Note added in proof: In order to avoid confusion, uniformity in the nomenclature of the Hb chains is desirable. Before such uniformity can be achieved, however, it will be necessary to determine which N-terminal sequence[9] corresponds to the chain which carries the localized defect.

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*EFFECT OF AGE ON THE ENZYME ACTIVITY IN ERYTHROCYTES**

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INTRODUCTION

The destruction of human red blood cells, under normal circumstances, is related to senescence.[1] This observation suggests the possibility that the aging of erythrocytes and their eventual disintegration may result from a progressive diminution in the activity of certain metabolic systems essential to the maintenance of the integrity of these cells. This investigation was designed to determine whether alterations in the activity of enzymes occur in associated with the in vivo aging of mature red blood cells.

It has been demonstrated by other investigators that the activities of cholinesterase glyoxalase, and catalase are elevated in the human erythrocyte populations containing a high percentage of reticulocytes.[2–4] In addition, Allison and Burn[4] found that cholinesterase and catalase levels fall progressively in transfused donor cells as they age in vivo. Recently, the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase, but not of purine nucleoside phos-

phorylase, were found to be high in erythrocytes of patients with an increased proportion of young red blood cells.[5]

The enzymes assayed in the present investigation include glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase. These dehydrogenases catalyze reactions providing the only known mechanism in mature red blood cells for oxidation of glucose to CO_2 with the generation of reduced triphosphopyridine nucleotide. In addition, phosphohexose isomerase and lactic acid dehydrogenase were studied. These two enzymes are involved in glycolysis via the Embden-Meyerhof pathway, the major source of energy in erythrocytes. Purine nucleoside phosphorylase, which catalyzes the phosphorolytic cleavage of purine nucleosides to ribose-1-phosphate and purine, has also been assayed.

In the present experiments, samples of erythrocytes with relatively young and old mean cell ages were obtained, employing techniques based on the fact that young mature red blood cells are more resistant to hypotonic lysis[6] and that they are less dense.[7, 8] Enzymatic assays performed on such samples of erythrocytes of differing mean cell age have revealed that glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase, but not lactic acid dehydrogenase or purine nucleoside phosphorylase, activities decrease markedly with the aging of red blood cells in vivo.

MATERIALS AND METHODS

Fifteen ml. of heparinized venous blood was drawn from subjects in good health. All blood samples were centrifuged,[9] and the plasma and buffy coat removed. The erythrocytes were washed with isotonic potassium chloride buffered at pH 7.4 and resuspended in this solution so as to provide a hemoglobin concentration of approximately 150 mg/ml. This procedure was performed at 0° C. These resuspended washed erythrocytes will be referred to as the "whole erythrocyte population." Aliquots of the whole erythrocyte population were removed for counting erythrocytes, leukocytes, and reticulocytes. Erythrocytes completely free of leukocytes and reticulocytes could not be obtained. It was found, however, that the activities of the enzymes per gram of hemoglobin were unaffected, provided that the contamination of erythrocytes by leukocytes and reticulocytes was less than 0.05 and 0.5 per cent, respectively.

Separation of Young and Old Erythrocytes.—The whole erythrocyte population was separated into fractions of relatively young and old mean ages by two different methods: (*a*) serial osmotic hemolysis and (*b*) centrifugation.

a) Serial osmotic hemolysis: One-ml. samples of the whole erythrocyte population were added to a tube containing 5 ml. of distilled water and to a series of tubes containing 5 ml. of sodium chloride solutions of varying degrees of hypotonicity. The tubes were mixed and allowed to stand at 0° C. for 10 minutes. The samples were centrifuged, and the supernatant solutions were removed and saved. The sedimented unlysed erythrocytes were washed promptly with 10 volumes of ice-cold isotonic potassium chloride buffered at pH 7.4 and resuspended in about 3 ml. of this solution. These erythrocyte samples and an aliquot of the whole erythrocyte population were hemolyzed by freezing and thawing twice.

The concentration of the sodium chloride solutions (usually 0.33–0.46 per cent sodium chloride) employed were empirically chosen to cause hemolysis of approxi-

mately 2, 5, 10, 90, 95, and 98 per cent of the added erythrocytes. The per cent hemolysis achieved by a given hypotonic sodium chloride solution was expressed as the ratio of the hemoglobin concentration in the supernatant solution to the hemoglobin concentration in the solution of water-lysed erythrocytes, multiplied by 100.

