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Research Article

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The rate of in vivo decline of pyruvate kinase (another age-dependent enzyme) was also measured and found very similar in the three groups.

The in vivo decline of glucose-6-phosphate dehydrogenase was found to follow an exponential rate, with a half-life of 62 days for controls and 13 days for Gd(—),A- erythrocytes. The activity in normal reticulocytes was estimated at 9.7 U and in Gd(—),A- reticulocytes at 8.8 U. These estimates were confirmed by direct measurements in reticulocytes isolated from patients with extreme reticulocytosis.

In Gd(—),Mediterranean erythrocytes activity could be demonstrated only in reticulocytes, which were estimated to average 1.4 U. The rate of decline is so extreme that no activity could be detected in mature erythrocytes.

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In Vivo Lability of Glucose-6-Phosphate Dehydrogenase in Gd^{A-} and $Gd^{Mediterranean}$ Deficiency

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ABSTRACT A decreased level of glucose-6-phosphate dehydrogenase might result from decreased rate of synthesis, synthesis of an enzyme of lower catalytic efficiency, increased lability, or a combined mechanism. To test the hypothesis of increased lability, the rate of decline of the enzyme in vivo was measured in three groups of individuals, controls, Gd(−),A–males, and Gd(−), Mediterranean males, by the slope of decline of activity in fractions containing erythrocytes of progressively increasing mean age. These fractions were obtained by ultracentrifugation on a discontinuous density gradient of erythrocyte suspensions free of contaminating platelets and leukocytes.

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In Gd(−),Mediterranean erythrocytes activity could be demonstrated only in reticulocytes, which were estimated to average 1.4 U. The rate of decline is so extreme that no activity could be detected in mature erythrocytes.

These data suggest that the glucose-6-phosphate dehydrogenase deficiency of both the Gd^{A-} and the $Gd^{Mediterranean}$ variant results from different degrees of in vivo instability of the abnormal enzyme.

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INTRODUCTION

An increased activity of glucose-6-phosphate dehydrogenase (G6PD) (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49) in the circulating erythrocytes during hyperactive erythropoiesis was observed by Marks (1). His findings suggested that the activity of this enzyme is higher in the younger erythrocytes and decreases with aging of the cells. Several investigators have since indirectly estimated the rate of decline of G6PD activity in vivo using the ratio of the activity of "young" and "old" erythrocytes separated by different techniques (2–5).

In order for these estimates to be accurate, the "young" and "old" erythrocyte fractions must be as free as possible not only of erythrocytes of intermediate age, but also of leukocytes and platelets. These blood elements are much richer than erythrocytes in enzymatic activities per unit volume (6) and tend to concentrate almost exclusively in the "young" erythrocyte fraction. Failure to remove leukocytes and platelets, therefore, results in spuriously higher enzymatic activities when these activities are measured as a function of hemoglobin (or of unit cell volume).

Centrifugation has been utilized in earlier experiments to isolate "young" and "old" erythrocytes; with this technique a ratio of 1.9 for the

G6PD activity of "young" and "old" erythrocytes was obtained (2). A higher ratio of 6.9 was observed after fractionation by differential osmotic lysis. With this technique, however, the "old" cells are obtained as a dilute hemolysate, resulting in spuriously low activity, because of inactivation of G6PD in absence of NADP (7). Powell, Brewer, DeGowin, and Carson with a technique of repeated centrifugation have recently reported a ratio of activity of 1.6 for normal erythrocytes and of 13.6 for erythrocytes from Negro deficient males (3). The activity of the "young" fraction of erythrocytes from Negro deficient males was still lower than that of the "old" fraction from normal erythrocytes. Centrifugation on phtalate esters has recently been utilized to reinvestigate the problem. With this technique, Brok, Ramot, Zwang, and Danon reported a ratio of activity of 2.8 between the top 1% ("young") fraction and the bottom 1% ("old") fraction isolated from normal blood after removal of leukocytes and platelets (4). Yoshida, Stamatoyannopoulos, and Motulsky isolated with similar technique the top 5% and the bottom 5% fractions and reported a ratio of 4.1 for blood from three normal individuals, and a ratio of 21.7 for blood from three Negro deficient males (5). These authors found a similar range of activities in the "young" cells of the normal individuals and of the Negro deficient males. The discrepancies between the findings of Powell et al. (3) and Broks et al. (4) and the findings of Yoshida et al. (5) can be easily explained since the latter used whole blood in their fractionation experiments, without removing leukocytes and platelets.

