# AGE RELATED CHANGES IN RED CELL ACTIVITIES OF GLYCOLYTIC ENZYMES, REDUCED GLUTATHIONE AND HEMOGLOBIN DENATURATION

by

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A THESIS

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## Abbreviations

| | |
|---|---|
| ADP | adenosine 5'diphosphate |
| ATP | adenosine 5'triphosphate |
| CNSHA | congenital nonspherocytic hemolytic anemia |
| 2,3-DPG | 2,3-diphosphoglyceric acid |
| DNA | deoxyribonucleic acid |
| EN | enolase |
| FAD | flavine adenine dinucleotide |
| F-6-P | fructose-6-phosphate |
| G-6-PD | glucose-6-phosphate dehydrogenase |
| G-6-P | glucose-6-phosphate |
| GSSG | glutathione oxidized form |
| GSSG-R | glutathione reductase |
| GSH | glutathione reduced form |
| GSH-Px | glutathione peroxidase |
| GOT | glutamic oxalacetic transaminase |
| GPI | glucose phosphate isomerase |
| GAPD | glyceraldehyde phosphate dehydrogenase |
| HK | hexokinase |
| HMS | hexosemonophosphate shunt |
| Hb | hemoglobin |
| $K_m$ | Michaelis constant |
| LDH | lactate dehydrogenase |

| | |
|---|---|
| MDH | malate dehydrogenase |
| nm | nanometer |
| NADP | nicotinamide adenine dinucleotide phosphate |
| NADPH | nicotinamide adenine dinucleotide phosphate, reduced |
| NAD | nicotinamide adenine dinucleotide |
| NADH | nicotinamide adenine dinucleotide, reduced |
| PFK | phosphofructokinase |
| PK | pyruvate kinase |
| PGK | phosphoglycerate kinase |
| 6-PGD | 6-phosphogluconate dehydrogenase |
| 6-PG | 6-phosphogluconic acid |
| PGA | phosphoglyceric acid |
| PEP | phosphoenolpyruvate |
| RBC | red blood cell |
| RNA | ribonucleic acid |
| Tris | tris(hydroxy methyl) amino methane |
| TEA | triethanolamine |
| TBH | tert-butylhydro-peroxide |
| TCA | trichloroacetic acid |
| $V_m$ | maximum velocity |

# INTRODUCTION

## I. The Aging Process in Human Erythrocytes.

The advantages of the red cell as an object of age related biochemical studies include its relatively simple structure, composition and metabolism. The erythrocyte depends on anaerobic metabolism of glucose since the TCA cycle enzymes are not present beyond the reticulocyte stage. It can not form glycogen or convert glucose to fatty acids. Finally red cells can be obtained repeatedly in large amounts and can be separated from other components of the blood. Human erythrocytes are highly specialized cells containing hemoglobin for the transport of oxygen. Maximal hemoglobin production occurs during early stages of red cell differentiation and ceases after the reticulocyte stage. The reticulocyte, which represents the youngest red cell in the circulation, has lost its nucleus and DNA and is not capable of RNA synthesis. In this period, the messenger RNA and associated ribosomes are capable of some protein synthesis primarily hemoglobin (1). After the reticulocyte stage, the cell loses its oxidative phosphorylation system, and is incapable of lipid and protein synthesis. The loss of these synthesis capabilities during the transition from reticulocyte to mature erythrocyte is associated with the metabolic changes seen during erythrocyte aging.

The finite life span of human erythrocytes is approximately 120 days. The senescent cell is removed from the circulation at this rather constant cell age. It is reasonable to assume that there are

biochemical and biophysical changes in the human erythrocyte during its life span (2). It has been shown that the integrity of the red cell depends on metabolic activities which are dependent on the enzyme systems. As the cell ages there is a general decline in enzyme activity resulting in loss of energy production. There is also a loss of surface lipid with age (3).

Changes in the surface properties of the red blood cell membrane can also be used to study red blood cell aging. The human erythrocyte has a net negative charge due to sialic acid. Seaman and Uhlenburg (4) found that the degree of charge reduction of the red blood cell surface following treatment with Vibrio cholerae neuraminidase depends on the animal species and found the greatest reduction in human erythrocytes. Membrane sialic acid and surface charge are decreased in older erythrocytes. Marikovsky (5) and Yaari (6) also found a difference in electrophoretic mobility of young and old cell populations. Durocher et al. (7) treated rat and rabbit red blood cells with Vibrio cholerae neuraminidase which is known to reduce the surface charge of the RBC membrane. The shorter $^{51}Cr$ labeled RBC survival of neuraminidase-treated erythrocytes compared to the normal control was found to be related to increased sequestration by the reticuloendothelial system. This is also supported by Landaw et al. (8, data unpublished). Recent studies were done to determine the relationship between RBC surface charge and deformability. Decreased surface charge resulted in

decreased filterability (9,10). A defect in shape, internal viscosity or intrinsic membrane deformability has been shown for nearly all types of hemolytic anemias studied to date.

The aging red cell appears to progressively lose the ability to maintain functional hemoglobin. Methemoglobin concentration increases in cells of greater age (11). 2,3-DPG and ATP bind tightly to deoxyhemoglobin and facilitate $O_2$ release (12,13). The diminished 2,3-DPG level in older cells appears to lead directly to a change in oxyhemoglobin dissociation (14).

The hexose monophosphate shunt (HMS) is critical for the function of hemoglobin and other red blood cell components during exposure to oxidant compounds. Defects in the several enzymes of the HMS may make the erythrocytes vulnerable to oxidative damage (15,16). The selective enzymatic oxidation of glutathione by low levels of $H_2O_2$ mediated by glutathione peroxidase and the subsequent regeneration of reduced glutathione by this shunt serves to protect the reactive sulfhydryl groups of hemoglobin and the cell membrane. Normal human hemoglobin has two easily titratable sulfhydryl groups at the β93 cysteine position. Oxidation of these groups tends to decrease the affinity of globin for heme (17). Since heme plays an important role in maintaining the structural configuration of globin (18), a decrease in the heme-globin affinity will lead to precipitation of denatured globin as Heinz bodies at the cell membrane.

Phenylhydrazine and related redox compounds are known to

cause sulfhydryl blockage and hemoglobin denaturation (19). In the presence of oxygen these compounds oxidize hemoglobin to methemoglobin. A second step is to oxidize reduced glutathione (GSH), with formation of oxidized glutathione (GSSG) and mixed disulfides with the β93 cysteine of hemoglobin. Both steps are reversible if reduced glutathione (GSH) can be regenerated by the HMS shunt. Failure of the regeneration systems as in G-6-PD deficiency or glutathione reductase deficiency result in oxidative injury to the hemoglobin molecule and its denaturation. As Heinz bodies are attached, the cell membrane is rigid and permeability to cations increases (20,21), and it is thought that these changes, particularly the increased rigidity, lead to sequestration and phagocytosis of the cells within the reticuloendothelial system.

II. Erythrocyte Separation Techniques.

Red cell populations of defined age may be obtained in several ways. One of the most commonly employed methods is density separation. The specific gravity of red cells increases with cell age, as shown by the early work of Halm and coworkers (22); all investigators agree that old cells are on the average more dense than young cells. This is used as a basis for separating old from young cells by centrifuging the cells, either in their own plasma or in a medium of known density. Both hypodense and isodense centrifugation methods are popularly used as techniques to separate red cells by age.

A hypodense medium is one in which the density of the medium

is less than the density of the erythrocytes. During centrifugation, while all erythrocytes go to the bottom of the tube, the more dense cells move to the bottom of the column of cells, forcing the less dense cells to the top. The upper layer of the red cell mass now contains more young cells and reticulocytes than the bottom layer which contains predominantly old cells.

An isodense medium is one in which the medium density is somewhere between that of the least dense and the most dense red cells. During centrifugation, erythrocytes will seek an equilibrium position. When only one density is present, the cells will float or sink, depending on their density relative to the density of the medium.

By the choice of the medium density, it is possible to separate a red cell population by density into any two subpopulations. Rebanding of the layers has shown that red cells do reach an equilibrium density position and has confirmed the reproducibility of this method. For these reasons, the use of isodense media for red cell age separation is currently the most popular method.

Media which have been used for isodense separation are aqueous (Bovine albumin, Ficoll, Stractan or Dextran) and non-aqueous (mixtures of dimethyl phthalate and dibutyl phthalate).

Isodense media can also be used as a continuous gradient or as a discontinuous gradient. A continuous density gradient is one in which the medium density increases continuously toward the bottom

of the tube. It will not produce discrete red cell density populations, except at the top and the bottom. There will be a continuous distribution of red cells throughout the medium. By contrast, a discontinuous gradient, in which the medium density decreases in a stepwise fashion, would produce a discrete red cell density population at each medium density interface. The red cells found between two medium densities would have a range of densities corresponding to the densities of the bordering media.

Danon and Marikovsky (23) used phthalate esters as media in a discontinuous gradient. There are two disadvantages of this lipid medium. First, it is known to change the surface properties of the red cell membrane (24). Second, phthalate esters give good separation only in capillary hematocrit tubes, used in a microcentrifuge. With this centrifuge, temperature cannot be adequately controlled.

Recently Schulman (25), Brinke and De Regt (26) used low molecular weight dextran as a discontinuous gradient to separate erythrocytes. Dextran is a polymer of glucose with a main chain of glucose units attached to each other by α-1,4 glycosidic bonds and side chain attached by α-1,3 or α-1,4 glycosidic linkage. While high molecular weight dextran increases red cell aggregation in vitro (27), dextran with a molecular weight less than 60,000 will not promote aggregation.

In this study, 40,000 molecular weight dextran was used as a separation medium to avoid aggregation and rouleaux formation.

Further reasons for the use of a discontinuous dextran density gradient included economy, ability to control osmotic conditions, simplicity of preparation, reproducibility and good age-related separation characteristics of the method.

III. Studies of Cells with Glycolytic Enzyme Defects.

A. Classification of congenital nonspherocytic hemolytic anemias.

Sterile, defibrinated blood stored at 37°C without additives for 48 hours may undergo "autohemolysis". Selwyn and Dacie (22) classified congenital nonspherocytic hemolytic anemia (CNSHA) into two types based on autohemolysis.

Type I. The autohemolysis is mild and is reduced by adding glucose or ATP during the incubation. Decreased intermediate phosphorylated compounds in the glycolytic pathway such as 2,3-DPG, ATP and inorganic phosphate were found in this type (28,29). This indicates there was a specific enzyme defect before the synthesis of 2,3-DPG. Decreased ATP substrate in CNSHA Type I affects the rate limiting enzymes, hexokinase and phosphofructokinase. Type I CNSHA include HK deficiency and PFK deficiency.

Type II. The autohemolysis is increased and is not corrected by glucose, but is corrected by ATP. In common with Type I CNSHA, there is a deficiency in erythrocyte ATP and an excess of inorganic phosphate. In Type II, however, there is an increase of 2,3-DPG (30,31). Pyruvate kinase deficiency is the most common

example of Type II, CNSHA.

B. Genetics of erythrocyte pyruvate kinase.

Erythrocyte PK deficiency is relatively common in Caucasians and cases have also been reported in Negroes, Orientals and American Indians. The incidence of the heterozygous state may be estimated by studying the occurrence of decreased enzyme activity. In a recent German survey, 1.4% of healthy young adults were apparently heterozygous (32), while testing of 400 consecutive newborn in Hong Kong indicated an incidence of 3.4% (33).

Family studies indicate that PK deficient hemolytic anemia is inherited as an autosomal recessive and both sexes are equally affected. Adults heterozygous for erythrocyte PK deficiency have only one-half normal PK activity in their red cells, and show no clinical disease (30). Several Chinese neonates with apparent heterozygosity, however, did have hyperbilirubinemia (33). In adults, severe and chronic hemolytic anemia has only been observed in homozygotes. There is no correlation of sex with severity of the disease or the enzyme level. Some mutant forms of the PK deficient cells show a decreased level of the pyruvate kinase activity but normal kinetics (Vmax and $K_m$PEP), others have abnormal kinetic characteristics with varying affinity of the enzyme for its substrates.

It is possible to distinguish the mutants of this enzyme into three groups on the basis of enzyme kinetics.

1. Decreased activity and normal $K_m$(PEP) (34,35)

2. Decreased activity and decreased $K_m$(PEP) (36,37)

3. Slightly decreased or normal activity and increased $K_m$(PEP) (38,39)

C. Genetics of erythrocyte glucose-6-phosphate dehydrogenase.

The genetic information for G-6-PD is carried on the X chromosome. Thus males carry a single G-6-PD gene; if this is mutant, it may be expressed as G-6-PD deficiency. The homozygous deficient females are rare. It may be anticipated that female heterozygotes for G-6-PD deficiency would exhibit only half the normal female level of the enzyme. In fact, such heterozygotes may have any level of G-6-PD from total absence to normal levels. This phenomenon is explained by the X inactivation hypothesis which proposes that at an early developmental stage one of the X chromosomes in each cell is blocked. This X inactivation is a random process and can produce an entire spectrum of phenotypes in heterozygotes.

The first variant G-6-PD was found in Negroes because of an associated primaquine induced hemolytic anemia. The $Gd^{A-}$ variant is common in Negroes but is rare in Caucasians (40). Exposure of these patients' rbc to primaquine causes loss of rbc reduced glutathione (GSH) (41). Further work showed that this and related abnormal events were caused by a striking diminution in G-6-PD activity in the patients' erythrocytes. Primaquine induced hemolysis in American Negroes has been found to be self-limited even when drug administration is continued. This occurs because the younger cells contain

sufficient enzyme to resist the effect of the hemolytic drug. In general, however, the clinical course of hemolysis tends to be more severe in cases of Favism; exposure to the fava bean produces no hemolysis in G-6-PD deficient Negroes but may produce hemolytic anemia in those with the Mediterranean type of G-6-PD deficiency.

More than 80 variants of abnormal G-6-PD have been reported in human populations. These variants are distinguished from one another by parameters such as the quantitative level of red cell G-6-PD activity and electrophoretic mobility. G-6-PD type B ($Gd^B$) is the most common in most populations, and represents the standard 100% of normal activity. Other biochemical parameters such as $K_m$(G-6-P, NADP), pH activity curve, heat stability and utilization of substrate analogues, such as 2-deoxy glucose-6-phosphate are used for differentiating variants of G-6-PD.

Beutler (42) and Yoshida et al. (43) classified the different variants of G-6-PD according to their degree of deficiency and clinical behavior into five groups.