Serial osmotic hemolysis achieves a separation of mature human erythrocytes into cell populations of relatively young and old mean cell ages. This was established by determining the concentration of erythrocytes of varying ages, as dated by Fe^{59} labeling in vivo, in the fractions of cells of different osmotic resistance.[10] It was found that the 10, 5, and 2 per cent least resistant cells were progressively more enriched with regard to old erythrocytes and impoverished with respect to young and middle-aged red blood cells. The unlysed cells of the 90, 95, and 98 per cent levels of hemolysis were progressively more enriched with young red cells and impoverished with respect to old and middle-aged erythrocytes. The supernatant solutions of the 2 ± 1, 5 ± 1, and 10 ± 1 per cent levels of hemolysis and the sedimented cells of the 90 ± 1, 95 ± 1, or 98 ± 1 per cent levels of hemolysis were chosen for assay of enzyme activities. These erythrocyte fractions will be referred to as the 2, 5, and 10 per cent least resistant and 10, 5, and 2 per cent most resistant cells, respectively. In addition, enzyme assays were performed on the water-lysed cells and the whole erythrocyte population.

b) Centrifugation: Six ml. of the whole erythrocyte population were centrifuged in 10×150-mm. tubes for 30 minutes at 2,000 *g*. The upper, middle, and lower 15 per cent of the packed column of erythrocytes were removed and hemolyzed by freezing and thawing twice. Enzyme assays were performed on these hemolyzates. It has been shown, employing Fe^{59} to date the age of erythrocytes, that the upper, middle, and lower layers of centrifuged human red cells have young, intermediate, and old mean cell ages, respectively.[8]

Enzyme Assays.—Glucose-6-phosphate dehydrogenase was assayed by a method based on that of Kornberg and Horecker;[11] 6-phosphogluconic dehydrogenase by a technique similar to that of Horecker and Smyrniotis;[12] and purine nucleoside phosphorylase by a modification of the method of Price *et al.*[13] The details of these enzyme assays have been described elsewhere.[5]

Phosphohexose isomerase was determined according to the method of Bodansky,[14] and lactic acid dehydrogenase by a procedure based on the technique of Hill and Levi.[15] The activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, purine nucleoside phosphorylase, and lactic acid dehydrogenase were expressed as the change in optical density per minute per gram of hemoglobin (ΔOD/min/gmHgb). A unit of phosphohexose isomerase activity was defined as the reciprocal of that amount of sample, expressed as grams of hemoglobin per milliliter reaction mixture, which, under the standard conditions of the assay, produced 25 μg. fructose as fructose-6-phosphate from glucose-6-phosphate. Hemoglobin concentrations were measured by pipetting a suitable quantity of hemolyzate into 10 ml. of 0.4 per cent NH_4OH and reading the optical density of the resulting solution in a spectrophotometer at 540 mμ.

Enzyme Recovery Studies.—To determine whether the fractionation of the whole erythrocyte population by serial osmotic hemolysis affected enzyme activity, the total activity of each enzyme was measured in the supernatant solution and sedimented cells of the various fractions. The sum of the enzyme activity in the

supernatant solution and that in the sedimented cells expressed as per cent of the enzyme activity in the whole erythrocyte population revealed the following mean values ± one standard deviation:

For glucose-6-phosphate dehydrogenase: 98.2 ± 5.3 per cent;
For 6-phosphogluconic dehydrogenase: 102.2 ± 5.3 per cent;
For purine nucleoside phosphorylase: 97.0 ± 5.1 per cent;
For phosphohexose isomerase: 101.8 ± 6.9 per cent;
For lactic acid dehydrogenase: 96.1 ± 6.3 per cent.

