

## **Heterogeneity of human platelets: *V. Differences in glycolytic and related enzymes with possible relation to platelet age***

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# Heterogeneity of Human Platelets

## V. DIFFERENCES IN GLYCOLYTIC AND RELATED ENZYMES WITH POSSIBLE RELATION TO PLATELET AGE

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**ABSTRACT** Human platelets were separated by density-centrifugation into heavy and light populations. Heavy platelets have an average volume approximately twofold greater than light platelets, and have previously been shown to be young platelets.

All 11 enzymes of the Embden-Meyerhof pathway plus the five related enzymes: phosphoglucomutase, glucose-6-P dehydrogenase, 6-P-gluconic dehydrogenase,  $\alpha$ -glycerol-P dehydrogenase, and glutathione reductase (TPNH) were examined in cell lysates from total, heavy, and light platelet populations. Apparent  $K_m$  for individual enzymes were measured in a total platelet population. Empirical  $V_{max}$  of the individual enzymes were measured in total, heavy, and light platelet populations. The three apparent rate-limiting enzymes for glycolysis were hexokinase, phosphofructokinase, and glyceraldehyde-3-P dehydrogenase.

Heavy platelets contained approximately twofold greater enzyme activity (per gram wet weight) than light platelets for 7 of the 16 enzymes measured: hexokinase, phosphohexoisomerase, phosphofructokinase, glyceraldehyde-3-P dehydrogenase, phosphoglycerokinase, lactic dehydrogenase, and phosphoglucomutase. Heavy platelets also contained 1.9-fold greater reduced glutathione (GSH), 1.7-fold greater DPNH, and 1.2-fold greater TPNH than light platelets. Heavy platelets contained 1.8-fold less lipid peroxidation products (malonyl aldehyde equivalents) than light platelets and were 2.4-fold more resistant to lipid peroxidation catalyzed by 0.1 mM FeCl<sub>3</sub>.

Sterile incubation of heavy platelets, *in vitro* for 17 hr, resulted in a significant loss of enzyme activity for

the "elevated" seven enzymes when compared with the remainder. Reducing agents such as GSH (0.1 mM), ascorbic acid (0.1 mM), and dithiothreitol (0.01 mM), when added to the incubation mixture, significantly reduced the *in vitro* loss of activity. *In vitro* incubation was also associated with a significant loss of GSH and DPNH and a 1.8-fold increase in lipid peroxidation products.

### INTRODUCTION

Human platelets have a significant aerobic glycolytic metabolism (1-3) and are dependent to a large extent upon the Embden-Meyerhof pathway for their energy requirement. Recently, they have been shown to be heterogeneous and can be separated into two extreme density populations, each representing approximately 15% of the total platelet population (4). The heavy platelet population has an average platelet volume which is approximately twofold greater than the light platelet population.

The large heavy platelet, when compared with the smaller lighter platelet, has been shown to have a greater glycogen, adenine nucleotide, and orthophosphate content (4); to have a greater rate of glycolysis, glycolysis (4), glycogenesis, glyconeogenesis (5), protein synthesis (4); and to have a greater resistance to osmotic shock (i.e., maintenance of ionic gradients [4]).

The large heavy platelet, when compared with the smaller lighter platelet, has also been shown to have a greater functional capacity with respect to more rapid aggregation with such physiologic aggregating agents as: adenosine diphosphate (ADP), thrombin, and epinephrine; associated with a greater release of adenine

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nucleotide and platelet factor 4, an antiheparin agent (6).

Cellular kinetic data in animals (4) as well as in humans (7) have indicated that the large heavy platelet is a young platelet and have suggested that the large heavy platelet becomes a smaller lighter platelet with age.

In the present report, the activity of glycolytic and related enzymes of these two platelet populations, as well as of the total platelet population, have been examined. The apparent  $K_m$ ,<sup>1</sup> empirical  $V_{max}$ , and rate-limiting enzymes of the total platelet population have been determined. The empirical  $V_{max}$  of the individual enzymes have been compared in heavy and light platelet populations. A significantly greater enzyme activity was found in the heavy platelet population for certain rate-limiting as well as other enzymes. Incubation experiments with intact heavy platelets were employed to compare in vitro enzyme lability with the  $V_{max}$ -enzyme differences found between heavy and light platelet populations in vivo. Enzyme lability was assessed with regard to protection by reducing agents, reductive capacity of the platelet, and resistance to lipid peroxidation.

## METHODS

*Preparation of washed platelets.* Human platelets were obtained, collected, and processed as described previously (4). The washed platelets were suspended in a human Ringer solution (8) and separated into heavy and light platelet populations as described previously (4).