1. Increased enzyme activity (44)
2. Very mild or no enzyme deficiency (45)
3. Moderate to mild enzyme deficiency (46,47)
4. Severe enzyme deficiency (48)
5. Severe enzyme deficiency associated with congenital nonspherocytic hemolytic anemia (49,50)

D. Age-related changes in glycolytic enzymes.

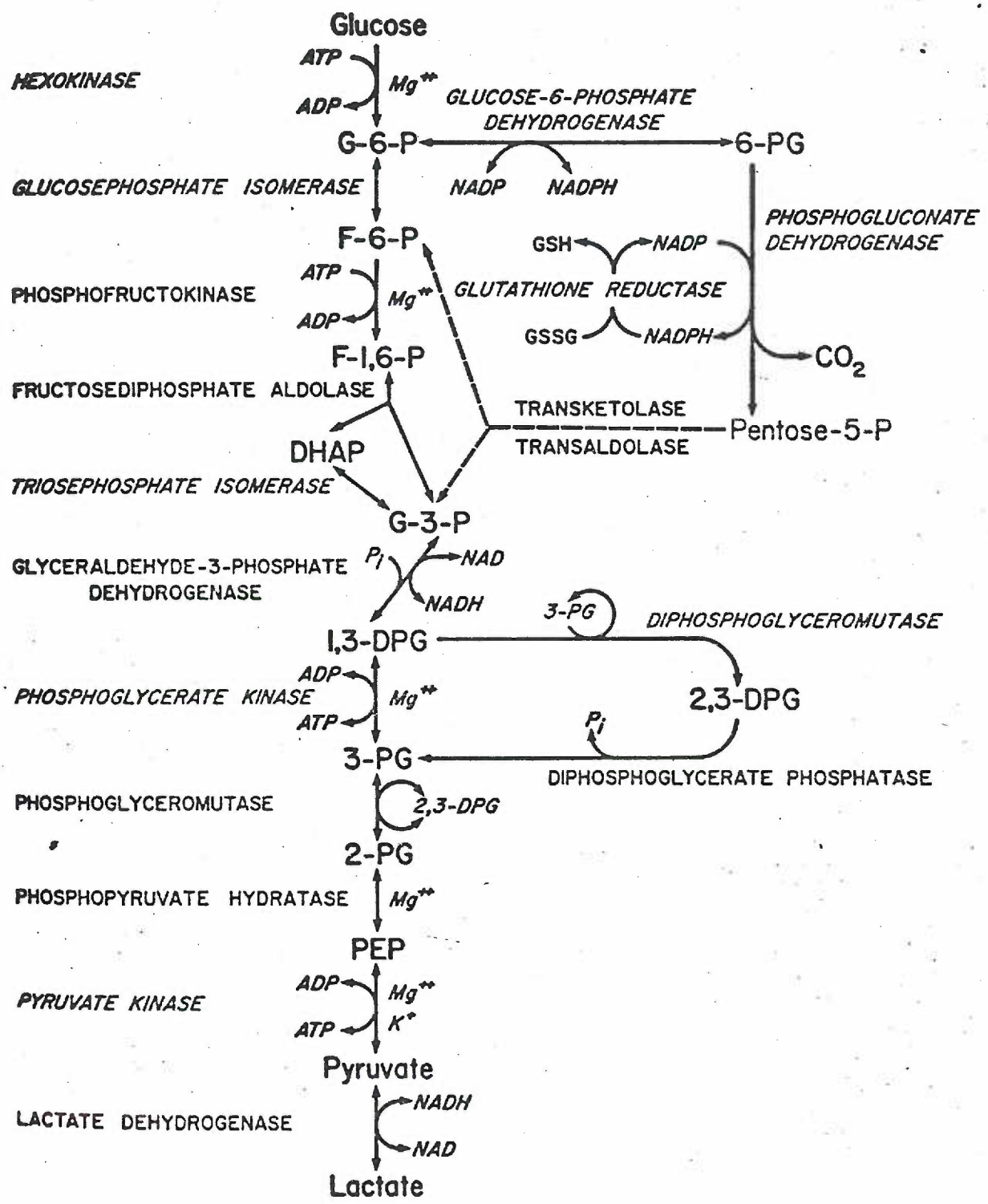
Many of the enzymes of the red cell show activity differences with cell age. Figure I indicates the major erythrocyte carbohydrate metabolic pathways and their participating enzymes. Earlier studies have shown extensive decreases in the catalytic activity of various enzymes with age. Hexokinase (51,52) is one of the most age-dependent of the red cell enzymes and has been used as an indicator of mean cell age. Although triose phosphate isomerase and glyceraldehyde phosphate dehydrogenase were reported to be decreased in old cells by Lohr et al. (53), Powell and Brewer (54) found no age related decrease in GAPD activity. Phosphoglycerate kinase (53) and pyruvate kinase (53, 55,56) activities decrease with age.

Of the hexose monophosphate shunt enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase have age-dependent activities in red blood cells (57,58). Glutamic oxalacetic transaminase activity shows the greatest decrease in activity in the course of maturation of the reticulocytes (59).

Congenital nonspherocytic hemolytic anemias provide an interesting group of conditions for aging studies. In some of these diseases, a genetic defect in one enzyme or in a membrane property is known, but the reason these lead to early cell death is not yet clear. In these patients, red cell survivals are shorter than normal. This may arise from primary defects at several levels of the intracellular systems. These include glycolytic metabolism, hemoglobin stability and membrane structure of red cells. Pyruvate

Figure I

The pathways of erythrocyte glycolysis (60).


Glucose
ATP
ADP
Mg++
HEXOKINASE
GLUCOSE-6-PHOSPHATE DEHYDROGENASE
G-6-P
6-PG
NADP
NADPH
GLUCOSEPHOSPHATE ISOMERASE
PHOSPHOGLUCONATE DEHYDROGENASE
F-6-P
GSH
NADP
GLUTATHIONE REDUCTASE
PHOSPHOFRUCTOKINASE
GSSG
NADPH
CO2
F-1,6-P
FRUCTOSEDIPHOSPHATE ALDOLASE
TRANSKETOLASE
TRANSALDOLASE
Pentose-5-P
DHAP
TRIOSEPHOSPHATE ISOMERASE
G-3-P
Pi
NAD
NADH
GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE
3-PG
DIPHOSPHOGLYCEROMUTASE
1,3-DPG
2,3-DPG
PHOSPHOGLYCERATE KINASE
3-PG
DIPHOSPHOGLYCERATE PHOSPHATASE
PHOSPHOGLYCEROMUTASE
2,3-DPG
2-PG
PHOSPHOPYRUVATE HYDRATASE
PEP
PYRUVATE KINASE
K+
Pyruvate
LACTATE DEHYDROGENASE
Lactate

kinase and glucose-6-phosphate dehydrogenase deficiencies are the prototypes of this group selected for study in the present work.

As outlined above, studies of hemolytic anemias may benefit from studies of red cell aging. If red cell studies reveal some abnormality in the glycolytic pathways, should this abnormality be considered as the specific abnormality of the disease or is it a reflection of the presence of a young cell population? In answering this question, it is necessary to correlate glycolytic enzyme activities with red cell age in normal and abnormal red cells. This is the approach taken in this study to resolve the question of the presence of a specific enzyme lesion or a non-specific age-related change in enzyme activity in a given hemolytic anemia.

# EXPERIMENTAL

## I. Materials.

### A. Samples.

1. 50 cc samples of control blood were collected from four healthy female and five healthy male volunteers.

2. Congenital nonspherocytic hemolytic anemia, type II, due to group I red cell pyruvate kinase deficiency with normal $K_m$ PEP.

The first RBC PK deficient patient was C. J., age 24. Her Hb was 8.8 gm %, Hct 29.5%, RBC 2.1 x $10^6$ cells/cu. mm., WBC 5,600 cells/cu. mm. and reticulocyte count 65.4%. The red cell pyruvate kinase was 12% of normal.

The second PK patient was her brother M. J., age 25. His Hb was 9.9 gm %, Hct 30.5%, RBC 2.3 x $10^6$ cells/cu. mm., WBC 14,900 cells/cu. mm. and reticulocyte count 33%. The red cell PK was 10.2% of normal.

3. G-6-PD deficiency type 4 (severe enzyme G-6-PD deficiency).

This group of G-6-PD deficiencies is always found in people from the Mediterranean area where it is relatively common. The phenotype of this G-6-PD deficient variant is $Gd^{Mediterranean}$. The sensitivity to hemolysis inducing agents and the severity of the individual episodes are more marked than with the other variants. Electrophoretic

mobility is the same as $Gd^B$ but other biochemical parameters such as Km (G6P, NADP), heat stability and pH optima are abnormal.

A. V., 24 years, was classified as having this variant on the basis of his Greek descent, the severity of his hemolytic episodes and sensitivity to fava bean. His Hb was 13.8 gm %, Hct 38%, RBC 4.6 x $10^6$ cells/cu. mm. and reticulocyte count 0.8%. His red cell G-6-PD was 20.4% of normal.

4. G-6-PD deficiency type 5 (severe enzyme G-6-PD associated with congenital nonspherocytic hemolytic anemia).

This is a rare set of variants found in people of West European descent. It is associated with chronic hemolytic anemia even in the absence of exposure to drugs. The enzyme activity is markedly decreased, the degree of deficiency varying with different types. $Gd^{Chicago}$ and $Gd^{Oklahoma}$ are examples of this type. While electrophoretic mobility is normal, all other biochemical parameters including Km G6P, Km NADP, and heat stability are abnormal.

S. Z., 21 years, has red cell G-6-PD, 4.3% of normal. Kinetic enzyme parameters of this patient have not yet been analyzed. On the basis of his chronic, severe congenital nonspherocytic hemolytic anemia, his Northern European ancestry and his low level of red blood cell G-6-PD activity he has been classified in this group.

At the time he was studied his Hb was 13 gm %, Hct 48%, RBC 3.5 x $10^6$ cells/cu. mm., WBC 7,000 cells/cu. mm. and reticulocyte count 5.6%.

B. Substrates and reagents.

The sodium salt of adenosine 5'-diphosphate (ADP) grade I, the sodium salt of adenosine 5'-triphosphate (ATP) grade I, the tetrasodium salt of D-fructose-1,6-diphosphate (FDP) grade II, the glycylglycine free base, the dithiothreitol (DTT) or Cleland's reagent, the flavine adenine dinucleotide (FAD), the purified oxidized glutathione (GSSG), the purified reduced glutathione (GSH), the DL Asparate (Asp), the monosodium salt of α-ketoglutarate, the tricyclohexylamine salt of phosphoenolpyruvate (PEP), the 2,3-diphosphoglyceric acid (2,3-DPG), the 2-phosphoglyceric acid (2-PGA), the 3-phosphoglyceric acid (3-PGA), rabbit muscle lactic dehydrogenase (LDH), pig heart malic dehydrogenase (MDH), yeast α glyceraldehyde dehydrogenase type III (GDH), rabbit muscle pyruvate kinase Type II (PK), Baker's yeast triose phosphate isomerase (TPI), Baker's yeast glucose-6-phosphate dehydrogenase (G-6-PD), rabbit muscle glyceraldehyde phosphate dehydrogenase (GAPD), the glutathione reductase (GSSG-R), the D(+) anhydrous glucose, grade III, the triethanolamine (TEA), tris and disodium EDTA, recrystallized orcinol were purchased from Sigma Chemical Co., Saint Louis, Missouri. The reduced form of disodium-nicotinamide adenine dinucleotide (NADH), the oxidized, and the reduced form of disodium-nicotinamide adenine dinucleotide phosphate (NADP, NADPH), the glucose-6-phosphate (G6P), the 6-phosphogluconate (6-PG), the 3-phosphoglycerate kinase grade A (3-PGK) and rabbit muscle aldolase were purchased from Calbiochem, La Jolla,

California.

Dextran T40 (MW = 40,000) lot numbers 4743 and 1600 were purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey. The tert-butyl hydroperoxide (TBH) was purchased from K and K Laboratories, Inc., Irvine, California. The metaphosphoric acid reagent was purchased from Matheson, Coleman and Bell, Norwood, Ohio; $MgCl_2$, $MgSO_4$, KCl, NaCl, $Na_3C_6H_5O_7$, $Na_2HPO_4$, $NaH_2PO_4$ were all reagent grade.

C. Solutions.

1. Stabilizing solution contains 2.7 mM neutralized EDTA and 0.7 mM 2-mercaptoethanol.

All the enzymes were diluted with stabilizing solution. The substrates were diluted with distilled water. All solutions were stored in the ice-bath.

2. Standard diluent (pH 7.4, osmolarity = 287 mOsm/kg of water, density = 1.005 gm $cc^{-1}$ at 25°C) consisted of 145.4 mM NaCl, 5.36 mM KCl, 1.08 mM $Na_2HPO_4$, 1.10 mM $KH_2PO_4$, 0.63 mM $MgSO_4 \cdot 7H_2O$ and 6.11 mM glucose. This was used for both dissolving the dextran and washing the red cells.

3. 0.9% sodium chloride solution.

4. Dextran solutions.

Stock solutions of 35% w/v dextran were prepared weekly by dissolving the dextran in standard diluent, using a magnetic stirring

bar and gentle heat until the dextran was in solution. After air bubbles disappeared, the solution was slowly diluted with 2-3 additions of standard diluent, and finally the volume was adjusted in a volumetric flask. It was stored at 4°C. The 35% dextran stock solution was diluted with standard diluent to make dextran concentrations from 24% to 32% w/v in 1% increments.

II. Methods.

A. Hematological techniques.

1. Instrumentation: Gilford 2000 multichannel absorbance recorder; Sorvall RC2-B centrifuge; Zeiss Model PMQ II spectrophotometer.

2. Hematocrit was determined by the standard microhematocrit technique using an International Model MB centrifuge.

3. Hemoglobin concentration was measured by the cyanmethemoglobin method in a Hemoglobinometer at 540 nm.

4. Red cell counts were obtained in a Celloscope Model 202 red cell counter with a 1:200,000 dilution and a 100 μ opening to minimize coincidence error. The solution used for dilutions contained 0.9% NaCl.

5. Reticulocyte counts were obtained after supravital staining with new methylene blue.

B. Measurement of dextran density.

The density of dextran was measured by first weighing a dry 2 ml pycnometer bottle on an analytical balance at 25°C. After the

addition of 2 ml of dextran solution to the pycnometer bottle, it was incubated at 25°C for 10 minutes and then weighed again. The difference between the first and the second weights was the weight of dextran in that concentration. The density of dextran was calculated as

$$\text{density} = \frac{\text{weight of dextran solution in gm}}{\text{volume of pycnometer in cc}} = (\text{gm/cc})$$

The volume of the pycnometer was determined by weighing the pycnometer with distilled water and dividing by the density of water, which equals 0.99707 gm $cc^{-1}$ at 25°C.

$$\text{Volume of pycnometer} = \frac{\text{weight of distilled water (gm) at 25°C}}{\text{density of distilled water (gm } cc^{-1})}$$

The density of each sample was measured twice.

C. Preparation of white blood cell and platelet free red cell suspension. (61)

Both normal and patient blood samples were diluted in the proportion of nine parts of whole blood to one part of 3.8% sodium citrate solution used as the anticoagulant. The white cells and platelets were removed by a single filtration through cotton at room temperature. Three gm of 100% cotton was boiled with distilled water for five minutes. This was repeated five times, each time decanting and replacing the distilled water. The cotton was then suspended and equilibrated to 0.9% NaCl. Finally, the cotton was transferred to a column of 150 mm diameter and washed twice with standard diluent.

A blood sample of 50 ml can be passed through the column in 30-40 minutes. The white blood cells and platelets adhere to the cotton. It must be emphasized that only pure cotton is suitable for this filtration method. Contamination of cotton results in poor separation of cells.

After removing the white blood cells and platelets, the red blood cells were washed with standard diluent and packed by centrifugation at 25°C, 2,000 rpm (650 xG) for 10 minutes in a Sorvall centrifuge. Contaminating white cells remaining in the red cell fraction were counted in a counting chamber after dilution with 2% acetic acid, or in the Coulter counter.