A technique of ultracentrifugation on a density gradient recently developed in this laboratory allows serial measurement of enzyme activity in groups of intact erythrocytes of progressively increasing mean age, in a system where contamination with leukocytes and platelets is reduced to an insignificant level (8). This method permits a quantitative estimation of the rate of decline in vivo of enzymatic activity, rather than an indirect estimate by the ratio of the activity of two fractions containing only relatively "young" and "old" erythrocytes as in the previously reported studies.

The present experiments were performed to establish the rate of decline of G6PD with aging of the cell in normal erythrocytes and to compare it with the rate of decline in erythrocytes from individuals genetically deficient in enzyme; both Negroes and Mediterraneans were studied.

According to the nomenclature recently recommended (9) the genotype for the most common enzyme is designated Gd^{B}, for the variant with normal activity observed in some Negroes Gd^{A}, for the deficient enzyme common in Negroes Gd^{A-} and for the deficient enzyme found in Mediterraneans $Gd^{Mediterranean}$. The corresponding notations for phenotype, in the male, are Gd(+),B; Gd(+),A; Gd(−),A −; and Gd(−),Mediterranean, respectively.

The type of enzyme deficiency commonly observed in Negroes (Gd^{A-}), is different in electrophoretic mobility (10) and in several other respects from the one observed in Mediterraneans ($Gd^{Mediterranean}$), (2). The G6PD activity in the erythrocytes of Gd(−),A− hemizygotes ranges between 5 and 15% of the normal, whereas in the erythrocytes of Gd(−),Mediterranean hemizygotes the activity is less than 5% and often undetectable (2). During severe hemolysis and hyperactive erythropoiesis, the enzyme level of Gd(−), A − erythrocytes rises, sometimes to normal (11), whereas in Gd(−), Mediterranean erythrocytes it remains low (12). Gd(−),A − hemizygotes are enzyme deficient only in the erythrocytes, whereas Gd(−), Mediterranean hemizygotes have decreased activity also in their leukocytes and platelets (2).

A low level of enzyme activity in the erythrocytes of genetically deficient individuals might be due to normal rate of synthesis of an enzyme of low catalytic efficiency, decreased rate of synthesis of a normally active enzyme, increased lability of the variant enzyme, or a combined mechanism. The observations in the Gd(−),A − males suggest that the deficiency could be due to a normal rate of synthesis of a more labile enzyme (13), whereas in the case of the Mediterraneans, the deficiency could be equally explained by any of the various alternatives. Measurements of the rate of decline of G6PD with aging of the cells provide a test for these hypotheses.

The G6PD locus is on the X chromosome so that blood from heterozygous deficient females contains a mixture of two populations of cells (14). Only hemizygous deficient males were therefore studied.

METHODS

Experimental procedures

Preparation of "pure" erythrocyte suspensions. The blood specimens were defibrinated with glass beads. They were used immediately or stored overnight at 4°C. The blood was diluted with 5 parts of cold saline (buffered with PO_4 to pH 7.4, containing 200 mg of glucose/100 ml) (8) and filtered twice in the cold through double layers of Whatman No. 2 filter paper. The erythrocytes were then washed three times in cold buffered saline and reconstituted to a hematocrit of approximately 70%. Experiments with ^{59}Fe-dated human and rabbit erythrocytes showed no age selective loss of cells through this procedure. The final erythrocyte suspensions were platelet free and contained less than 2% of the original number of leukocytes and the same percentage of reticulocytes as the original blood.