*Separation of platelets into heavy and light populations.* Briefly, this entails layering of 6–10 ml of a 7–10% platelet suspension of platelets, washed at 4°C, over 0.5 ml of a fixed density oil mixture. Two fixed density mixtures with a sp gr of 1.032 and 1.042, respectively, are employed. Platelets remaining on the surface of the lower sp gr mixture (<1.032) and platelets sedimenting to the bottom of the heavier sp gr mixture (>1.042) were harvested.

Previous control experiments revealed that passage of platelets through this oil mixture had no discernible effect on platelet adenine nucleotide content, lactate production, or glucose uptake (4). Neither was there any effect on the enzyme activity measured in the present report.

*Extraction procedure for enzyme determinations.* All operations were performed at 0–4°C unless otherwise noted. The separated heavy and light platelet populations were washed once in Human Ringer-0.1 mM ethylenediaminetetraacetate (EDTA), approximately 20 vol of wash to 1 vol of platelets, and then processed for extraction. Two extraction procedures were employed: (a) *Freeze-grinding.* A platelet pellet was obtained by centrifugation in a plastic tube at 3000 *g* for 15 min. The platelet pellet was frozen by completely immersing the plastic tube in liquid nitrogen, –196°C. The tube was then cracked open and the frozen pellet ground to a fine powder with cold mortar and pestle

<sup>1</sup>Abbreviations used in this paper: DPNH, diphosphopyridine nucleotide; GSH, reduced glutathione;  $K_m$ , Michaelis constant; TPNH, triphosphopyridine nucleotide;  $V_{max}$ , maximum velocity.

containing 30 mg of sea sand. The fine powder was extracted in 3 vol/g wet weight (or platelet volume) of 50 mM tris buffer, pH 7.5, 5 mM EDTA, 5 mM mercaptoethanol. This mixture was allowed to thaw to 4°C, with grinding and then centrifuged at 4000 *g* for 30 min. The sediment was discarded and the supernate designated “4000 *g* homogenate.” A portion of this homogenate was then recentrifuged at 105,000 *g* for 60 min in a Beckman L2 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The sediment was suspended in its original volume of the buffer extractant with a Dounce homogenizer and designated “particulate fraction.” The supernate was designated, “105,000 *g* supernate.” (b) *Ultrasonic disruption.* A platelet pellet was obtained as above, and suspended in 3 vol per platelet volume of human Ringer solution containing 5 mM EDTA, 5 mM mercaptoethanol, pH adjusted to 7.5. This suspension was sonicated at maximal intensity at 4°C with a Bronwill Biosonik sonicator microprobe (Bronwill Scientific, Inc., Rochester, N. Y.) for two 10-sec intervals. This disrupted cellular suspension was then separated into a 4000 *g* homogenate, 105,000 *g* supernate, and 105,000 *g* particulate fraction, as above.

*Enzymatic assays.* Enzymatic assays employed classical techniques for coupling of enzymatic reactions to pyridine nucleotide oxidation-reduction changes at 340  $\mu$  and 30°C (Table I). A Gilford power supply and photo-cell (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), Beckman spectral system and Honeywell recorder (Honeywell, Inc., Denver, Colo.) were employed to measure initial enzyme rates. These were linear with time and “extract” concentration, and were zero in the absence of substrate(s). Optimal dilutions for concentration-dependent linearity were made with the tris buffer extractant mentioned and are given in Tables IA and B. Enzyme rates were assayed in microcuvettes of 0.5 ml volume in 50 mM tris buffer, 5 mM mercaptoethanol pH 7.5 at 30°C. All rates were assayed in triplicate, employing 10, 20, and 30- $\mu$ l portions of tissue extract.

Control experiments revealed no significant differences between enzyme activity extracted from washed platelets when compared with platelets extracted immediately after their sedimentation from plasma. The following enzymes were found to be labile (with the two extraction conditions employed) if not assayed within 2 hr after extraction: phosphofructokinase, glyceraldehyde-3-P dehydrogenase, phosphoglucumutase, and phosphoglyceromutase. The remainder were measured the next morning, after storage at 4°C, within a time interval of < 20 hr. These manipulations provided enzyme rates which are comparable with enzyme rates obtained immediately after extraction.

*Reduced pyridine nucleotide and glutathione measurements.* DPNH and TPNH were assayed spectrophotometrically by the methods of Ciotti and Kaplan (9), employing alcohol dehydrogenase for DPNH and glutathione reductase for TPNH. Recovery of added DPNH or TPNH to the cell extract was better than 95%. Reduced glutathione (GSH)<sup>2</sup> was measured by a minor modification of the method of Beutler, Duron, and Kelly (10) reacting, 5,5'-dithiobis(2-nitrobenzoic acid) with a neutralized perchloric acid extract

<sup>2</sup>It should be recognized that this method, although extensively employed for the measurement of GSH, is not specific for this compound and will measure any sulfhydryl-reacting soluble peptide. However, as will be noted, the purpose of this measurement was to determine the relative “reductive capacity” of the cell sap in heavy vs. light platelets, rather than GSH specifically.