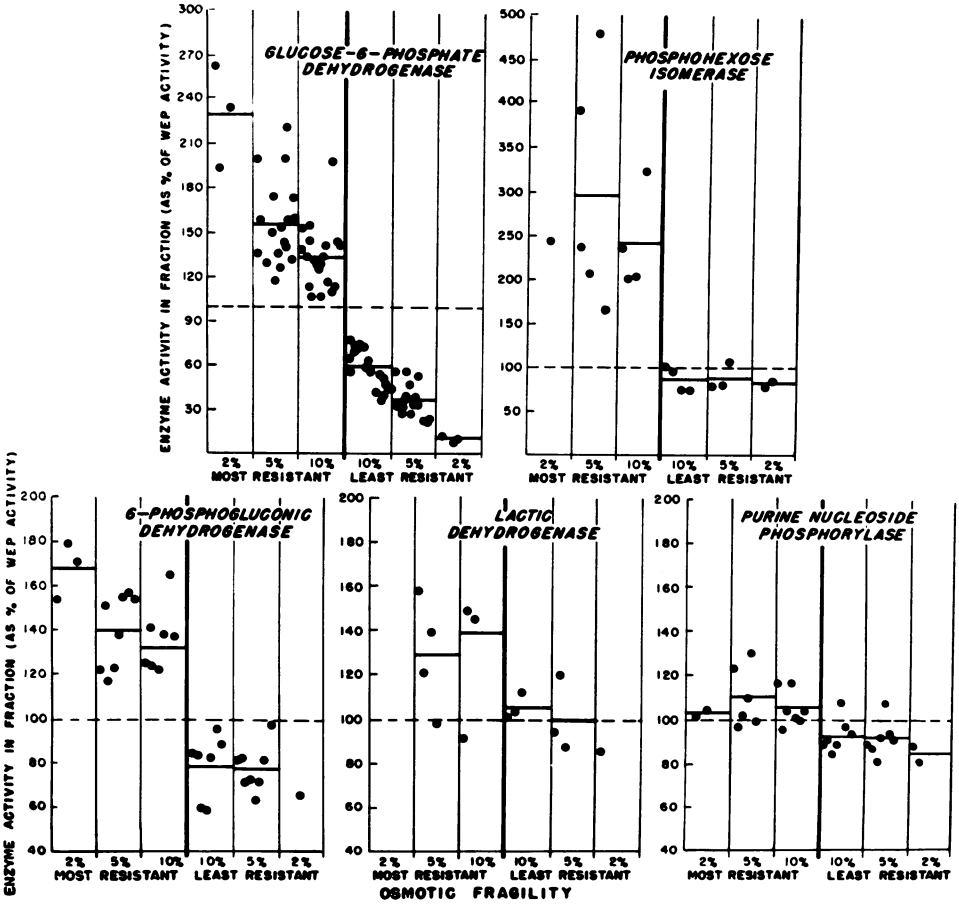

FIG. 1.—Enzyme activity in erythrocyte fractions of varying osmotic fragility. This figure contains all the data obtained for enzyme activity in erythrocytes fractionated by serial osmotic hemolysis. For each enzyme, the activity found in the various fractions (2, 5, and 10 per cent least resistant and 10, 5, and 2 per cent most resistant fractions) is expressed in terms of the per cent of the activity of the whole erythrocyte population (WEP). The cross-bars indicate the mean values. These data represent 22 studies for glucose-6-phosphate dehydrogenase, 6 studies for phosphohexose isomerase, 11 studies for 6-phosphogluconic dehydrogenase, 5 studies for lactic dehydrogenase, and 9 studies for purine nucleoside phosphorylase.

There were no significant differences in activities of these enzymes in hemolyzates prepared by freezing and thawing compared with hemolyzates prepared by water

lysis. In addition, partially purified preparations of glucose-6-phosphate dehydrogenase,[11] 6-phosphogluconic dehydrogenase,[12] and purine nucleoside phosphorylase[16] added to hemolyzates of various fractions gave theoretical values within the analytical error of the enzymatic essays.

RESULTS

Enzyme Activity in Erythrocytes Fractionated by Serial Osmotic Hemolysis.—In red blood cells fractionated by serial osmotic hemolysis, the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase were markedly higher in the more resistant than in the less resistant erythrocyte fractions. Purine nucleoside phosphorylase and lactic acid dehydrogenase levels were reduced only 20–40 per cent in the least resistant cell fractions (Fig. 1).

TABLE 1

ENZYME ACTIVITY IN ERYTHROCYTE FRACTIONS OF VARYING OSMOTIC FRAGILITY: ILLUSTRATIVE EXAMPLES OF SINGLE FRACTIONATION FOR EACH ENZYME

			FRACTION OF ERYTHROCYTES†					
			Most Resistant (Per Cent)			Least Resistant (Per Cent)		
ENZYME*	SUBJECT	WEP*	2	5	10	10	5	2
G-6-P. D.	LZ	16.3	38.3	26.1	23.2	7.3	3.9	1.7
6PG. D.	DE	20.7	35.4	32.1	...	17.0	14.8	...
PHI	ZA	14.2	...	68.0	46.0	10.7	...	11.3
PNP	LI	173	179	166	178	145	139	138
LDH	PE	141	...	154	161	124	133	...