Density gradient fractionation was performed as previously described (8), except that the gradient was prepared in a tube 2.5 × 7.5 cm; 2 ml of "pure" erythrocyte suspension was layered on it and the centrifugation was performed for 90 min at 25,000 rpm with the SW25 rotor in the Spinco model L ultracentrifuge. Three to five layers of albumin were used to obtain four to six fractions. In each experiment, an erythrocyte suspension from a control individual was fractionated simultaneously with erythrocyte suspensions from one or two deficient individuals.

Hemoglobin concentration was measured after conversion to cyanmethemoglobin according to vanKampen and Zijlstra (15).

Reticulocyte counts were performed on dry preparations obtained after supravital staining with new methylene blue. 1000 cells were counted.

Slide elution test for G6PD was performed as described by Gall, Brewer, and Dern (16) except that at the end of the 3 hr incubation, one-half volume of human type AB plasma was added to the vials to improve cell morphology. 1000 cells were counted.

Enzymatic measurements were performed at 25°C at 340 mμ for 20 min in a Gilford model 2000 spectrophotometer. Each assay was performed in duplicate on a hemolysate obtained by freezing the erythrocytes once and diluting with distilled water, unless otherwise specified.

The assay system for G6PD contained Tris/HCl, pH 8.4, 0.12 mole/liter; glucose-6-phosphate, 1.6 mmoles/liter; NADP, 1 mmole/liter; nicotinamide, 6 mmoles/liter; $MgCl_2$. 3.5 mmoles/liter; and hemolysate, 0.15–0.3 mg of Hgb/ml.

The assay system for pyruvate kinase (PK) (ATP: pyruvate phosphotransferase, EC 2.7.1.40) contained TES (*n*-tris (hydroxymethyl) methyl-2-aminoethan sulfonic acid) buffer, pH 7.4, 31.4 mmoles/liter; Phosphoenolpyruvate, 28 mmoles/liter; NADH, 0.1 mmole/liter; ADP, 2.6 mmoles/liter; lactic dehydrogenase, 4 U/ml; $MgCl_2$, 2.3 mmoles/liter; and hemolysate, 0.1–0.25 mg of Hgb/ml.

Enzyme activities were expressed as μmoles of substrate converted per gram of Hgb per minute at 25°C.

Electrophoresis of G6PD was performed on starch gel, according to Kirkman and Hendrickson (10).

Correlation between specific gravity and age of the cells

Position in the gradient. When normal human erythrocytes are separated according to specific gravity a normal distribution is obtained (17). The mean specific gravity, although constant for each individual, varies in different individuals, but the variance of the distribution is always very similar (18). Hence, when several individuals are studied, the cumulative distribution function rather than the absolute specific gravity must be used for pooling of data.

The sample percentile of each fraction (f_j) was calculated from the formula:

$$\text{Sample percentile} = \left[\frac{H_{(f_1)}}{H_{(t)}} + \cdots + \frac{H_{(f_{j-1})}}{H_{(t)}} + \left(\frac{H_{(f_j)}}{H_{(t)}} \times \tfrac{1}{2}\right)\right] \times 100,$$

where $H_{(f)}$ = hemoglobin content of fraction (mg), $H_{(t)}$ = total hemoglobin content in all fractions (mg), and, (f_1) ⋯ (f_j) = consecutive fraction (f_1) to (f_j) from top to bottom. As the absolute hemoglobin content of the erythrocyte is a constant throughout life-span (8), milligrams of hemoglobin are directly proportional to the number of cells. Therefore, the sample percentile as calculated indicates the midpoint position of each fraction in the gradient expressed as percentage of the total number of cells.

Relationship between sample percentile and cell age. Experiments in the rabbit with isotopically dated erythrocytes have shown that the younger cells are lighter and the older, heavier (18). The distribution of groups of cells of the same age is normal and the mean sample percentile in the gradient of each group is related to its age. The variance was found to be independent of age, when a nonreutilized label (glycine-2-^{14}C) was used (8). These data are consistent with the hypothesis that erythrocytes emerge from the bone marrow as a normally distributed population with regard to specific gravity and the increase in specific gravity with aging involves the entire distribution equally. The same type of relationship between specific gravity and cell age was found for human erythrocytes (19), in which case the distribution is symmetrical, as the random destruction found in rabbit erythrocytes is absent or minimal in man (20).