TABLE IA  
Emden-Meyerhof Pathway Enzymes

Enzyme	Substrates	Auxiliary reagents*	Dilution	Apparent $K_m$ M
Hexokinase	20 mM glucose 12 mM MgATP	TPN + glucose-6-P dehydrogenase	1:10	$3.0 \times 10^{-5}$ $7.0 \times 10^{-4}$
Phosphohexoisomerase	10 mM fructose-6-P	TPN +; B.S.A. glucose-6-P dehydrogenase	1:80	$7.7 \times 10^{-5}$
Phosphofructokinase	6 mM fructose-6-P 1 mM MgATP	DPNH; B.S.A. Triose-P isomerase 40 $\mu$ g/ml aldolase $\alpha$ -glycerol-P dehydrogenase	1:20	$5.7 \times 10^{-5}$ $5.0 \times 10^{-6}$
Aldolase	10 mM fructose-1,6-diP	DPNH Triose-P isomerase $\alpha$ -glycerol-P dehydrogenase	1:100	$2.5 \times 10^{-5}$
Triose-P isomerase	2 mM glyceraldehyde-3-P	DPNH $\alpha$ -glycerol-P dehydrogenase	1:200	$2.7 \times 10^{-4}$
Glyceraldehyde-3-P dehydrogenase	4 mM glyceraldehyde-3-P 20 mM Pi 0.5 mM DPN +		1:20	$1.6 \times 10^{-5}$ $1.7 \times 10^{-4}$ $2.4 \times 10^{-5}$
Phosphoglycerokinase	6 mM 3-P-glycerate 1 mM MgATP	DPNH glyceraldehyde-3-P dehydrogenase	1:40	$4.0 \times 10^{-5}$ $5.9 \times 10^{-6}$
Phosphoglyceromutase	6 mM 2-P-glycerate 1 mM MgATP	DPNH glyceraldehyde-3-P dehydrogenase 2 $\mu$ g/ml phosphoglycerokinase, 50 mM KCl	1:20	$6.5 \times 10^{-5}$ $8.0 \times 10^{-5}$
Enolase	2 mM 2P-glycerate	DPNH 2 mM MgCl 10 mM MgADP 2 $\mu$ g/ml pyruvate kinase lactic dehydrogenase	1:100	$4.7 \times 10^{-5}$
Pyruvate kinase	5 mM P-enolpyruvate 10 mM MgADP	DPNH lactic dehydrogenase	1:200	$6.0 \times 10^{-4}$ $6.2 \times 10^{-4}$
Lactic dehydrogenase	2 mM pyruvate 0.3 mM DPNH		1:200	$3.6 \times 10^{-5}$ $4.6 \times 10^{-5}$

\* All assays were run in tris buffer 50 mM, pH 7.5, 5 mM mercaptoethanol at 30°C. Concentrations of auxiliary reagents consisted of: 0.5 mM TPN +; 0.3 mM DPNH; 4 mg/ml bovine serum albumin; 2  $\mu$ g/ml glucose-6-P dehydrogenase; 4  $\mu$ g/ml triose-P isomerase; 4  $\mu$ g/ml  $\alpha$ -glycerol-P dehydrogenase; 2  $\mu$ g/ml glyceraldehyde-3-P dehydrogenase; 1  $\mu$ g/ml lactic dehydrogenase. Pyruvate kinase rates were not appreciably affected by the addition of 10 mM KCl.

obtained from a frozen platelet pellet (3). Recovery of added GSH to this extract was better than 95%.

*Lipid peroxidation.* The oxidative degradation of unsaturated fatty acids was estimated by a product of lipid

peroxidation recognized as malonyl dialdehyde (11-13). This was assayed by the 2-thiobarbituric acid technique with final color development in alkaline solution (14). Clarification of "tissue" turbidity was achieved with the use of Bio-solv