* Abbreviations include: G-6-P. D., glucose-6-phosphate dehydrogenase; 6PG. D., 6-phosphogluconic dehydrogenase; PHI, phosphohexose isomerase; PNP, purine nucleoside phosphorylase; LDH, lactic acid dehydrogenase; WEP, whole erythrocyte population.

† Units of enzyme activity are: G-6-P. D., 6PG. D., PNP and LDH: Δ OD/min/gm Hgb; PHI: 10^3 units/gm Hgb.

The most resistant and least resistant erythrocyte fractions have relatively younger and older mean cell ages, respectively. Accordingly, the enzyme activities may be related directly to the relative mean age of the red blood cells in the various fractions. Among different subjects, there was an overlap in the range of enzyme activity for erythrocyte fractions of differing mean cell age (Fig. 1). This variation may be attributed in part, to the fact that erythrocyte fractions of a given osmotic fragility prepared from red cell populations of different subjects varied in the degree of relative enrichment with young and old cells. However, in a given fractionation study, it was consistently observed that the older the relative mean cell age of the erythrocyte sample, the lower the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase (Table 1).

Of the five enzymes studied, glucose-6-phosphate dehydrogenase activity showed the most marked difference between the most and least resistant red blood cell fractions. The mean values for enzyme activity in the 5 per cent most resistant compared to the 5 per cent least resistant erythrocyte fractions differed by a factor of 4.0 for glucose-6-phosphate dehydrogenase, 3.3 for phosphohexose isomerase, 1.9 for 6-phosphogluconic dehydrogenase, 1.2 for lactic acid dehydrogenase, and 1.2 for purine nucleoside phosphorylase. Comparing enzyme activity in the young cell fractions with that in the whole erythrocyte population, the decrease in phosphohexose isomerase was most striking (Fig. 1).

Enzyme Activity in Erythrocytes Fractionated by Centrifugation.—The levels of

glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase, but not of purine nucleoside phosphorylase, were higher in the upper, less dense, than in the lower, more dense, erythrocyte fraction (Table 2). Among individuals there was again an overlap in the range of these dehydrogenase activities between the least and the most dense erythrocyte fractions. For a given erythrocyte fractionation study, however, the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase were consistently higher in the less dense red blood cells.

The fractions of erythrocytes of relatively young and old mean cell ages separated by centrifugation showed smaller differences in glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase activities than did the fractions of differing mean cell age prepared by serial osmotic hemolysis. These observations with regard to enzyme activity are consistent with the finding based on Fe^{59} specific activity that, of the two methods employed in this study, serial

TABLE 2

ENZYME ACTIVITY IN ERYTHROCYTE FRACTIONS OF VARYING DENSITY

FRACTION OF CELLS*	ENZYME ACTIVITY† ΔOD/MIN/CM HGB) G6P. D.	6PG. D.	PNP	PHI (10 Units/ GM HGB)
WEP	17.3 (12.8–21.9)	14.2 (12.1–16.4)	144 (135–160)	16.2 (14.2–18.2)
Top 15 per cent cells	20.3 (14.4–24.7)	16.7 (13.1–19.8)	145 (136–158)	19.2 (16.1–22.3)
Bottom 15 per cent cells	14.9 (11.9–19.0)	13.0 (10.6–16.4)	141 (124–154)	12.9 (10.7–14.1)

* WEP represents the whole washed erythrocyte population. The top 15 per cent cells and bottom 15 per cent cells refer to the upper and lower 15 per cent of a column of packed erythrocytes (see text).

† The values for the enzyme activity represent the mean and range (indicated in parentheses) of 15 studies for G6P. D., 3 studies for 6PG. D., 5 studies for PNP, and 2 studies for PHI. Abbreviations are as defined in n.* of Table 1.

osmotic hemolysis achieved a more effective fractionation of the whole erythrocyte population.

