytosol.

## ACKNOWLEDGMENT

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