TABLE IB  
Other Glycolytic and Related Enzymes\*

Enzyme	Substrates	Auxilliary reagents	Dilution	Apparent $K_m$ M
Phosphoglucumutase	10 mM glucose-1-P	TPN <sup>+</sup> ; B.S.A. Glucose-6-P dehydrogenase 1 mM MgCl	1:20	$3.5 \times 10^{-5}$
Glucose-6-P dehydrogenase	10 mM glucose-6-P 0.5 mM TPN <sup>+</sup>		1:20	$7.7 \times 10^{-5}$ $2.8 \times 10^{-5}$
6-P-gluconic dehydrogenase	10 mM 6-P-gluconate 0.5 mM TPN <sup>+</sup>	1 mM MgCl	1:10	$2.7 \times 10^{-5}$ $8.2 \times 10^{-6}$
$\alpha$ -Glycerol-P dehydrogenase	5 mM dihydroxyacetone-P 0.3 mM DPNH		1:5	$3.0 \times 10^{-5}$ $2.2 \times 10^{-6}$
Glutathione reductase	10 mM GSSG 0.3 mM TPNH		1:4	$6.1 \times 10^{-3}$ $2.3 \times 10^{-5}$

\* All assays were run in tris buffer 50 mM, 5 mM mercaptoethanol, pH 7.5 at 30°C. Concentrations of auxilliary reagents consisted of: 0.5 mM TPN<sup>+</sup>; 4 mg/ml B.S.A.; 2  $\mu$ g/ml glucose-6-P dehydrogenase.

Both phosphoglucumutase and 6-P-gluconic dehydrogenase had suboptimal rates in the absence of Mg<sup>++</sup> which increased 12-fold and 3.3-fold, respectively, in the presence of 1 mM MgCl<sub>2</sub>. Phosphoglucumutase rates were not affected by the addition of 0.2 mM glucose-1,6-diphosphate. Glutathione reductase rates were inhibited by the addition of 0.004–0.4 mM FAD.

(15). A standard curve was obtained with malonyl dialdehyde bis(diethylacetal). Products of lipid peroxidation were enhanced by the use of FeCl<sub>3</sub> as catalyst (16). A 0.5% vol suspension of washed platelets was incubated for 1 hr at 37°C in the presence and absence of 0.1 mM FeCl<sub>3</sub>. These conditions were found to be optimum for formation of products of lipid peroxidation.

**Protein measurement.** Protein was measured by the Biuret method (17), employing salt-free bovine serum albumin as standard. No significant difference was noted in the protein content of the 105,000 g supernate when either extraction procedure was employed (four experiments). The heavy platelet population, 105,000 g supernate, contained 22% more protein than the light population with both extraction procedures, i.e., 16.5 vs. 13.5 mg/ml (eight experiments,  $P < 0.01$ ). This small difference in protein content was similar to that reported previously (4).

**Incubation experiments.** Heavy platelets were resuspended in their own (ACD-A) plasma (previously gassed with 5% CO<sub>2</sub>-95% O<sub>2</sub>) and incubated for 17 hr at 37°C as a 0.5 vol per cent (physiologic) platelet suspension in the presence of penicillin, 100 U/ml, and additional glucose of 150 mg/ml. The pH of the incubation mixture remained fairly constant at about pH 7.

**Measurement of platelet volume and relative population volume.** Platelets were enumerated via phase microscopy as described previously (5). Platelet volume was determined by centrifugation at 2000 g in 12.5-cm glass capillary tubes, (obtained from Clay Adams, Parsippany, N. J.) as described previously (2). The relative volume difference between heavy and light platelet populations was obtained by dividing the number of platelets per packed cell volume of light platelets by the number of platelets per packed cell volume of heavy platelets. The average volume of heavy platelets was approximately twice that of light platelets,

with a range of 2- to 2.4-fold. Extracellular space measurements of the packed cell volumes of washed heavy, total, and light platelets were determined with inulin (<sup>14</sup>C) and revealed no significant difference between populations. Extracellular space averaged 12.2±2.1% (SD, eight experiments). Radioactivity was measured in a Beckman LS 100 scintillation spectrometer with isoset adjusted to give 96% counting efficiency. Platelets were dissolved in 0.5 ml of Hyamine hydroxide and quantitatively transferred to a scintillation cocktail. Control experiments revealed no interference in radioactivity measurements by platelet concentrations employed in the determination of extracellular space. Counts per minute were 5–100 times background and counting error was adjusted to less than 3%.

**$K_m$  measurements.** Apparent  $K_m$  were measured with dilutions of tissue extract (105,000 g supernate) which varied from 200- to 10,000-fold. It is very unlikely, therefore that any significant interference could have been encountered from tissue glycolytic intermediates or other products. Six substrate concentrations were employed to determine velocity measurements for the apparent  $K_m$  measurement. Lineweaver-Burke plots were linear for all enzymes measured except for glutathione reductase (TPNH),  $\alpha$ -glycerol-P dehydrogenase (dihydroxyacetone-P), and phosphofructokinase (MgATP) where nonlinearity was noted at high substrate concentration. These  $K_m$  were measured at the cosubstrate concentration designated in Table I.