D. Test for agglutination of red blood cells due to dextran.

Five to ten ml of blood (nine parts) was mixed with 3.8% sodium citrate solution (one part) as an anticoagulant. After the removal of white cells and platelets with a single filtration, the remaining red cells were washed three times with the standard diluent. A drop of the final packed red cell preparation was added to three drops of 35% and of 15% dextran. Satisfactory results were no visible agglutination or rouleaux formation under microscopic examination of a wet drop preparation at either dextran concentration.

E. Methods of separation of red cells (Fig. II).

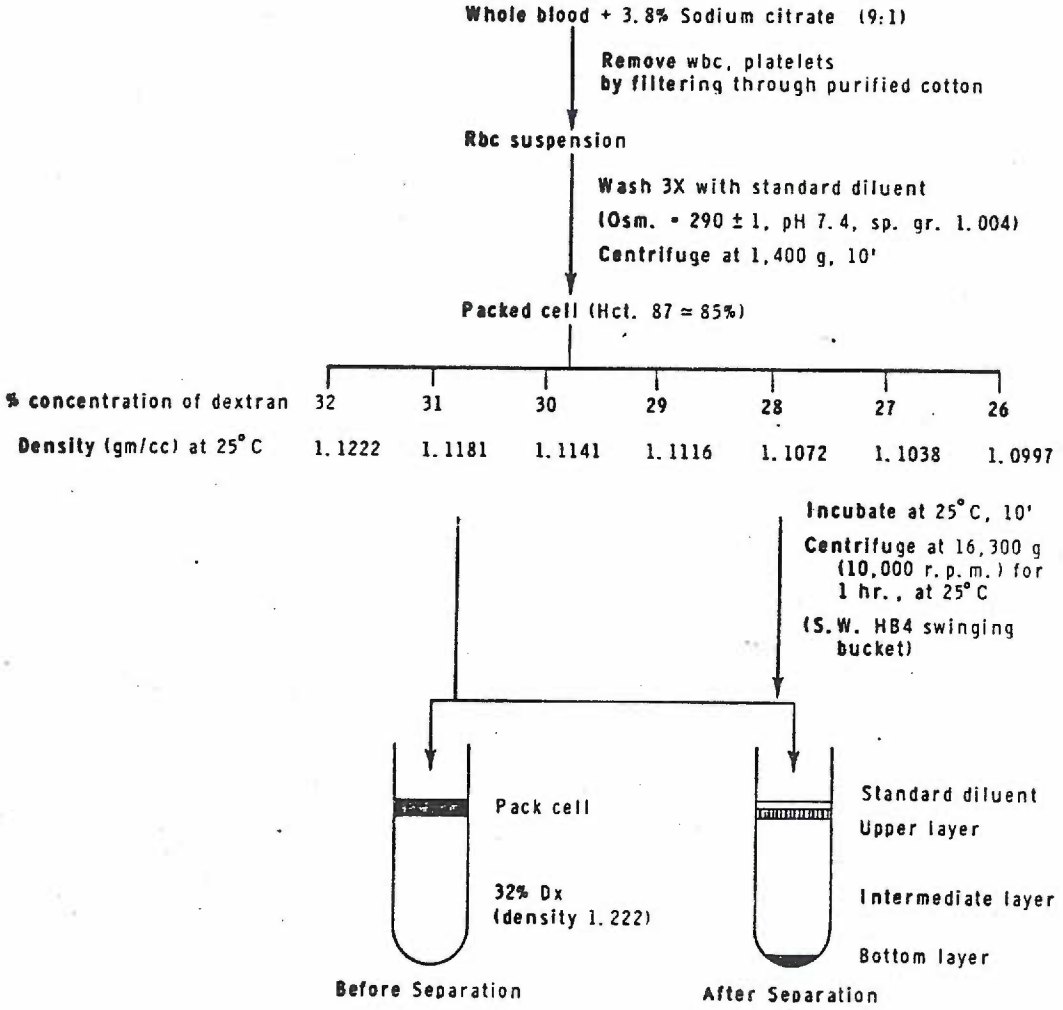
Gradients were prepared by layering 25 ml of a given dextran concentration by syringe into polypropylene centrifuge tubes of 50 ml total volume. Each gradient was loaded with three ml of

Figure II

Procedure for the separation of red blood cell by a dextran gradient.

**PROCEDURE FOR THE SEPARATION OF RBC BY A DEXTRAN GRADIENT**

(Dextran T40, average M.W. = 40,000)



packed red cells using a siliconized one ml volumetric pipet, and was then centrifuged in a Sorvall HB-4 swinging-bucket rotor at 16,300 G (10,000 rpm) at 25°C for one hour.

After centrifugation the supernatant standard medium layer on the top of the gradient, which contained no red cells, was taken off and discarded. The red cells in the top, intermediate, and bottom layers were removed by syringe as demonstrated in Figure II.

Fractions were washed two times in standard diluent and red cell count, Hb, Hct, and reticulocyte count were determined for each layer and the fraction of the total red cell population in each fraction was calculated.

1. Definition of red cell populations (Figure III).
    a. The mixed cells represent the whole red cell population after removal of white blood cells and platelets.
    b. The young cells were defined as approximately the lightest 10% of red blood cells. This was the top layer for density gradients of low specific gravity.
    c. The old cells were defined as approximately the heaviest 10% of red blood cells. They represent the bottom layer for a density gradient of higher specific gravity.
    d. The intermediate cells represent a cell population

Figure III

The procedure for separation of the erythrocyte into young, intermediate and old cell populations in normal subjects.

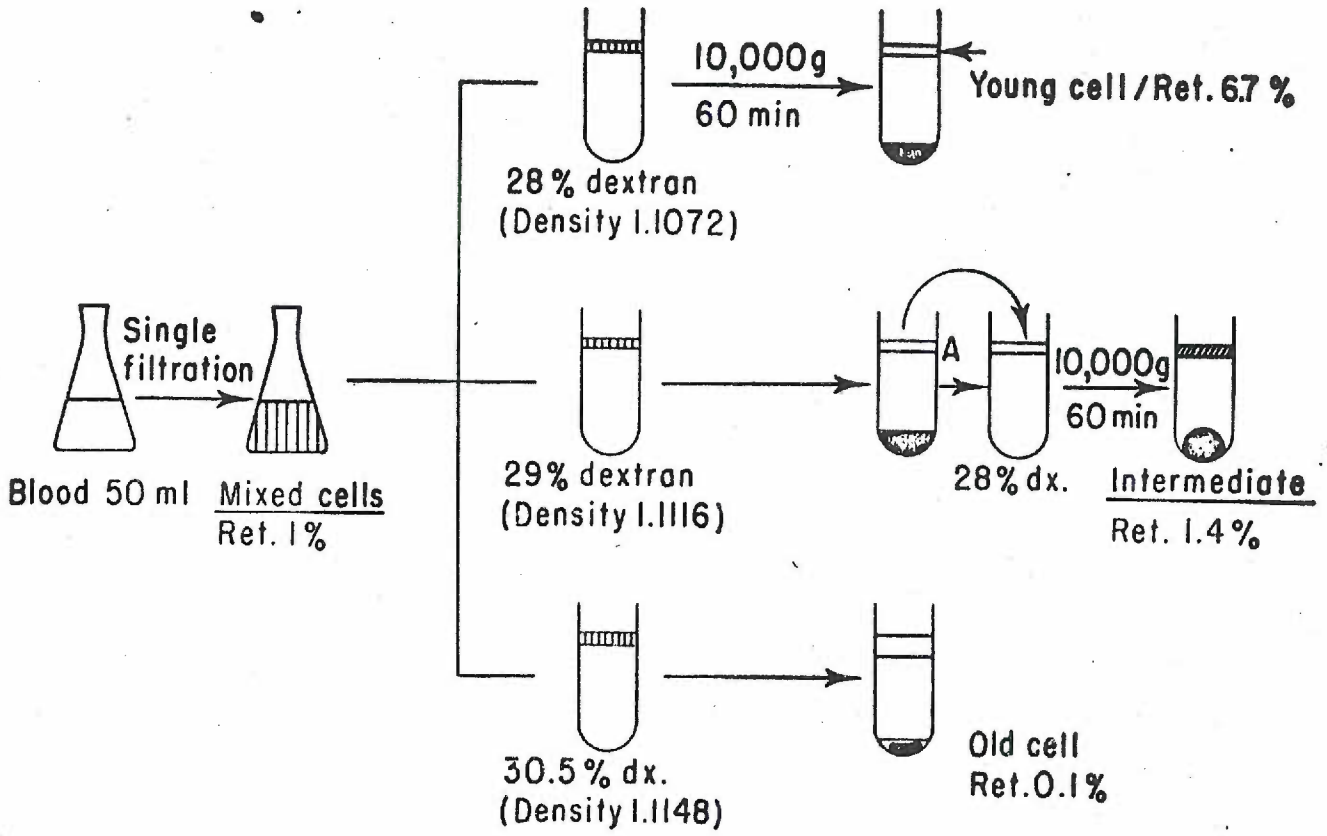



**Figure III:** The procedure for separation of the erythrocyte into young, intermediate and old cell populations in normal subjects

which was obtained by density separations on a gradient intermediate between the gradient used to obtain young and old cells.

As shown in Figure III, in normal subjects 28% dextran was used to separate the young cells, 30.5% dextran was used to separate the old cells, and the intermediate population represents red cells which remain at the top of a 29% dextran and sediment through a 28% dextran solution. For separation of the intermediate red cells in G-6-PD, type 5 (severe G-6-PD deficiency with congenital nonspherocytic hemolytic anemia), 28% dextran was used to separate the young cells and 32% dextran was used to separate the old cells. The intermediate population of this patient was obtained by using 30% dextran as the first medium and transferring the top fraction from 30% dextran to 29% dextran. The final cell pellet represented the intermediate population.

2. Rebanding experiment.

Duration and gravity force of centrifugation are the major factors influencing the efficiency of red cell separation. A stable equilibrium separation is achieved if centrifugation force and time are sufficiently large. The method to be described was designed to demonstrate that equilibrium had been achieved by a given procedure and that the final red cell position was reproducible relative to the red cell density and medium density. It is similar to the rebanding procedure developed by Bishop and Prentice (62) to

confirm equilibrium in a continuous gradient experiment.

One ml of normal packed red cells is layered on 28%, and on 30.5% dextran, and spun at 10,000 rpm (16,300 g) for 45 min. at 25°C. After separation the upper (young) layer from 28% dextran, and the lower (old) layer from 30.5% dextran were washed once with standard diluent. Packed young cells were then transferred to a new 28% dextran solution while old cells were transferred to a 30.5% dextran solution. Both were spun again. After the second centrifugation all the cells in the 28% dextran remained in the top fraction and all the cells in the 30.5% dextran concentration went to the bottom of the tube. This can be taken as evidence that the gravity force of 16,300 g and a time of 45 minutes with a control temperature of 25°C will give a separation that has reached equilibrium.

F. Enzyme assays.

The glutamic oxalacetic transaminase was assayed by the method of Blume (61). Assays for all the other enzymes listed below were done according to Beutler (63).

1. Hexokinase (HK)
2. Glucose phosphate isomerase (GPI)
3. Phosphofructokinase (PFK)
4. Triosephosphate isomerase (TPI)
5. Glyceraldehyde-3-phosphate dehydrogenase (GAPD)
6. Phosphoglycerate kinase (PGK)
7. Enolase (EN)

8. Pyruvate kinase (PK)
9. Glucose-6-phosphate dehydrogenase (G-6-PD)
10. 6-Phosphogluconic dehydrogenase (6-PGD)
11. Glutathione reductase (GSSG-R)
12. Glutathione peroxidase (GSH-Px)
13. Glutamic oxalacetic transaminase (GOT)

G. Preparation of hemolysates.

The mixed, the young and the old red cells were resuspended in the standard diluent in the dilution 1:4. Red cell counts and hemoglobin determinations made from those suspensions were used to express the enzyme activities in terms of units/$10^{10}$ RBC or units/gm Hb.

Hemolysate was made by mixing one volume of the above red cell suspension with nine volumes of stabilizing solution. This hemolysate was rapidly frozen one time in methanol at -20°C for 15 minutes. After thawing at room temperature, this hemolysate was ready for assay.

H. Assay at 340 nm.

Velocities of change in optical density were followed in matched (0.5 cm or one cm light path) quartz cuvettes. For one ml reaction mixtures a one cm light path was used; 0.5 cm light path cuvettes were used for reactions in which there were significant amounts of Hb as in the GOT reaction. The change in optical density with time (ΔOD/min) at 340 nm are recorded in a Gilford 2000 multi-channel absorbance recorder. The temperature of the cuvette chamber

was maintained with a Heto circulating water bath at 37°C. The test samples and blanks were run in triplicate. If the optical density differences between sample and blank varied excessively between samples, the assay was repeated.

I. RNA determination.

The transition between reticulocyte and mature stages of erythrocytes is accompanied by loss of RNA. RNA content may, therefore, be used to define the reticulocyte content of a given cell population. The absence of DNA in erythrocytes and reticulocytes simplifies the problem of extraction of nucleic acid from nonnucleated erythroid cells, and the nucleic acid in the cells is referred to as the RNA.

The RNA contents of the red cell populations were determined after Burka (64). In this method a colorimetric measurement is used to quantitate the content of pentose in the ribonucleic acid molecule. Pentoses on treatment with hot concentrated HCl are converted to furfural. The condensation of furfural with orcinol (5-methyl 1,3 dihydroxy benzene) in the presence of ferric ion gives a green complex with an absorption maximum at 660 nm. Burka used yeast RNA as a standard while D-ribose was used in this work.

J. Glutathione stability test.

Glutathione (GSH) is a tripeptide of glycine, glutamic acid and cysteine, which is synthesized by two enzymatic steps, glutamyl-cysteine synthetase, (I), and glutathione synthetase, (II).

$$ATP + \text{L-glutamate} + \text{L-cysteine} \xrightarrow{Mg^{++}} ADP + P_i + \text{L-glutamyl-L-cysteine} \quad (I)$$

$$ATP + \gamma\text{-L-glutamyl-L-cysteine} + \text{glycine} \xrightarrow{Mg^{++}} ADP + P_i + GSH \quad (II)$$

In normal red cells the level of reduced glutathione (GSH) is only slightly decreased after incubation with APH. Cells deficient in glutathione reductase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase activities show a large drop in reduced glutathione under APH stress. The level of reduced glutathione in young, old, and mixed cell populations was determined following Beutler (63).

K. Labie heat stability test (65).

Normal hemoglobin has a slight tendency to precipitate when heated at 65°C, whereas unstable hemoglobin will precipitate at a higher rate. Hemoglobin solutions (40 mg/ml), buffered at pH 7.5 in 0.02 M sodium phosphate, were incubated at 65°C. Aliquots were taken at defined intervals, cooled in an ice-bath and centrifuged. The remaining soluble hemoglobin was estimated in the supernatant.

# RESULTS AND INTERPRETATION

## I. Criteria for Old and Young Cell Populations.

In Figure IV the volume of red cells in the bottom layer, as a percentage of total red cell volume, was plotted against varying dextran densities for three normal subjects. It is assumed that a negligible number of cells is contained within the dextran. From these curves, density gradients were determined to give samples with approximately 10% of the cells in the top layer (young populations) or 10% in the bottom layer (old cells). Twenty-eight percent dextran (density 1.1072 gm $cc^{-1}$) was chosen as the medium to separate the top layer (10% young) and 30.5% dextran (density 1.1148 gm $cc^{-1}$) was used as the medium to separate the bottom layer.