DISCUSSION

The present study indicates that the levels of certain enzymes in mature human erythrocytes decrease with the aging of these cells in vivo. The activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase fall more markedly than do those of purine nucleoside phosphorylase and lactic acid dehydrogenase. In addition, among these enzymes, differences were apparent in the pattern of this decline in activity. Thus the level of glucose-6-phosphate dehydrogenase was progressively lower, the older the red cell fraction, while phosphohexose isomerase activity decreased primarily in the younger cell fractions. It must be emphasized that the differences between young and old red blood cells with respect to the activities of these enzymes are very much greater than the differences measured in the fractions of cells of varying osmotic resistance. These fractions are merely enriched with young or old erythrocytes.

It is of interest that the decrease in enzyme levels with erythrocyte aging in vivo are in striking contrast to the lack of change in enzyme activity in cells stored in vitro. It has been found that red cells stored at 4° C. in acid-citrate-dextrose solu-

tion, or acid-citrate-dextrose-inosine solution for as long as 7 months showed no change in the levels of glucose-6-phosphate dehydrogenase- 6-phosphogluconic dehydrogenase, purine nucleoside phosphorylase, or phosphohexose isomerase.[17]

The explanation of the decline in enzyme activity with erythrocyte aging in vivo is not apparent from our present knowledge. A possible interpretation of these observations is that, following the loss of nucleic acids, the erythrocyte cannot synthesize proteins. The previously synthesized complement of enzymes may then fall progressively as the cell becomes older. There is good evidence that the mature erythrocyte cannot synthesize heme.[18] The data with regard to their ability to synthesize stromal proteins are inconclusive.[19] There have been no studies on enzyme synthesis in mature red blood cells. If mature erythrocytes do indeed synthesize enzymes, then the diminution in activity of enzymes with age may be related to alterations in rates of turnover of these proteins. These problems are being investigated.

Evidence has accumulated to indicate that the maintenance of the integrity of the erythrocyte membrane is dependent on various enzymatically catalyzed reactions.[20] In view of the present finding that the levels of certain erythrocyte enzymes are related to the age of these cells, it is reasonable to suggest that the diminution in activity of critical enzymes may determine the life-span of the red blood cell in vivo.

SUMMARY

The activities of certain enzymes have been studied in mature human erythrocytes of relatively young and old mean cell ages. The samples of young and old red blood cells were separated by methods based on the fact that young, compared to old, erythrocytes are less osmotically fragile and are less dense. The activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase decrease more markedly with the aging of red blood cells in vivo than do those of lactic acid dehydrogenase and purine nucleoside phosphorylase. It is suggested that the decline in activity of critical enzymes is a determinant of the life-span of erythrocytes.

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SPONTANEOUS NUCLEATION IN SUPERSATURATED VAPORS

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At the end of the last century C. T. R. Wilson[1, 2, 3, 4] established that a given degree of supersaturation was required to produce homogeneous nucleation in a vapor. The technique used was the adiabatic expansion of the vapor in the presence of a non-condensable gas, and the test for the nucleation was the instantaneous (or very rapid) formation of a visible cloud of liquid droplets. Wilson recognized that some ions were always present to contribute to the nucleation, and the principal use of the cloud chamber soon came to be a method of making ion tracks visible. Various experimenters have tried to remove ions from the chamber by electric fields. The most satisfactory method of reducing the number of ions present, as judged by our experience with the maximum degree of supersaturation obtainable, is by the use of clean apparatus and a gas such as hydrogen or helium, which contains a small number of electrons per molecule and contributes a small amount of intrinsic ionization.

Spontaneous nucleation must be regarded as superposed on ionization, and its observation must therefore be indirect, depending on such factors as the rate of growth of the cloud of droplets in density. The density of the cloud, however, depends very much upon the critical drop size, which may be defined as the size of drop which gives the maximum vapor pressure at a given temperature. The existence of such a critical drop size is demonstrated by an equation for quasi-thermodynamic equilibrium derived by one of the authors.[5] The equation contains two terms which are functions of drop radius. One contains the free surface energy which tends to make the drop unstable, and the other expresses the stabilizing effect of the entropy of the drop as a polyatomic molecule of gas. The two opposing terms lead to a maximum for the free energy of the drop as a function of radius. So long as the critical size is large, the drop containing 100 molecules or more, the equation is quite exact. When the drop size is small—for example, in the case of mercury because of the large surface tension of mercury—the concept of the free surface energy of the drop calculated as a sphere becomes very approximate indeed.

In order to test the application of this equation, studies were made upon the supersaturation of a number of liquids of different surface tensions and vapor pressures.