**Empirical  $V_{max}$ .** This was the highest enzyme rate obtained with increasing substrate concentration when cosubstrate concentration (when indicated) was held at the concentration cited in Table IA and B.

**Materials.** Distilled deionized water was used at all times. All chemicals were reagent grade. Enzyme reagents were obtained from either Sigma Chemical Co., St. Louis, Mo., or Boehringer Mannheim Corp., New York unless otherwise

indicated. Beef heart lactic dehydrogenase was obtained from Worthington Biochemical Corp., Freehold, N. J. N-dibutyl phthalate was obtained from Fisher Scientific Co., Pittsburgh, Pa. Apiezon A oil was obtained from James G. Biddle Co., Plymouth Meeting, Pa. Hyamine hydroxide was obtained from Packard Instrument Co., Inc., Downers Grove, Ill. Dithiothreitol was obtained from Calbiochem, San Diego, Calif. Mercaptoethanol was obtained from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y. 5,5-dithiobis(2-nitrobenzoic acid) was obtained from Sigma Chemical Co. Bio-solv was obtained from Beckman Instruments Inc., Fullerton, Calif. Malonyl dialdehyde bis(diethyl-acetal) was obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. Chloroquine hydrochloride was obtained from Winthrop Laboratories, New York. Cortisone acetate was obtained from Merck & Co., Inc., Rahway, N. J., (carboxylic-<sup>14</sup>C) inulin, 8.3 mCi/mM, was obtained from Nuclear-Chicago Corp., Des Plaines, Ill.

## RESULTS

### Enzyme measurements of the total platelet population

Optimum enzyme activity was dependent upon the extraction procedure employed and the duration of time between extraction and assay of enzyme rate (see Methods). The sonic lysate extraction procedure gave highest rates for most of the enzymes measured, the exceptions being glucose-6-P dehydrogenase, 6-P-gluconic dehydrogenase, and lactic dehydrogenase. Higher rates were obtained for these three enzymes with the freeze-grinding procedure (1.8, 1.2, and 1.3-fold higher respectively, > 2 sd difference). The empirical  $V_{max}$  with  $\pm$ sd range is reported for the extraction procedure giving the highest rate. These experiments represent the mean of 8-10 different determinations from platelet

TABLE II  
Enzyme Activity of Total Platelet Population Cell Sap

Embden-Meyerhof pathway enzymes	Mean $V_{max}$ *	Range $\pm$ 2 sd
Hexokinase	200	172-228
Phosphohexoisomerase	3202	2922-3482
Phosphofructokinase	552	422-682
Aldolase	913	685-1141
Triose-P isomerase	8152	7532-8772
Glyceraldehyde-3-P dehydrogenase	499	415-583
Phosphoglycerokinase	1592	1458-1726
Phosphoglyceromutase	733	625-841
Enolase	6230	5621-6839
Pyruvate kinase	4019	3163-4875
Lactic dehydrogenase†	4397	3797-4997
Other related enzymes		
Phosphoglucomutase	550	504-596
Glucose-6-P dehydrogenase†	579	556-602
6-P-gluconic dehydrogenase†	295	272-318
$\alpha$ -Glycerol-P dehydrogenase	15	12-19
Glutathione reductase	161	139-183

\* Micromoles/gram wet weight per hour at 30°C.

† Extracted by freeze-grind method; other enzymes extracted by sonic lysate method.

TABLE III  
Platelet Population Enzyme Activity\*

Embden-Meyerhof pathway enzymes	Light	Total	Heavy	Heavy/light ratio
Hexokinase	128	200	239	1.9
Phosphohexoisomerase	2310	3202	4577	2.1
Phosphofructokinase	411	552	801	2.0
Aldolase	1008	913	1008	1.0
Triose-P isomerase	7860	8152	7860	1.0
Glyceraldehyde-3-P dehydrogenase	357	499	709	2.0
Phosphoglycerokinase	894	1592	1619	1.8
Phosphoglyceromutase	688	733	708	1.0
Enolase	6560	6230	6410	1.0
Pyruvate kinase	5510	4019	5568	1.0
Lactic dehydrogenase	3010	4397	6260	2.1
Other related enzymes				
Phosphoglucomutase	346	550	713	2.1
Glucose-6-P dehydrogenase	600	579	630	1.1
6-P-gluconic dehydrogenase	308	295	322	1.0
$\alpha$ -glycerol-P dehydrogenase	15	15	17	1.1
Glutathione reductase	152	161	161	1.1

\* Micromoles/gram wet weight per hour at 30°C.

pools obtained from 2-4 different U of platelet-rich plasma, Table II. The three apparent "rate-limiting enzymes" of the Embden-Meyerhof pathway (i.e., enzymes with lowest activity) were hexokinase, phosphofructokinase, and glyceraldehyde-3-P dehydrogenase.