The same approach was applied to patients with G-6-PD or PK deficiency. Due to increased erythrocyte production and a consequent shift in mean cell age and mean cell density, the dextran concentrations required for appropriate separation of enzyme-deficient RBC populations were different than for normal.

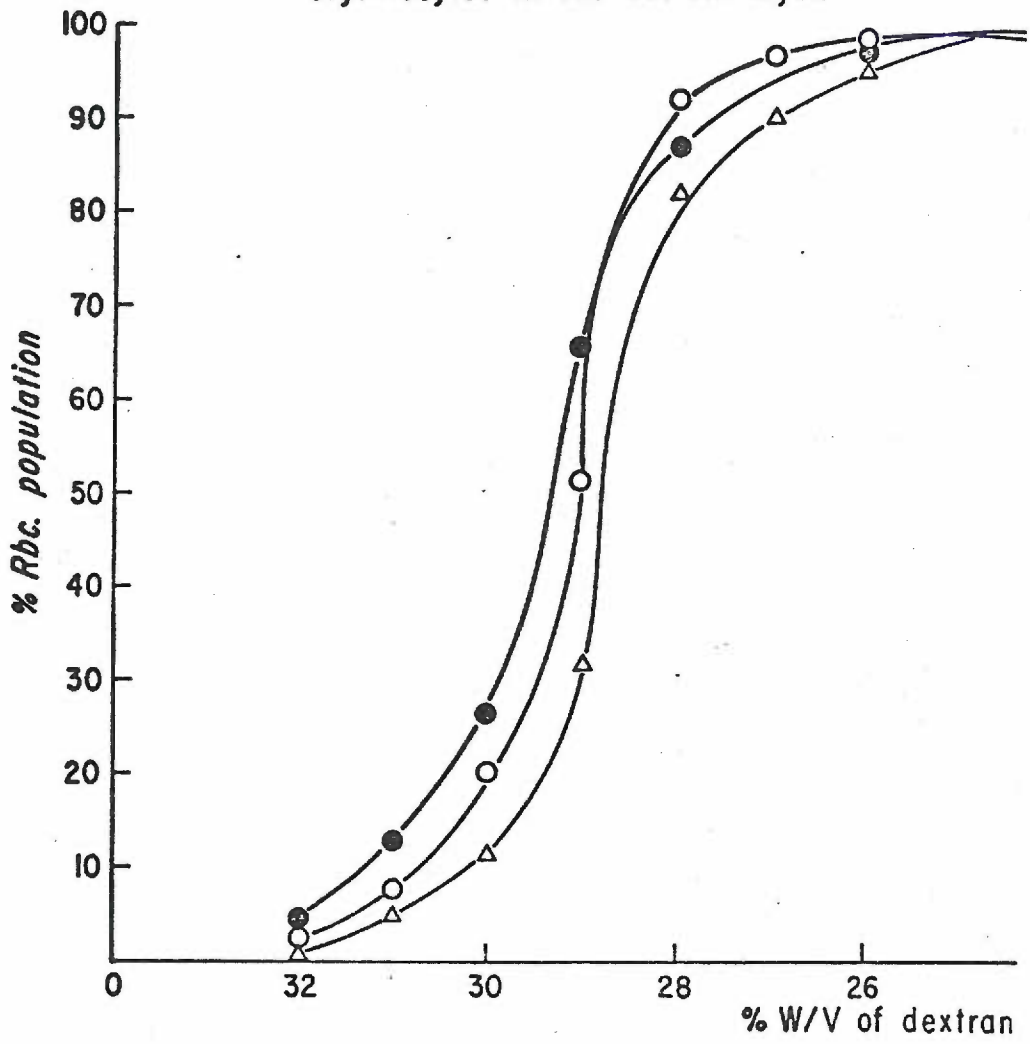
In both PK type 1 patients, 27% dextran (density 1.1038 gm $cc^{-1}$) was used to separate the young cells and 30% dextran (density 1.1141 gm $cc^{-1}$) was used to separate the old cells.

In the G-6-PD deficient patient, type 4, 29% dextran (density 1.1116 gm $cc^{-1}$) was used to separate the young cells and 31% dextran (density 1.1181 gm $cc^{-1}$) was used to separate the old cell population.

Figure IV

Density distribution of normal human erythrocytes in the bottom layer of three different subjects.

Density distribution of normal human erythrocytes in the bottom layer.



In the G-6-PD deficient patient, type 5, 28% dextran (density 1.1072 gm $cc^{-1}$) and 32% dextran (density 1.1222 gm $cc^{-1}$), respectively, were used to separate the young and old cells.

Table 1 presents the observed densities for dextran concentrations from 20 to 32%; these were prepared by diluting a 35% by weight dextran stock solution with standard diluent. The density of the stock solution was 1.1327 gm $cc^{-1}$. The average observed density of each concentration is compared to the calculated density and is found to be 0.005±0.001 gm $cc^{-1}$ less than calculated. The relationship between concentration of dextran in %w/v and density in gm/cc is graphically shown in Figure V. The Y intercept represents the density of standard diluent and agrees with the density as measured by pycnometry.

II. Studies of Enzymes in Normal Erythrocytes.

Tables 2 and 3 show the results of assays of 13 enzymes in a group of normal subjects. These enzymes may be placed in two groups on the basis of activity changes with cell age. The first enzyme group (Table 2 and Figure VI) shows a significant change in enzyme activity with increased red cell age. This group includes GOT, HK, PFK, G-6-PD and EN. The greatest ratio between young and old cell activities was found for GOT in which the ratio was seven. The rest, including HK, PFK, PK, G-6-PD, and EN, shows a young-to-old ratio of about two. Enzyme activity in the mixed cell population lies between the activities for young and old cells.

Table 1. Comparison of observed and calculated densities

| % Concentration of dextran | Observed densities (gm/cc) at 25°C | | | Calculated density* (gm/cc) at 25°C |
|---|---|---|---|---|
| | 1 | 2 | Average | |
| 32 | 1.1219 | 1.1224 | 1.1222 | 1.1270 |
| 31 | 1.1182 | 1.1184 | 1.1183 | 1.1232 |
| 30 | 1.1142 | 1.1141 | 1.1142 | 1.1204 |
| 29 | 1.1112 | 1.1120 | 1.1116 | 1.1160 |
| 28 | 1.1070 | 1.1073 | 1.1072 | 1.1124 |
| 27 | 1.1037 | 1.1039 | 1.1038 | 1.1088 |
| 26 | 1.0997 | 1.0996 | 1.0997 | 1.1048 |
| 25 | 1.0967 | 1.0967 | 1.0967 | 1.1001 |
| 24 | 1.0932 | 1.0931 | 1.0932 | 1.0972 |
| 20 | 1.0788 | 1.0788 | 1.0788 | 1.0816 |

*The difference between observed and calculated densities was 0.005±0.001.

$\rho^{T}_{dx}$ = density of dextran solution gm/cc = $\dfrac{M_{dx} + \rho^{T}_{sol}(V_T - \bar{V}_{dx}M_{dx})}{V_T}$

$M_{dx}$ = gm dextran in total volume of dextran solution (gm)

$\rho^{T}_{sol}$ = density of solvent at temp T (gm/cc)

$V_T$ = volume of total dextran solution (cc)

$\bar{V}_{dx}$ = partial specific volume of dextran = 0.611 cc/gm

Figure V

The relation between concentrations (%w/v) and densities (gm/cc) of dextran.

The relation between concentrations (%W/V) and densities (Gm/CC) of dextran

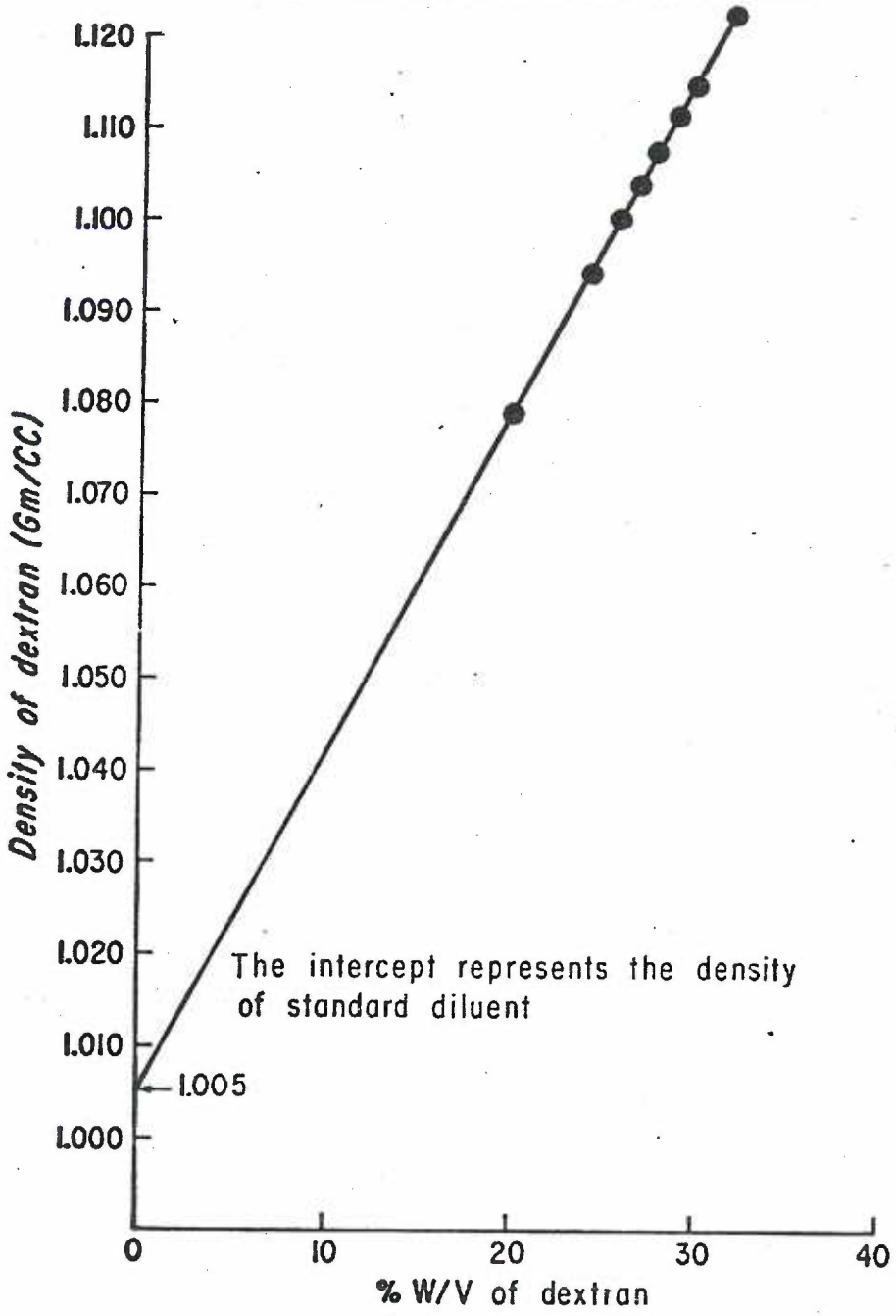


Table 2. Erythrocyte enzymes showing cell age related activity changes* (mean units per $10^{10}$ RBC ± 2S.D.)

| | GOT[1] | HK | PFK | PK | G-6-PD | EN |
|---|---|---|---|---|---|---|
| Sample | 9 | 9 | 7 | 9 | 9 | 3 |
| Young | 12.21±4.9 | 0.3±0.1 | 4.45±1.8 | 2.55±0.5 | 2.22±0.5 | 2.47±0.3 |
| Mixed | 3.49±1.6 | 0.2±0.1 | 2.27±1.6 | 1.60±0.8 | 1.40±0.6 | 1.53±0.5 |
| Old | 1.72±1.0 | 0.13±0.1 | 1.82±1.4 | 1.20±0.4 | 1.21±0.5 | 1.03±0.2 |
| Ratio Y/O | 7.1 | 2.3 | 2.5 | 2.1 | 1.8 | 2.4 |

[1]expressed as unit/gm Hb

*P <0.005

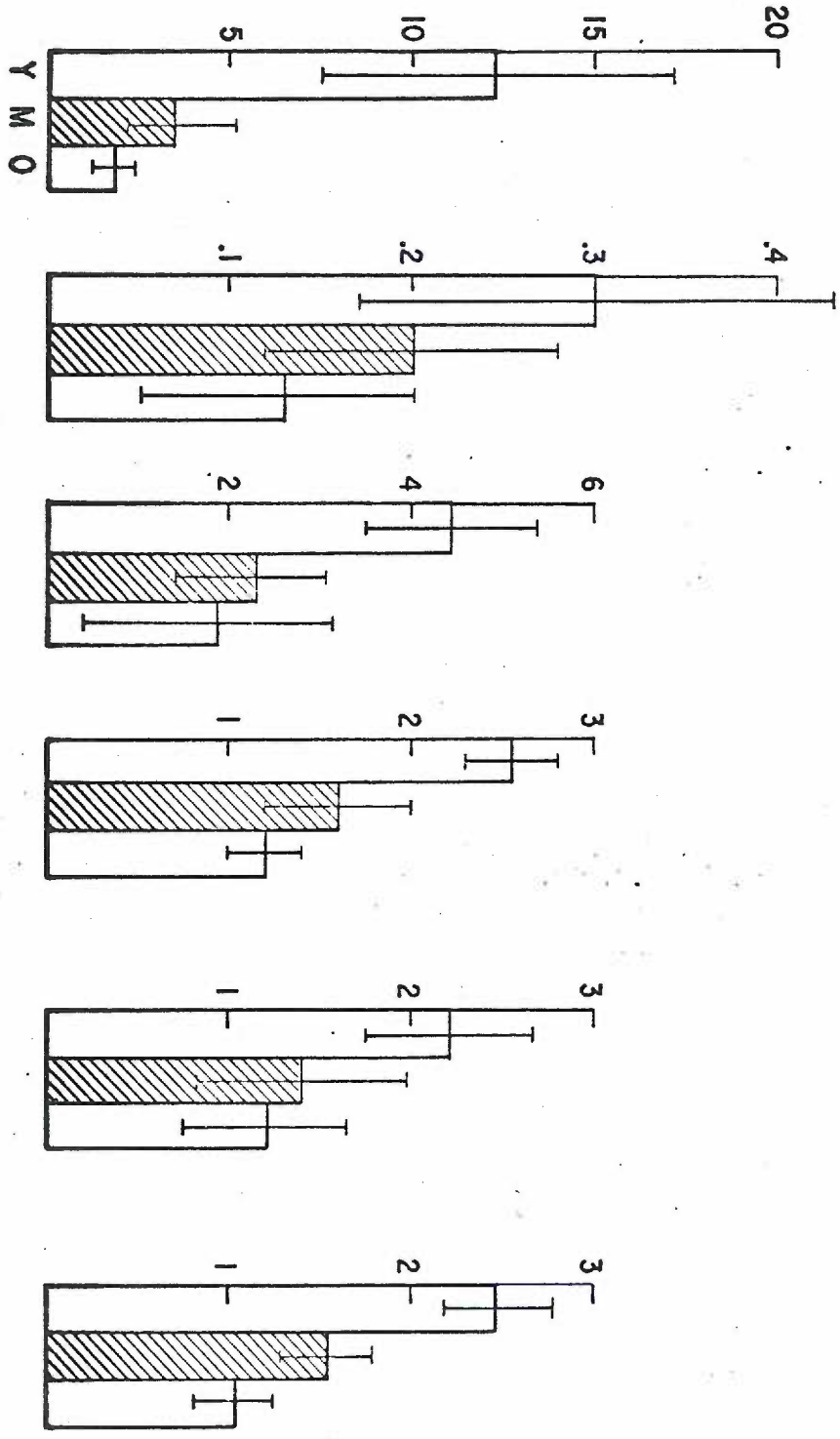
Table 3. Erythrocyte enzymes showing no significant change** in activity with cell age (mean units per $10^{10}$ RBC ± 2S.D.)

| | TPI | GAPD | GPI | PGK | 6-PGD | GSSG-R | GSH-Px |
|---|---|---|---|---|---|---|---|
| Sample | 9 | 9 | 9 | 9 | 9 | 9 | 4 |
| Young | 140.69±51 | 23.59±7.7 | 8.06±3.3 | 27.68±2.1 | 0.31±0.1 | 1.62±0.4 | 6.48±1.5 |
| Mixed | 138.71±49 | 19.55±7.8 | 6.63±2.6 | 26.54±2.7 | 0.28±0.1 | 1.19±0.4 | 6.15±0.7 |
| Old | 134.01±48 | 21.16±7.4 | 6.43±2.1 | 23.33±4.2 | 0.29±0.1 | 1.21±0.6 | 6.12±1.1 |
| Ratio Y/O | 1.1 | 1.1 | 1.3 | 1.2 | 1.1 | 1.3 | 1.1 |

**P >0.1

Figure VI

Red blood cell enzymes showing a significant decrease in activity between young (left) and old (right) cell populations. Activity for mixed cells are shown by the cross-hatched area. The bars represent the mean ± 2S.D.