*Cellular distribution of enzyme activity.* The 4000 g homogenate rates equaled the sum  $\pm$ 10% of the 105,000 g supernatant and sediment rates for all the enzymes measured. The 105,000 g supernatant rates represented approximately 97-100% of total enzyme activity for 13 of the 16 enzymes measured. Three of the enzymes measured had significant particulate enzyme rates, with both extraction procedures which represented 10% or more of the total enzyme activity. These were hexokinase (8.0-10.7%),  $\alpha$ -glycerol-P dehydrogenase (16.9-23.2%), and glutathione reductase (12.5-12.5%). Values given are the mean of three experiments for freeze-grind and sonic lysate procedures, respectively.

### Comparison of heavy, light, and total platelet populations

6 of the 11 Embden-Meyerhof pathway enzymes: hexokinase, phosphohexoisomerase, phosphofructokinase, glyceraldehyde-3-P dehydrogenase, phosphoglycerokinase, and lactic dehydrogenase were present at an approximately twofold greater activity in heavy platelets compared with light platelets, Table III and Fig. 1. Similar results were obtained for one of the other five enzymes measured, phosphoglucomutase, Table III.

Fig. 1 compares the empirical  $V_{max}$  of the total platelet population with the ratio in  $V_{max}$  between the heavy and light platelet populations. It is of interest that the three "rate limiting" enzymes mentioned above are

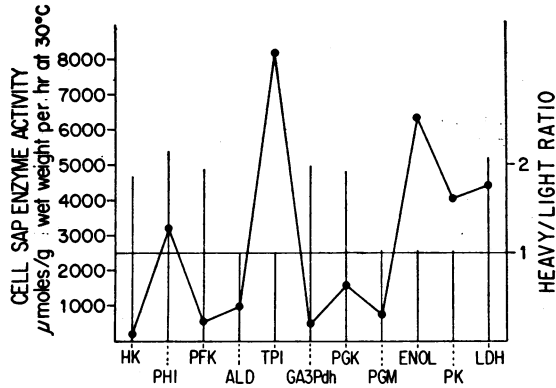


FIGURE 1 Empirical  $V_{max}$  of enzymes of the Embden-Meyerhof pathway. Data for total platelet population (●—●) are compared with the ratio of  $V_{max}$  of enzymes of heavy platelets and light platelets (vertical lines). Rates are given as micromoles/gram wet weight per hr at 30°C and represent the average of 8–10 experiments.

among the enzymes with twofold greater activity in heavy than in light platelets.

#### In vitro incubation of heavy platelets

*Effect on empirical  $V_{max}$ .* Washed heavy platelets were incubated in their own plasma at 37°C (see Methods) in order to compare in vitro lability of the enzymes measured with in vivo differences between heavy and light platelets (i.e., heavy and light platelets extracted at time zero). Fig. 2 reveals the pattern of enzyme decay for all 11 Embden-Meyerhof pathway enzymes. The basal heavy/light ratio is given in parentheses. As can be noted, the group of six enzymes with twice the activity in heavy vs. light platelets, are the enzymes which are more labile when heavy plate-

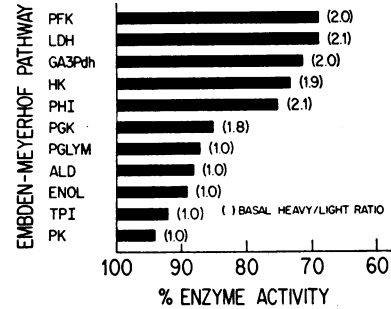


FIGURE 2 In vitro incubation of heavy platelets for 17 hr. Empirical  $V_{max}$  was measured at zero time and after 17 hr of sterile incubation in plasma with 0.5 vol per cent platelet suspension. Per cent remaining enzyme activity is compared with initial heavy/light ratio. Data represent the average of four incubation experiments.

lets are incubated in vitro at 37°C for 17 hr. The same also applied for phosphoglucosmutase of the other enzyme group. Similar results were obtained after 40 hr of in vitro incubation (four experiments).

#### Studies on the mechanism of increased enzyme lability in vitro

*Possible release of intracellular enzymes into plasma.* The increased lability of this group of seven enzymes could not be attributed to disruption of platelets with release of enzymes into the plasma, since plasma enzyme activity before and after incubation represented less than 1% of total platelet activity at time zero (average of three experiments). It is possible however, that “released” plasma enzymes may have been inactivated during the period of incubation.

*Possible release of lysosomal enzymes.* The increased

TABLE IV

*Effect of Cortisone Acetate, Chloroquine Hydrochloride, Dithiothreitol, GSH, or Ascorbic Acid on In Vitro Enzyme Lability of Heavy Platelets after Incubation at 37°C for 17 Hr*

Enzymes	Control* (4)	Cortisone (2)	Chloroquine (1)	Dithiothreitol (3)	GSH (2)	Ascorbic acid (2)	GSH + ascorbic acid (2)
Hexokinase	73	58	64	123	75	91	89
Phosphohexoisomerase	75	68	64	109	100	85	104
Phosphofructokinase	69	57	58	126	74	87	79
Glyceraldehyde-3-P dehydrogenase	71	61	58	107	80	77	84
Phosphoglycerokinase	85	73	63	101	112	104	104
Lactic dehydrogenase	69	60	48	82	94	89	88
Phosphoglucosmutase	76	65	62	119	81	79	86

\* Per cent of initial enzyme activity after 17 hr incubation at 37°C in plasma, 100 U/ml penicillin, and additional 150 mg/ml glucose. Number of experiments is given in parentheses. Concentration of effector agents: cortisone acetate,  $10^{-4}$  M; chloroquine hydrochloride,  $10^{-4}$  M; dithiothreitol,  $10^{-5}$  M; GSH,  $10^{-3}$  M; ascorbic acid,  $10^{-3}$  M.

lability could not be attributed to intracellular release of lysosomal enzymes, since addition of lysosomal stabilizers (18), such as cortisone acetate or chloroquine hydrochloride had no beneficial effect on the lability of this group of seven enzymes, Table IV.

*Evidence for oxidative stress during in vitro incubation.* However, addition of reducing agents such as dithiothreitol, GSH, or ascorbic acid were successful in preventing the decay of enzyme activity noted at the end of 17 hr of in vitro incubation at 37°C, Table IV. Dithiothreitol at 10<sup>-5</sup> M was the most effective agent employed.

*Intracellular reductive capacity of platelets.* The cellular reductive capacity of heavy, total, and light platelet populations were next examined by measurement of the intracellular reducing agents: GSH, DPNH, and TPNH, at zero time and after in vitro incubation at 37°C. Table V reveals a greater content of GSH, DPNH, and TPNH in heavy platelets compared with light platelets of 1.9, 1.7, and 1.2-fold respectively ( $P < 0.01$ ). After in vitro incubation of heavy platelets at 37°C (1 hr for GSH and 17 hr for DPNH and TPNH), GSH, DPNH, and TPNH declined 41, 26, and 10% respectively ( $P < 0.01$ ).

*Lipid peroxidation products and resistance to lipid peroxidation.* A 1.8-fold greater content of lipid peroxidation products was noted in light platelets compared with heavy platelets (Table V). After incubation for 17 hr at 37°C heavy platelet lipid peroxidation products increased 1.8-fold (Table V). When exposed

to the oxidative catalytic action of FeCl<sub>3</sub>, heavy platelets were 2.4-fold more resistant to the formation of lipid peroxidation products than were light platelets (Table V).

## DISCUSSION

These data reveal the heterogeneity of human platelets with respect to glycolytic and other enzyme activity; and with respect to reductive capacity and ability to resist oxidative stress. They are consistent with previously reported data on the heterogeneity of human platelet glycolysis, glycolysis, adenine nucleotide content, and resistance to osmotic shock (maintenance of cationic gradients [4]). They lend support to previously reported data on enhanced glycogen synthetase and fructose-1,6-diphosphatase activity of heavy platelets with associated increased glycogen content, glycogenesis, and glyconeogenesis (5). Thus heavy platelets are more active metabolically, have a greater reductive capacity and are more resistant to "oxidative" stress than light platelets. This cannot be attributed to the increased platelet volume of heavy platelets since these differences are obtained from data expressed per gram wet weight or per unit volume (or milligram protein).

The three "rate-limiting" glycolytic enzymes: hexokinase, phosphofructokinase, and glyceraldehyde-3-P-dehydrogenase ("sulfhydryl-dependent" enzymes) were found to be present at twofold the activity in heavy platelets compared with light platelets. The group of enzymes with greater activity was also found to be more "labile" after simulated in vivo conditions i.e., in vitro incubation in plasma at 37°C for 17 hr. Similar results were obtained at 40 hr of incubation. The mechanism of the increased lability is apparently related to oxidative processes within the cell or on the membrane, since reducing agents can partially or completely reverse this effect in heavy platelets. This is consistent with the increased resistance to lipid peroxidation in heavy platelets. It is conceivable that free radical formation might be responsible for the oxidative changes noted during in vitro incubation of heavy platelets; as well as partially responsible for the enzyme differences noted in "initially extracted" heavy and light platelets. Differences in enzyme activity might also be related to the greater reductive capacity and resistance to lipid peroxidation of heavy platelets compared with light platelets. Free radical formation is further supported by the apparent increase in lipid peroxidation after incubation of heavy platelets, associated with a decrease in enzyme activity. Lipid peroxides and their products have been shown to be metabolic poisons capable of forming inactivating complexes with proteins (19, 20) and essential enzymes (21-23). Oxidation of sulfhydryl groups (20, 22), microsomes, mitochondria (24-27),