GOT
HK
PFK
PK
G-6PD
EN
Y
M
O
5
10
15
20
.1
.2
.3
.4
2
4
6
1
2
3
1
2
3
1
2
3

The group of enzymes which showed no significant decrease in activity from young to old cells are described in Table 3 and Figure VII. In Table 4 there was a decrease in GSH between young and old cells both with and without incubation with acetylphenylhydrazine; it was not statistically significant, however.

III. Studies of Enzyme Deficient Erythrocytes.

A. Enzyme Assays.

The results in Table 5 show the enzyme activities in young and old cells for normal and PK deficient subjects. There are two points with respect to PK. First, in both PK, type 2, deficient patients the level of red cell pyruvate kinase was lower than normal in all cell populations. Further, the ratio of young to old activities was also lower than normal.

Second, the gene defect in PK deficiency did not affect other enzymes. For example, the levels of enzyme activity for PFK and HK are higher than normal in both patients, as expected with a younger than normal mean red blood cell age. Enzymes in the hexose monophosphate shunt, G-6-PD and 6-PGD, have increased activities. The level of GOT activity in young, mixed, and old cells were higher than normal but the Y/O ratio was nearly four-fold lower than normal. The reticulocyte count in the light (young) cells is 64 times higher than in dense (old) cells in normal subjects. In both PK patients each cell population had a high reticulocyte count. The reticulocyte ratio for young/old cells was also lower than normal. In the subjects with type 2 PK enzyme deficiency hemolysis is characterized by anemia, reticulocytosis, and Heinz body formation on exposure to

Figure VII

Red blood cell enzymes showing no significant decrease with age.

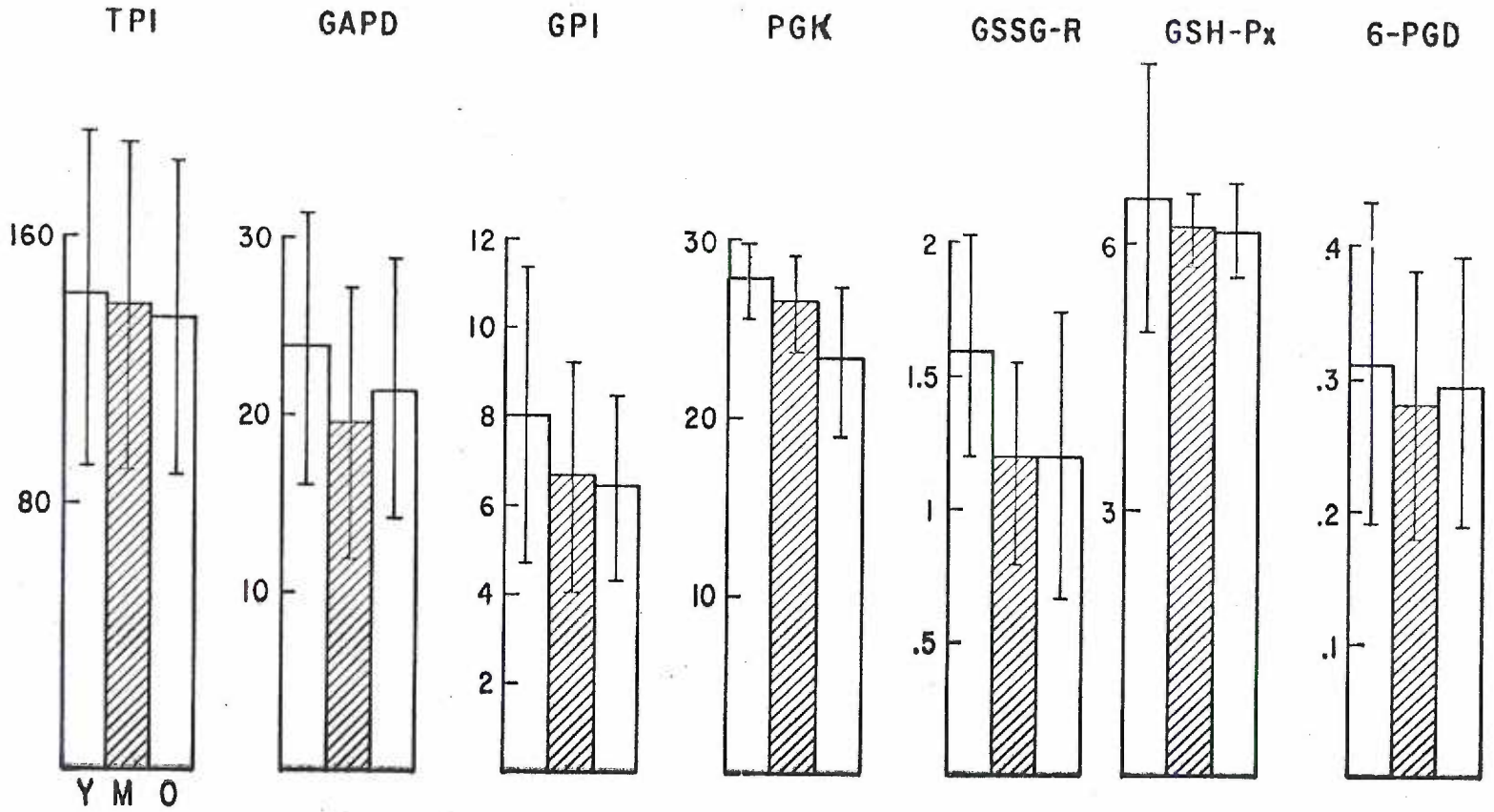

TPI
GAPD
GPI
PGK
GSSG-R
GSH-Px
6-PGD
160
80
30
20
10
12
10
8
6
4
2
30
20
10
2
1.5
1
.5
6
3
.4
.3
.2
.1
Y M O

Table 4. Comparison of glutathione stability in young and old red cells (mean ± two standard deviations of duplicate glutathione stability tests) from five normal subjects.

| | Glutathione (GSH) mg/100 ml red cells | |
|---|---|---|
| | Untreated | Incubated with acetylphenylhydrazine |
| Normal (n=5) | | |
| Young | 76.11±19.0* | 77.68±18.5** |
| Old | 65.17±34.2 | 63.58±23.2 |

* P >0.1
**P <0.05

Table 5. Results of erythrocyte enzyme assays** for normal and PK deficiency type 1 samples.

| | GOT | HK | PFK | PK | G-6-PD | 6-PGD | Retic% |
|---|---|---|---|---|---|---|---|
| Samples | 9 | 9 | 7 | 9 | 9 | 9 | 9 |
| | | | Normal | | | | |
| Young | 12.21±4.9* | 0.3±0.1 | 4.45±1.8 | 2.55±0.5 | 2.22±0.5 | 0.31±0.1 | 6.4 |
| Mixed | 3.49±1.6 | 0.2±0.1 | 2.27±1.6 | 1.60±0.8 | 1.40±0.6 | 0.28±0.1 | 1.0 |
| Old | 1.72±1.0 | 0.13±0.1 | 1.82±1.4 | 1.20±0.4 | 1.21±0.5 | 0.29±0.1 | 0.1 |
| Y/O | 7.1 | 2.3 | 2.5 | 2.1 | 1.8 | 1.1 | 64 |
| | | | PK type 1 (C.J.) | | | | |
| Young | 59.93 | 1.69 | 14.41 | 0.50 | 4.50 | 0.30 | 98 |
| Mixed | 40.59 | 1.05 | 13.81 | 0.41 | 3.20 | 0.42 | 55 |
| Old | 29.46 | 0.75 | 13.36 | 0.34 | 2.24 | 0.13 | 38 |
| Y/O | 2.03 | 2.25 | 1.08 | 1.47 | 2.01 | 2.31 | 2.57 |
| | | | PK type 1 (M.J.) | | | | |
| Young | 62.93 | 1.88 | 16.89 | 0.70 | 10.01 | 1.91 | 82 |
| Mixed | 36.94 | 1.31 | 13.83 | 0.38 | 5.13 | 1.51 | 33 |
| Old | 30.78 | 0.77 | 13.18 | 0.34 | 4.37 | 0.81 | 29 |
| Y/O | 2.04 | 2.44 | 1.28 | 2.05 | 2.29 | 2.86 | 2.82 |

*mean activity ± 2S.D.

**P <0.005

acetylphenylhydrazine or supravital dyes. The method used for reticulocyte count determination cannot separate the Heinz bodies from reticulocytes because both are stained with new methylene blue. Thus, the reticulocyte count is falsely high. The RNA determination which should not be affected by the Heinz body formation accurately reflects the true relative population of young cells.

Table 6 and Figure VIII show the enzyme activities in young, intermediate and old cells in three normal subjects and in two subjects with G-6-PD deficiency. Because the activity of 6-PGD was lower than the normal value reported by Blume and Beutler (61,63), it was thought that the phosphate buffer in the standard diluent interferred with this assay. Therefore, in this set of experiments 0.9% NaCl was used to wash the cells after separation had been completed. A higher 6-PGD activity was obtained and is comparable to reported results.

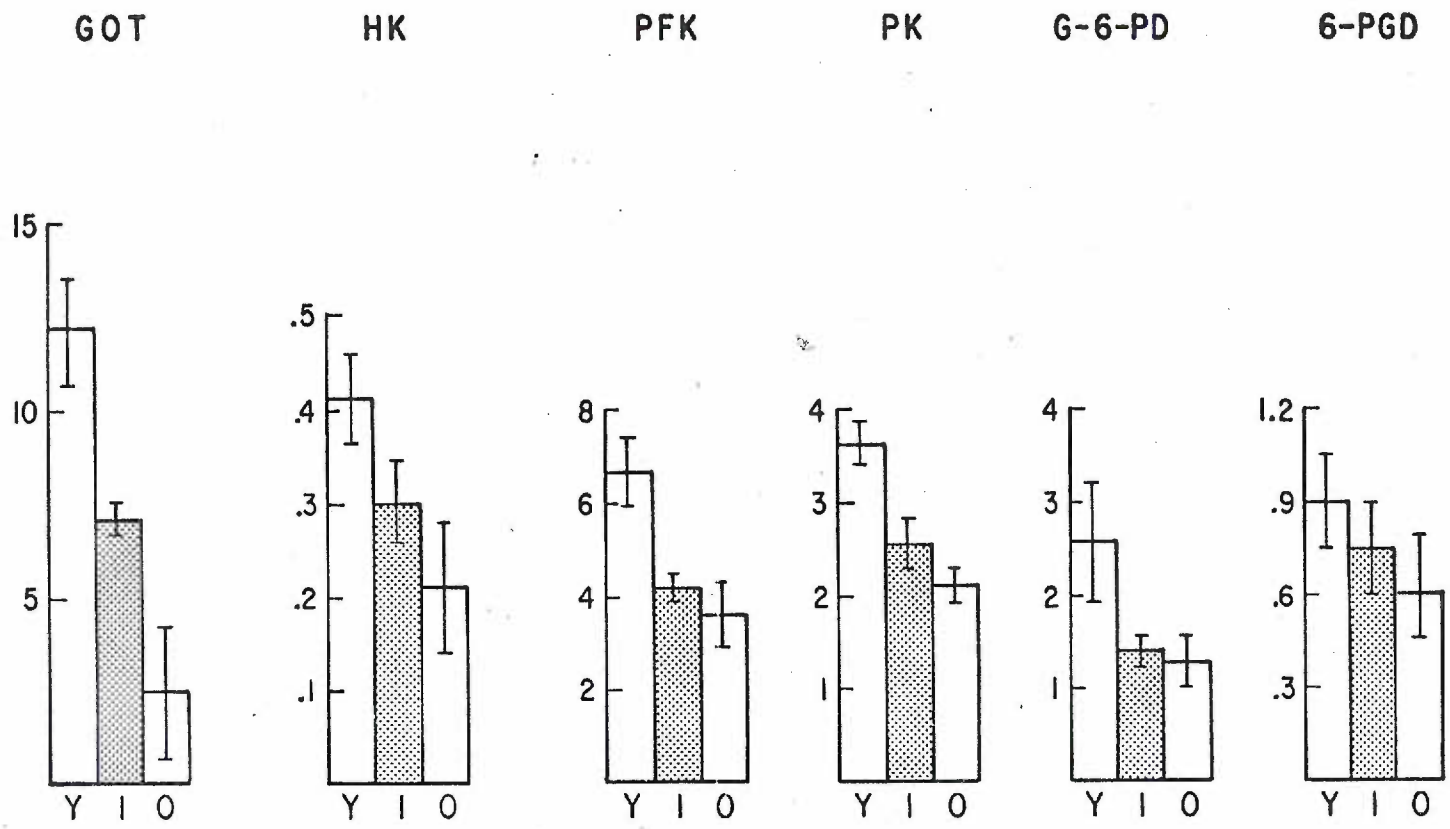
The ratio of young to old activities for HK, PFK, PK, G-6-PD is almost two times normal; the ratio for GOT is 4.9. The G-6-PD type 4 sample was analyzed before the technique for intermediate cell population separation was developed.

Most of the enzyme activities in Figure VIII showed a significant decrease in old cells when compared with young cells. Only 6-PGD, while showing a decreased activity with age, did not show a significant difference. The enzyme activity in intermediate cells was significantly lower than in young cells in GOT, HK, PFK, and G-6-PD.