TABLE V  
Reductive Capacity and Resistance to Lipid Peroxidation  
of Different Platelet Populations

	Heavy	Total	Light	Heavy/light† ratio	Heavy‡ incubation
GSH (5)*	10.2	7.20	5.42	1.9	6.29
DPNH (4)	0.049	0.035	0.029	1.7	0.036
TPNH (4)	0.069	0.063	0.057	1.2	0.062
				Light/heavy¶ ratio	
Lipid Perox. (4)‖	2.19		3.95	1.8	3.85
+ FeCl <sub>3</sub> (5)	5.39		12.94	2.4	

\* Micromoles/gram wet weight of initially extracted GSH, DPNH, and TPNH of various platelet populations; number of experiments given in parentheses.

† Probability of heavy/light ratio being greater than 1 was significant at the  $P < 0.01$  level. SEM was less than 10%.

‡ Heavy platelets were suspended in plasma (0.5 vol per cent) and incubated for 1 hr (GSH) or 17 hr (DPNH, TPNH, lipid peroxidation) at 37°C, before extraction.

‖ Nanomoles/gram wet weight of malonyl dialdehyde equivalents. Washed platelets were initially extracted; or suspended in human Ringer solution (0.5 % vol) containing 0.1 mM FeCl<sub>3</sub> and incubated for 1 hr at 37°C before extraction; number of experiments given in parentheses.

¶ Probability of light/heavy ratio being greater than 1 was significant at the  $P < 0.01$  level. SEM was less than 10%.



and swelling and lysis of mitochondria (27) have been reported secondary to lipid peroxidation. Indeed free radical formation has been incriminated as one of the mechanisms responsible for the process of aging (28, 29).

If lability of rate-limiting glycolytic and other enzymes secondary to oxidative stress is associated with in vitro platelet senescence, it might also be associated with in vivo senescence. In this context, increased resistance to lipid peroxidation has recently been reported in "young" rat platelets obtained after the administration of rabbit anti-rat platelet antibody (15). Furthermore, Murphy and Gardner (30) have recently presented evidence, from platelet preservation studies, that in vitro aging approximates in vivo aging. It should be emphasized, however, that the association of rate-limiting enzyme lability with in vitro incubation and oxidative stress does not prove a causal relationship.

Previous cellular kinetic data in rabbits and humans have suggested a transition of the heavy large, young platelet to a lighter smaller, older platelet (4, 7). McDonald, Odell, and Goslee (31) have noted a decrease in rat platelet size with aging. Minter and Ingram (32) have noted an increase in dog platelet size and density after the thrombopoietic stimulus of acute blood loss. In a more recent publication, Minter and Ingram (33) have referred to the heavy large platelets of acute blood loss in dogs as young "stress" platelets, rather than "physiologic" young platelets. Their evidence, although indirect, does not support a transition of heavy large "stress" platelets to lighter smaller platelets. The heavy large platelets in our study were isolated during basal conditions and not obtained under thrombopoietic stress. It is conceivable that two types of heavy large platelets apply: one that is physiologically young, the other that is "stress" young.

If heavy large young platelets do indeed become lighter smaller older platelets as has been suggested (4, 7, 31) it would be tempting to speculate that this could occur as a result of, or associated with, loss of glycolytic "rate-limiting" enzyme activity. This in turn would contribute to lower energy stores, i.e., glycogen and adenine nucleotide content. The platelet, being an aerobic glycolytic tissue is dependent to a large extent upon the Embden-Meyerhof pathway for its energy requirement (1-3). This energy is required for the maintenance of cationic gradients (34), maintenance of steady-state conditions (3), and maintenance of platelet discoid shape (3, 35). Consequently, any impairment in energy storage or utilization would be a decided disadvantage to the cell with respect to viability. Energy is also required for platelet function: ADP-induced platelet-plug formation (36), clot retraction (36), incorporation of P<sup>32</sup> into phospholipid (37) and

phospholipid synthesis (38), active transport of amino acids (39) and serotonin (40).

The presently reported biochemical data on glycolytic enzymes, reductive capacity, and resistance to oxidative stress of different platelet populations indicate the metabolic heterogeneity of human platelets and lend indirect support to the concept of human platelet senescence.

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