Table 6. Enzyme activities ± 2S.D.* in three normal subjects and two G-6-PD deficient patients.

| | GOT | HK | PFK | PK | G-6-PD | 6-PGD | Retic% |
|---|---|---|---|---|---|---|---|
| Samples | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| | | | Normal | | | | |
| Young | 12.14±1.4 | 0.41±0.1 | 6.63±0.8 | 3.63±0.2 | 2.57±0.6 | 0.89±0.1 | 6.4 |
| Inter-mediate | 7.19±0.3 | 0.30±0.1 | 4.20±0.3 | 2.57±0.2 | 1.39±0.1 | 0.75±0.1 | 1.6 |
| Old | 2.47±1.8 | 0.21±0.1 | 3.63±0.7 | 2.11±0.2 | 1.28±0.3 | 0.62±0.1 | 0.1 |
| Y/I | 1.7 | 1.3 | 1.6 | 1.4 | 1.9 | 1.2 | 4 |
| Y/O | 4.9 | 2.0 | 1.8 | 1.7 | 2.0 | 1.4 | 64 |
| | | | G-6-PD type 4 (severe enzyme deficiency) (A.V.) | | | | |
| Young | 13.95 | 0.30 | 6.57 | 3.95 | 0.43 | 0.21 | 6.7 |
| Old | 2.83 | 0.09 | 2.20 | 1.96 | 0.24 | 0.02 | 0.2 |
| Y/O | 4.9 | 3.8 | 3.0 | 2.0 | 1.8 | 10.5 | 34 |
| | | | G-6-PD type 5 (severe enzyme deficiency associated with CNSHA) (S.Z.) | | | | |
| Young | 36.72 | 0.86 | 4.80 | 6.29 | 0.11 | 1.00 | 10.6 |
| Inter-mediate | 31.67 | 0.55 | 3.47 | 4.48 | 0.07 | 0.80 | 5.5 |
| Old | 26.18 | 0.27 | 1.22 | 3.37 | 0.06 | 0.71 | 2.4 |
| Y/I | 1.2 | 1.6 | 2.3 | 1.4 | 1.6 | 1.3 | 1.9 |
| Y/O | 1.4 | 3.2 | 6.4 | 1.9 | 1.8 | 1.4 | 4.4 |

*P <0.005


GOT
HK
PFK
PK
G-6-PD
6-PGD
15
10
5
.5
.4
.3
.2
.1
8
6
4
2
4
3
2
1
4
3
2
1
1.2
.9
.6
.3
Y I O
Y I O
Y I O
Y I O
Y I O
Y I O

Only GOT and PK showed significant differences in all three cell populations.

The loss of activity appears to proceed at all cell ages, as shown in Figures IX and X for the activity of HK, 6-PGD, G-6-PD, PK, GOT, and PFK in the three populations and G-6-PD type 5, respectively.

B. RNA as evidence for cell age.

The RNA data for the PK patients (Table 7) shows the anticipated absolute increase in RNA, and the Y/O ratio is comparable to that for normals. Both G-6-PD patients show an absolute decrease in G-6-PD activity from normal and like PK patients show a normal relative decrease in enzyme activity with age. They both also show an absolute increase in RNA.

C. Hemoglobin denaturation.

Table 8 and Figures XI, XII, and XIII show percent hemoglobin denaturation of three cell populations; mixed, young and old in five normal subjects. While there is no statistically significant difference in hemoglobin denaturation with age in these samples, there is a consistent pattern of increased denaturation in the older population.

One PK patient (see also Figure XIV) showed an increased rate of Hb denaturation when compared to a control. This increased rate of denaturation is particularly evident at the first five and ten minute periods when both mixed and PK red cells had nearly four times the denaturation rate of normal cells.

Figure IX

Mean activities of PFK (●—●), GOT (o—o), PK (▲—▲), G-6-PD (△—△), 6-PGD (■—■), and HK (□—□) in young (Y), intermediate (I), and old (O) red cells for three normal subjects.

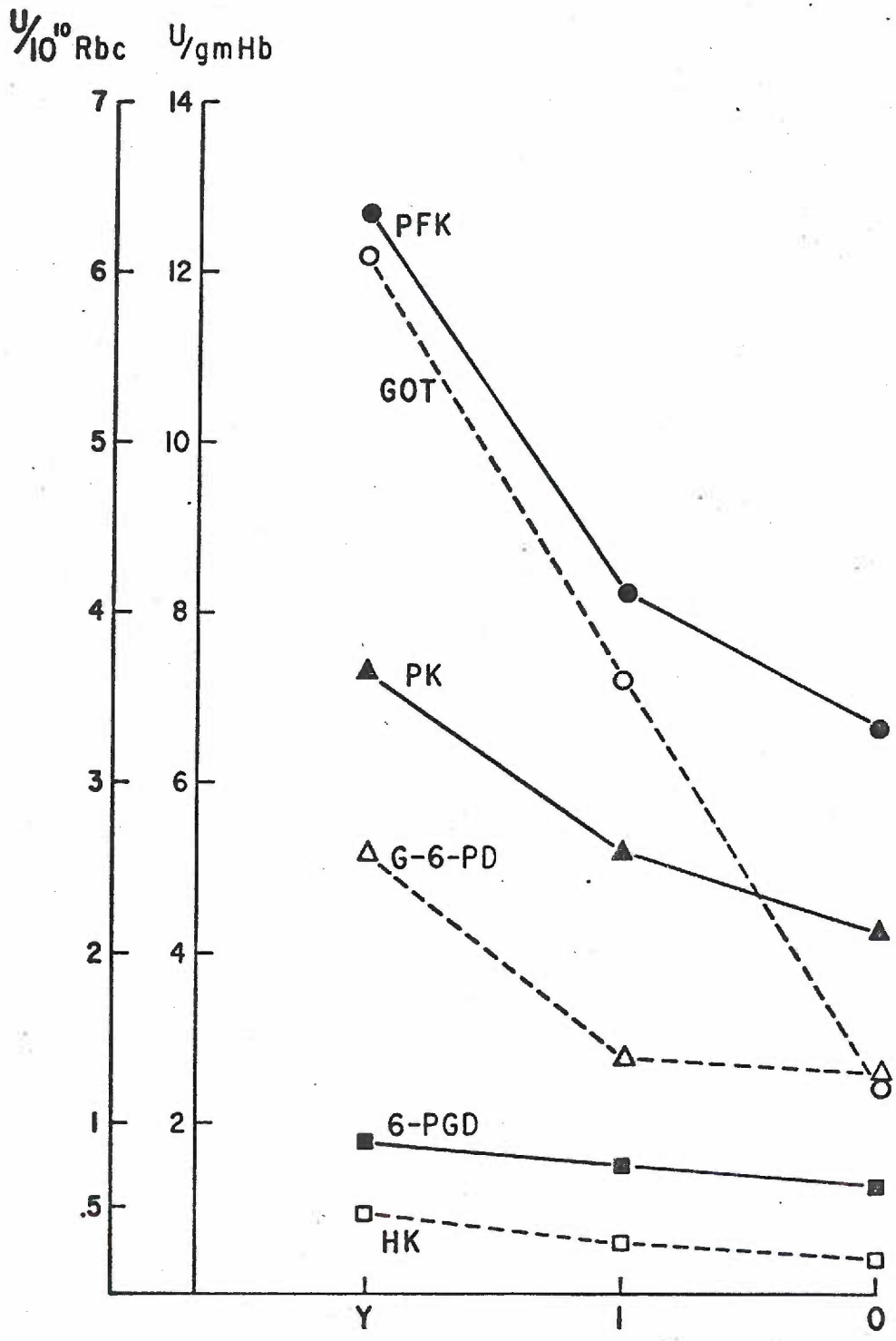

U/10^10 Rbc
U/gm Hb
7
6
5
4
3
2
1
.5
14
12
10
8
6
4
2
PFK
GOT
PK
G-6-PD
6-PGD
HK
Y
I
0

Figure X

Activities of PFK (●—●), GOT (o—o), PK (▲—▲), G-6-PD (Δ—Δ), 6-PGD (■—■), and HK (□—□) in young (Y), intermediate (I), and old (O) red cells of a G-6-PD type 5 patient.

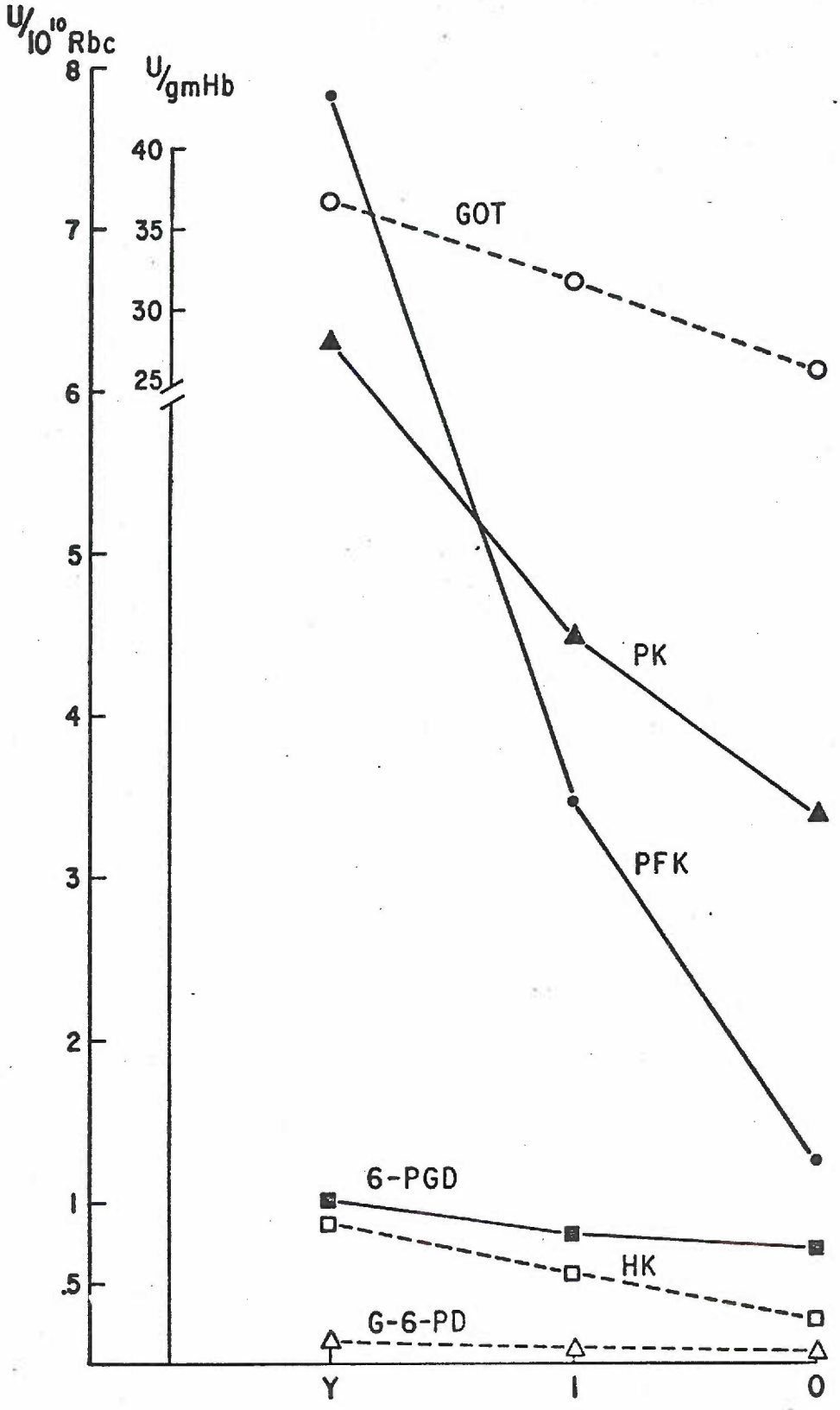

U/10^10 Rbc
U/gmHb
8
7
6
5
4
3
2
1
.5
40
35
30
25
GOT
PK
PFK
6-PGD
HK
G-6-PD
Y
I
0

Table 7. Reticulocytes, RNA, Pyruvate kinase (PK), Glucose-6-phosphate dehydrogenase (G-6-PD) in erythrocyte fractions of varying density.

+ mean of two results given in parentheses. * mean ± 2S.D.

| | Reticulocytes (%) | RNA mg/100 ml packed cells | PK $U/10^{10}$RBC | PK activity as % of young cells | G-6-PD $U/10^{10}$RBC | G-6-PD activity as % of young cells |
|---|---|---|---|---|---|---|
| Controls (n=3) | | | | | | |
| Young | 6.5/6.2(6.4)+ | 19.8/20.3(20.1) | 3.6±0.2 | 100 | 2.6±0.6 | 100 |
| Mixed | 1.0±1.0* | 15.0±2.5 | | | | |
| Old | 0.1/0.9(0.5) | 8.2/8.5(8.3) | 2.1±0.2 | 58.1 | 1.3±0.3 | 49.8 |
| Y/O | 12.7 | 2.4 | | | | |
| PK type 2 (C.J. and M.J.) | | | | | | |
| Young | 98/82(90) | 87.3/100(93.6) | 0.5/0.7(0.6) | 100 | | |
| Old | 38/29(33.5) | 29.5/35.2(32.3) | 0.3/0.3(0.3) | 56.7 | | |
| Y/O | 2.7 | 2.9 | | | | |
| G-6-PD type 4 (A.V., severe enzyme deficiency) | | | | | | |
| Young | 6.7 | 33.3 | | | 0.4 | 100 |
| Old | 0.2 | 13.7 | | | 0.2 | 55.8 |
| Y/O | 33.5 | 1.8 | | | | |
| G-6-PD type 5 (S.Z., severe enzyme deficiency associated with CNSHA) | | | | | | |
| Young | 10.6 | 47.7 | | | 0.1 | 100 |
| Old | 2.4 | 12.4 | | | 0.1 | 55.5 |
| Y/O | 4.4 | 3.9 | | | | |

Table 8. Denaturation of Hb with time in mixed, young, and old cells of five normal subjects and two patients.

| | Mean percent denaturation ± 2S.D. | | | | |
|---|---|---|---|---|---|
| Time (min) | 5[+] | 10[+] | 20[**] | 30[**] | 40[**] |
| Mixed | 2.16±1.3 | 7.84±2.0 | 14.82±3.1 | 30.45±6.6 | 46.47±9.7 |
| Young | 1.15±0.7 | 3.51±2.6 | 18.24±5.6 | 28.12±8.1 | 43.74±13.1 |
| Old | 2.84±2.3 | 6.39±2.2 | 20.04±7.0 | 32.77±10.5 | 45.50±9.0 |
| PK type 1 (C.J.) | | | | | |
| Control[*] | 2.04 | 6.12 | 12.24 | 25.51 | 36.22 |
| Mixed | 8.45 | 16.41 | 25.12 | 39.80 | 49.25 |
| Young | 7.07 | 19.00 | 29.79 | 44.95 | 54.04 |
| G-6-PD type 4 (A.V.) | | | | | |
| Control[*] | 0.95 | 4.40 | 11.19 | 21 | 33.9 |
| Mixed | 0.75 | 2.3 | 6.4 | 15 | 24.7 |
| Young | 0.76 | 1.9 | 8.4 | 12.2 | 19.1 |
| Old | 0.38 | 0.8 | 4.9 | 16.6 | 24.2 |

*A mixed population of normal red cells was used as a control done at the same time as the PK and G-6-PD deficient blood.

[+]P <0.025
[**]P >0.1

Figure XI

Limits of % Hb denaturation with time (mean ± 2S.D.) for the mixed red cells of five normal subjects.

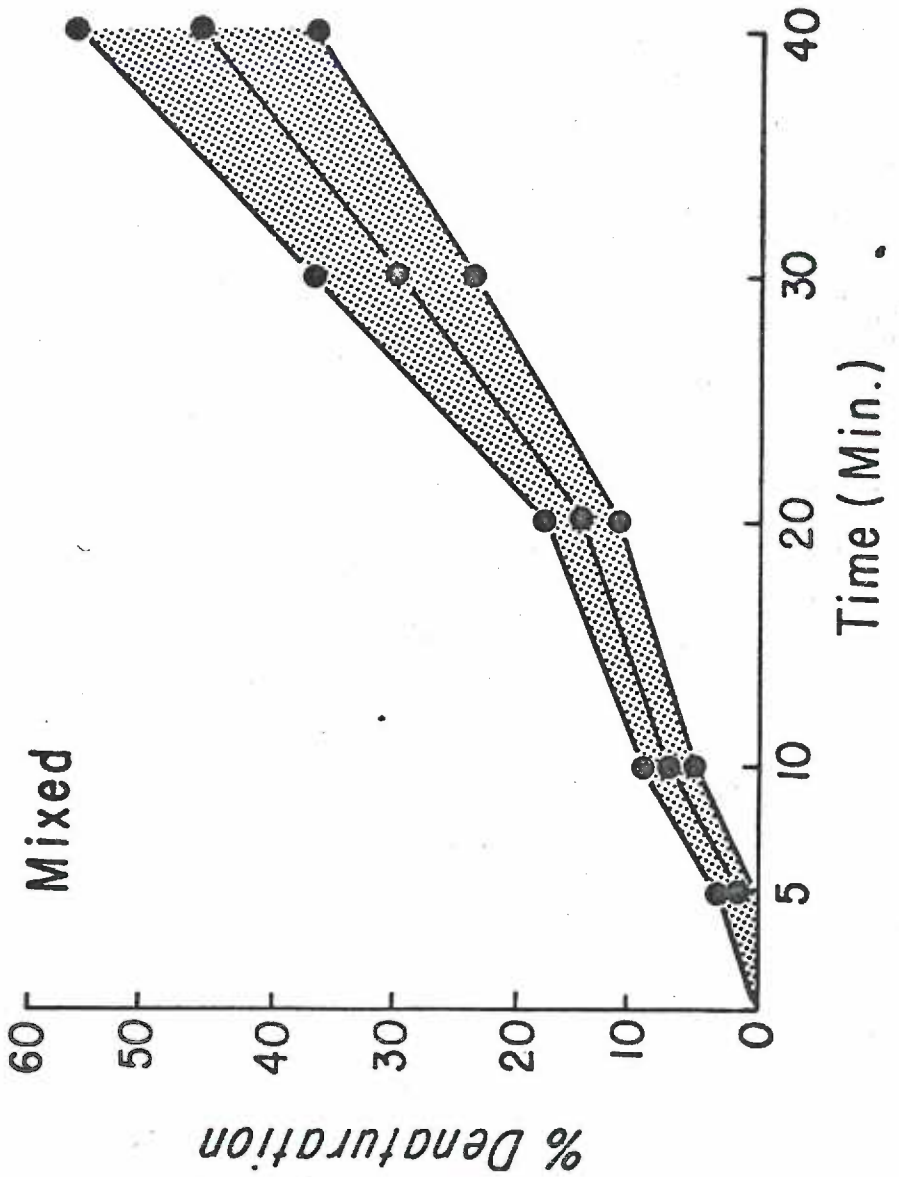

Mixed
% Denaturation
0
10
20
30
40
50
60
5
10
20
30
40
Time (Min.)

Figure XII

Limits of % Hb denaturation with time (mean ± 2S.D.) for the youngest ≈10% of red blood cells of five normal subjects.

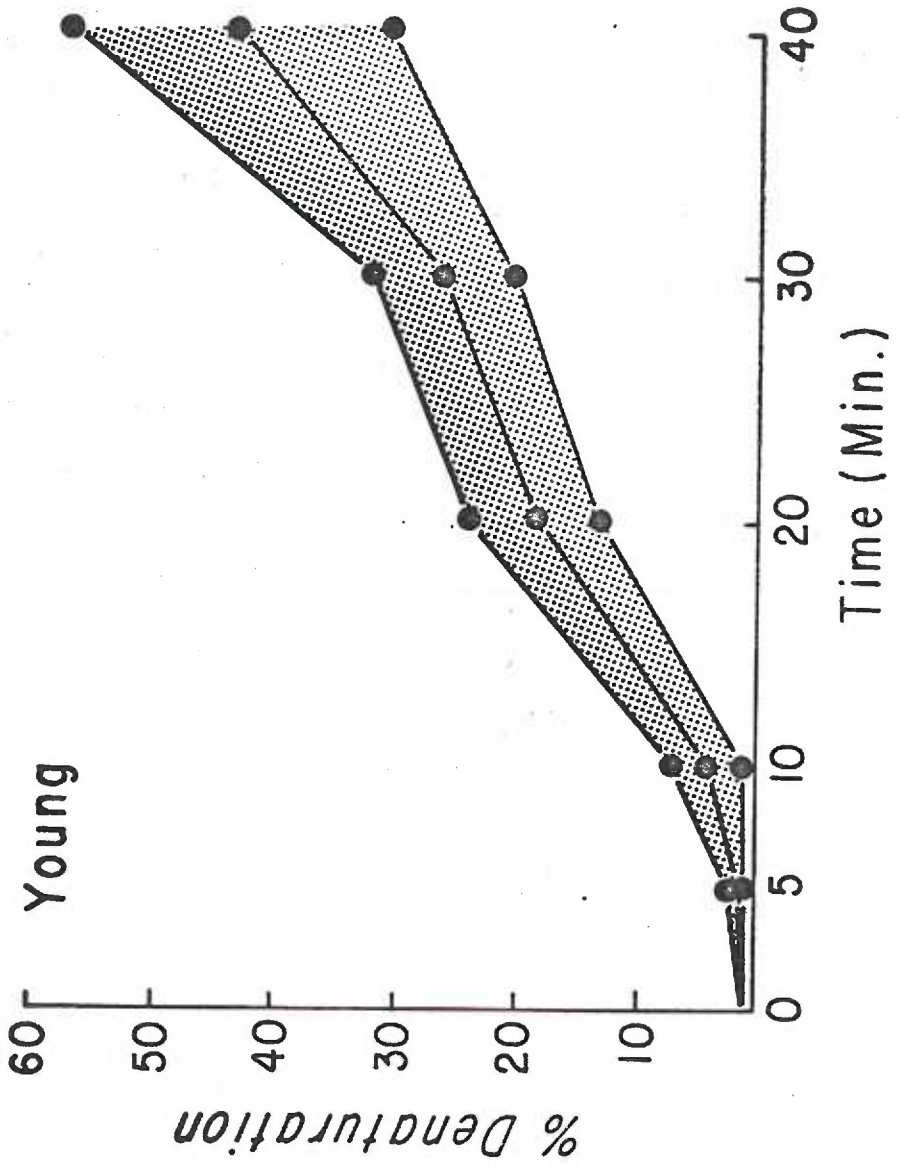

Young
% Denaturation
60
50
40
30
20
10
0
5
10
20
30
40
Time (Min.)

Figure XIII

Limits of % Hb denaturation with time (mean ± 2S.D.) for the oldest ~10% of red blood cells of five normal subjects.

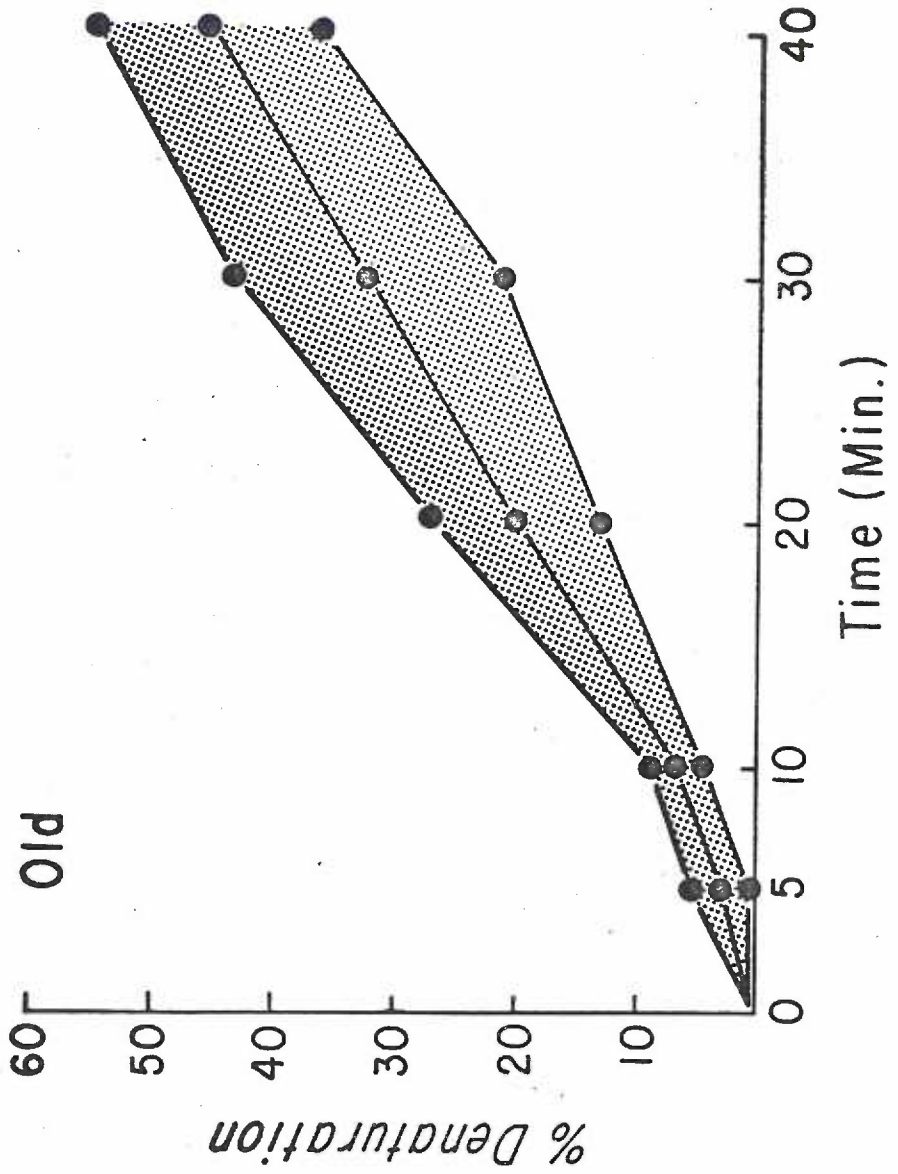
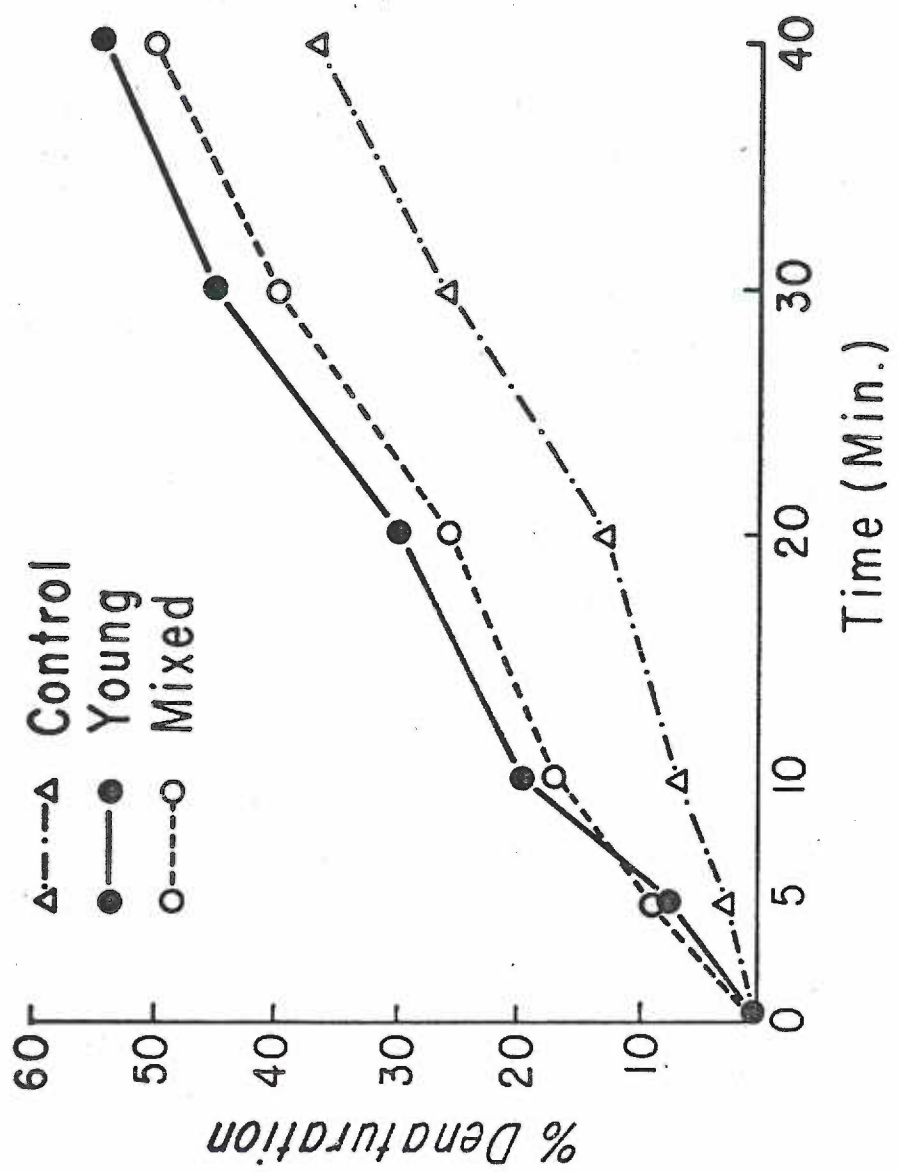

Old
% Denaturation
60
50
40
30
20
10
0
5
10
20
30
40
Time (Min.)

Figure XIV

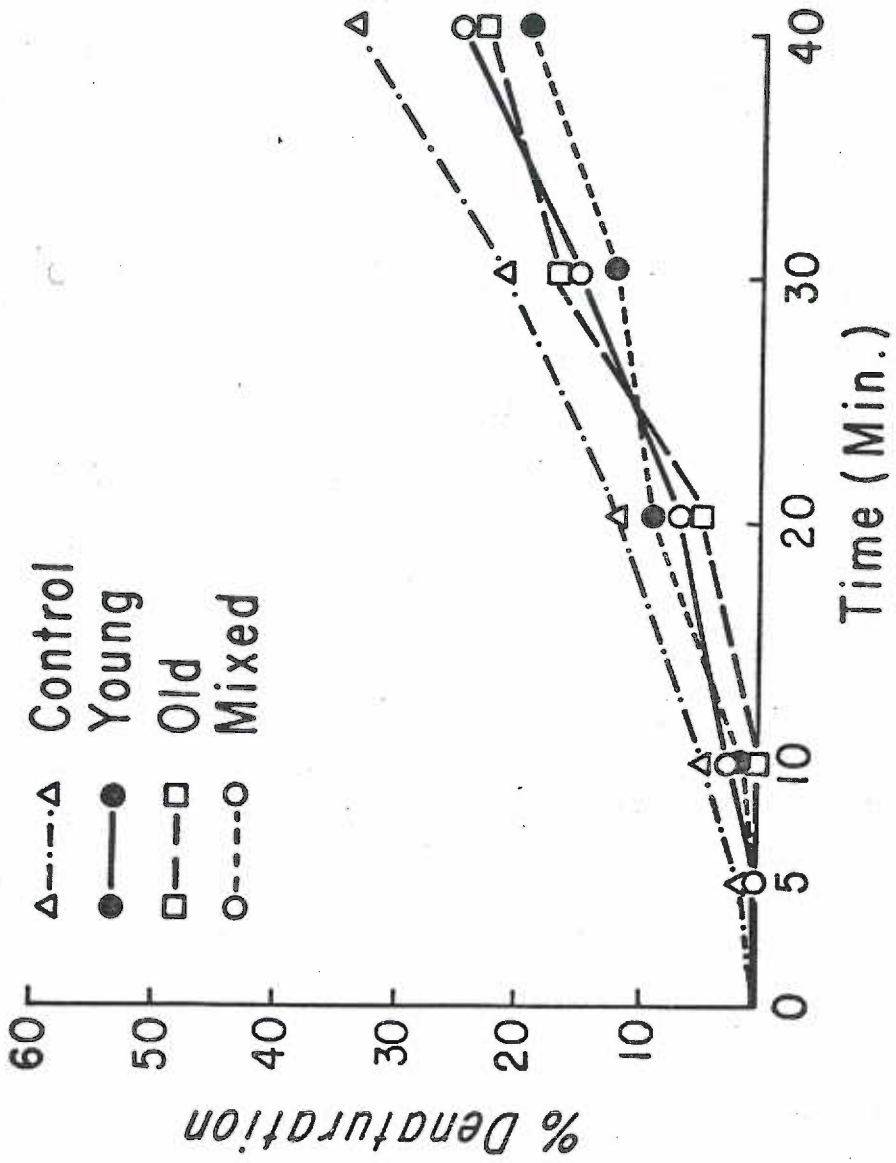
Values of % Hb denaturation with time for normal red blood cells (control Δ·-·-Δ) and PK deficient red blood cells type 1 (young ●—● and mixed o----o).


Control
Young
Mixed
% Denaturation
60
50
40
30
20
10
0
5
10
20
30
40
Time (Min.)

For the G-6-PD type 4 (see Figure XV) the percent denaturation of Hb in the mixed, the young and the old in the first five minutes were within the normal range.

Figure XV

Values of % Hb denaturation with time for normal red blood cells (control Δ·-·-Δ) and G-6-PD deficient red blood cells type 4 (young ●—●, old □--□ and mixed o----o).


Control
Young
Old
Mixed
% Denaturation
Time (Min.)
0
5
10
20
30
40
10
20
30
40
50
60

# DISCUSSION

The red cell has been a favorite experimental system for a number of physiological studies, including red cell aging as reviewed by Fornaini (2) and Harris (66). To separate erythrocytes of different ages several techniques are now available with improvements constantly being developed. A discontinuous gradient offers several advantages over a continuous one: first, one obtains separate layers with a minimal intermixing between layers and secondly, a relatively larger quantity of red cells can be centrifuged, depending on the ratio of gradient volume to volume of centrifuged sample. The red cells behave in the gradient as osmometers (67). Deviation from isotonicity of the medium can cause the specific gravities of the cells to change through loss or gain of water, which would result in changes of specific gravity probably unrelated to cell age (68), and hemolysis if the medium isotonicity is too low. Anticoagulants also influence the RBC separation. Heparin may cause an increase in rouleaux formation (69) while oxalate and EDTA have effects on both osmolarity and pH of the suspending medium (70).

Red cell aging is associated with physical, chemical and metabolic changes. Increased density (71,72) and decreased electric charges (5,6) were found in old cells. The electrolyte $Na^+$ concentration is increased together with a decreased concentration of $K^+$ and $Ca^{++}$ (73,74). Lohr et al. (53) and Brok et al. (75) found a

decrease in phosphate esters, mainly nucleotides, phospholipids, and cholesterol (76). There are also age-related changes in the catalytic activity of various enzymes (53-59). The activities of the following enzymes have previously been found to decrease progressively with age: hexokinase, aldolase, triose-phosphate isomerase, pyruvate kinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and glutamic oxalacetic transaminase. However, the differences in activities between young and old cells found by these authors were not striking and varied depending on the method of separation and the enzyme assay used.

In this experiment there are six enzymes, including GOT, HK, PFK, PK, G-6-PD, and EN which showed a significant change in enzyme activity with increased red cell age. GOT has a particularly dramatic drop in activity during the life span of the red cell (59, 77). The Y/O GOT activity ratio of 4.9 obtained in this study is in general agreement with the value of 3.5 obtained by Sass. It may be that part of the difference can be accounted for by the assay technique. We used the Blume method with enzymatic assay at 340 nm while Sass used the Karmen method at 540 nm.

G-6-PD was assayed by Arnold et al. (78) in centrifugation-separated red cell populations. They found that enzyme activity decreased 46.9% from the top (young) 10% to the bottom (old) 10%. Our results in Table 7 (49.8%) for G-6-PD is in good agreement with their results.

Brok et al. (75) studied HK activity using a phthalate ester density separation to obtain 5 to 10% young and old populations. They found that HK activity in old cells was 61.8% of the young cell activity. This compares well with our value of 51.2%.

Paglia and Valentine (56) used 28% bovine serum albumin density gradients to separate the cells of various ages. They found that for the lightest and most dense fractions, the Y/O ratio for G-6-PD activity was 1.7. We obtained a ratio of 2.0 for G-6-PD. The Y/O ratio for PK that we observed as 1.7 also agreed with their ratio of 1.6.

The other seven enzymes which decrease with age at an insignificant rate were TPI, GAPD, GPI, PGK, GSSG-R, GSH-Px and 6-PGD. The loss of activity appears to proceed at all cell ages, as shown in Figure IX. GOT shows a dramatic linear drop of activity loss during red cell aging. In contrast the enzymes PFK, PK and G-6-PD showed different non-linear patterns of loss of activity with increasing red cell age. Evaluation of the kinetics of 6-PGD and HK was difficult due to the smaller changes in activity with age.

In attempting to interpret the kinetic loss of enzyme activity during red cell aging, it is important to consider the major alterations in metabolic capability by the maturing red cell during the transition from reticulocyte to erythrocyte. Most red cells enter the circulation as reticulocytes but within one or two days, the young cell loses its characteristic cytoplasmic RNA and

changes from reticulocyte to erythrocyte. Alterations in the intracellular environment occurring during the transition from reticulocyte to erythrocyte include loss of mitochondria and may also be associated with a loss of activity of certain enzymes in the glycolytic pathway and HMS shunt. For this reason the enzyme activity may drop rapidly during the transition from reticulocyte to erythrocyte, and at a much slower rate after that.

Another possible interpretation might be that some enzyme activities such as PFK, PK and G-6-PD follow an exponential decline with cell age and the decrease in enzyme activity with time may be a first order process.

$$\frac{dA}{dt} = -\lambda A \qquad (1)$$

When A is the enzyme activity at any given time (t) and $\lambda$ is a decay constant, the integral form of equation (1) is:

$$A_t = A_o e^{-\lambda t}$$

$A_t$ is the enzyme activity at time t during cell aging.

$A_o$ is the enzyme activity of the erythrocyte when it enters the circulation.

Because old cells have less enzyme activity than young cells, the enzyme activity of the old red cell population would follow a less steep decline than the young cells since the rate of change in activity is proportional to the amount of enzyme present.

In the present study both homozygous PK patients had decreased enzyme activity in cells of all ages which paralleled the amounts found in normal subjects. It is known that pyruvate kinase is a key enzyme in glycolysis and catalyzes the conversion of PEP and ADP to pyruvate and ATP. Reduced activity of PK causes a defect in the production of energy and results in a congenital hemolytic anemia (79). The red cells of one patient (C.J.) contained an enzyme activity of 0.41 $U/10^{10}$ RBC which is 12% of normal. The other patient (M.J.) had a RBC activity of 0.38 $U/10^{10}$ RBC, which is 10.2% of normal.

The normal young cells have PK activities two times higher than the old cells, while the PK patients' young cells have PK 1.7 times higher than old cells. There is a comparable rate of decline in activity with age in both cell populations. PK activity in young deficient cells was 0.6 $U/10^{10}$ RBC while normal levels were much higher at 2.55 $U/10^{10}$ RBC. These results can be explained if the rate of activity decline is comparable in both PK deficient and normal red blood cells.

The kinetic behavior of pyruvate kinase from red cells of these patients (C.J. and M.J.) has been examined by Campos, Koler and Bigley (34). Both $K_m$ PEP and $K_m$ ADP showed no difference from normal for both substrates and the variant also showed normal allosteric properties. A high reticulocyte count is characteristic of PK deficiency and was found in both PK deficient subjects (see Table

5). In normal blood samples a fall in reticulocyte count is closely paralleled by a progressive decrease in RNA content (80,81). Both of the PK deficient patients had many more reticulocytes with new methylene blue than we expected. This elevated reticulocyte count was not accompanied by an equivalent increase in RNA content. This discrepancy can be accounted for by the fact that PK deficient subjects show an increased tendency to form Heinz bodies in the presence of supravital dyes such as new methylene blue. The Heinz bodies cannot be differentiated easily from reticulocytes by our technique, giving a falsely positive high reticulocyte count. For this reason the RNA level rather than the reticulocyte count was used to characterize the separated red cell populations. Reticulocytes in the relatively young cell populations of red cells maintain ATP by oxidative phosphorylation. These ATP levels may themselves stimulate ATP synthesis by a feedback mechanism (82). The ATP content of PK deficient cells starts at a lower level than in normal and declines at a rate comparable to normal cells. If ATP level controls life span, it is possible that the PK deficient cells will then have a reduced life span but the older 10% of the deficient cells will have the same minimal ATP levels as the oldest 10% of normal cells.

We have observed that easily denatured hemoglobin is detectable in the red cells of patients with red cell PK deficiency. The percent of denaturation was higher even in the young cells.

Van Berkel et al. (83) also observed decreased glutathione peroxidase activity due to decreased GSH in the RBC of some of their PK deficient patients.

In the hexose monophosphate shunt (Figure I) while no ATP is formed, NADP is reduced to NADPH. Three groups of enzymes are required to complete this pathway: 1) The G-6-PD and 6-PGD steps reduce NADP to NADPH. 2) The glutathione reductase system to oxidize NADPH to reduce GSSG to GSH, and 3) The glutathione peroxidase that oxidizes GSH for the reduction of peroxide. If any of these three enzyme systems is not functioning properly there is a susceptibility for drug-induced hemolysis. In the case of red cells with G-6-PD deficiency, the concentration of NADPH is not sufficient to overcome the oxidative stress resulting in a decrease in reduced glutathione in the red cells (84,85) and formation of Heinz bodies (86). This would explain the hemolysis of severe G-6-PD deficiency type 4. In contrast to G-6-PD deficiency type 5, hemolytic anemia may occur even in the absence of exposure to any oxidative stress. There is marked shortening of red cell life span under these conditions. In G-6-PD Negro variants the hemolytic anemia is self-limited in that only the oldest red cells are susceptible to hemolysis and their selective destruction leaves in the circulation only the younger cells with their higher level of G-6-PD activity. Ir contrast, in the Mediterranean type even in the young cells the decrease in enzyme activity was found and hemolysis was not self limited.

In this study of different subjects, there was an overlap in the range of enzyme activity for erythrocyte fractions of differing mean cell age. This variation may be attributed in part to variable efficiencies of separation. Erythrocyte fractions of a given density prepared from red cell populations of different subjects may vary in the degree of relative enrichment with young and old cells.

The finite red cell life span may well be a consequence of diminished or altered enzyme activities that is a reflection of erythrocyte aging. Our data shows several systems that may be related to cell death. We showed a drastic decline of rate-limiting enzymes in both the glycolytic pathway and the hexose monophosphate shunt with increasing cell age. Glutathione stability and hemoglobin stability were also observed to be decreased in the oldest fraction. All of these changes associated with cell aging are accelerated in the study of anemic patients. Together these changes probably represent a serious metabolic handicap for the senescent erythrocyte and may be a major factor in determining its life span both in normal and enzyme-deficient subjects.

# CONCLUSION AND SUMMARY

Human erythrocytes were separated into age groups according to their specific gravity using the discontinuous dextran gradient method. Reticulocyte count and RNA assay were used to indicate the efficiency of the separation.

Normal human erythrocytes, pyruvate kinase deficiency type 1 with decreased activity and normal $K_m$PEP, G-6-PD deficiency type 4 (severe enzyme deficiency), and G-6-PD deficiency type 5 (severe enzyme deficiency associated with congenital nonspherocytic hemolytic anemia) were separated into three age groups on the basis of density: young, intermediate and old. The activity of 13 red blood cell enzymes was determined in these red blood cell populations: GOT, HK, GPI, PFK, TPI, GAPD, PGK, EN, PK, G-6-PD, 6-PGD, GSSG-R and GSH-Px. In one group of enzymes (GOT, HK, PFK, EN, PK, G-6-PD) activities were found to be significantly decreased in older red cells. All enzymes showed a young-to-old ratio of about 2 except GOT which was 4.94. A second group of enzymes which showed no significant red cell age related decrease in activity included GPI, TPI, GAPD, PGK, 6-PGD, GSSG-R and GSH-Px. Enzymes in this group showed a decrease in activity between young and old cells, which was not statistically significant. The enzyme activity in the intermediate fraction was between that for young and old cells.

In this study the first group of red cell enzymes show at least two distinct patterns of age related activity loss. The first

of these is a steady fall in activity with increasing red cell age. This pattern is shown by GOT. The second group of enzymes showing a different pattern of loss of activity included PFK, PK and G-6-PD. It appears that this loss of activity is due to a rapid fall in enzyme activity during transition from reticulocyte to erythrocyte and follows an exponential decline with cell age.

In pyruvate kinase deficiency type 1 with decreased activity and normal $K_m$PEP, the average red cell pyruvate kinase in both patients studied was 11% of normal. Their young cells contained only one-fifth the normal red blood cell PK activity. Their levels of enzyme activity in glycolytic pathway steps before pyruvate kinase (PFK and HK) are normal, as are enzymes in the hexose monophosphate shunt.

Glucose-6-phosphate dehydrogenase type 4 and 5.

The red blood cell G-6-PD for the type 4 patient was 20.4% of normal, while the patient's young cell G-6-PD level was only one-sixth of the normal for young red blood cell activity. The G-6-PD deficiency type 5 (associated with CNSHA) patient had a cell G-6-PD level only 4.5% of normal. G-6-PD in young cells was 23.3 times less than normal young cell activity.

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