Within the gradient, therefore, contamination by overlapping groups of cells of intermediate age becomes negligible only in a very small percentile of cells at the extremes of the distribution (at the top and at the bottom). These fractions can be considered "100%" young and, respectively, "100%" old. The mean age at the center of the gradient is equal to one-half of the total length of the life span, as overlapping younger cells are symmetrically balanced by overlapping older cells. Hence, any parameter of the erythrocytes related with age must decrease linearly within the gradient from top to bottom with the probits of the sample percentile and reach a value at the 50% point equal to that of the unfractionated sample.

This theoretical relationship was accordingly tested against several alternative hypotheses and was found to give the best correlation in all groups studied.

Statistical analysis. The accuracy of the estimate for reticulocyte count slide elution test as well as for enzymatic assay decreases with decreasing concentration or activity. In order to correct for this, each value was assigned a weight inversely proportional to its standard error (21). The standard error was estimated in the case of cell counts as the reciprocal of the square root of the absolute number of cells counted and in the case of enzymatic assays as the reciprocal of the square root of the absolute OD measured at 340 mμ.

Regression and covariance analyses were performed by standard techniques (21) with an Olivetti 101 table computer. Points with a value of 0 were assigned a value equal to 1/10 of the threshold of the technique. The threshold was estimated at 0.1% in the case of cell counts and at 0.1 μmole/g of Hgb per min in the case of enzymatic assays.

Experimental groups

Three groups were studied: controls, Negro enzyme-deficient males, and non-Negro enzyme-deficient males. All individuals studied, unless otherwise specified in the text, had normal hemoglobin and hematocrit levels and no history of anemia for at least 6 months.

Controls. The controls were 10 Gd(+),B males; 1 Gd (+),A male; and 8 Gd(+),B females. They were laboratory personnel, medical students, and members of the Bellevue Hospital house staff.

Enzyme-deficient subjects. The subjects were all males; 15 Negroes had phenotype Gd(−),A−; 8 individuals had phenotype Gd(−),Mediterranean: of these 3 were Jewish, 3 Sardinian, 1 Southern Italian, and 1 Greek.

All individuals were unrelated except for a Sardinian father and son, in which case the son's G6PD deficiency was obviously inherited from his mother.

RESULTS

The mean values and their standard errors in the three experimental groups are shown in Table I.

Beside the abnormalities in G6PD activity and slide elution test, which are inherent in the classification, both groups of enzyme-deficient individuals had reticulocyte counts higher ($P < 0.001$) than the control group. PK activity was higher in both groups, but the differences did not attain statistical significance for the size of the sample.

Effect of leukocytes and platelets on the G6PD assay. This effect was estimated by comparing the activity measured in whole blood to that of the "pure" erythrocyte suspensions (Table I). In the controls and in the Negroes, an average of 0.4 U was attributable to leukocytes and platelets. This was only 8% of the total activity for the controls, but it corresponded to almost one-half of the total activity in the Negroes. In the Mediterraneans, the activity attributable to leukocytes and platelets averaged 0.21 U; this however, was almost the total activity present in whole blood; in

TABLE I

Comparison of Mean Values from the Three Experimental Groups

Group	Reticulocytes	PK units	G6PD units in erythrocyte suspensions	G6PD units in whole blood	Slide elution, % cells stained
	%				
Controls					
No. tested	19	10*	19	10	10
Mean	0.81	3.72	4.95	5.35	>95
SEM	0.07	0.24	0.16	0.36	
Gd(−),A−					
No. tested	15	6*	15	15	7
Mean	1.69‡	4.21	0.56	0.99	20.8
SEM	0.77	0.10	0.05	0.11	2.06
Gd(−), Mediterranean					
No. tested	8	3*	8	8	8
Mean	1.48‡	3.90	0.024	0.126	2.5
SEM	0.22	0.26	0.02	0.04	0.70

* This subgroup was not significantly different from the corresponding entire group with regard to percentage and slope of decline of reticulocytes.

‡ Different from control ($P < 0.001$).

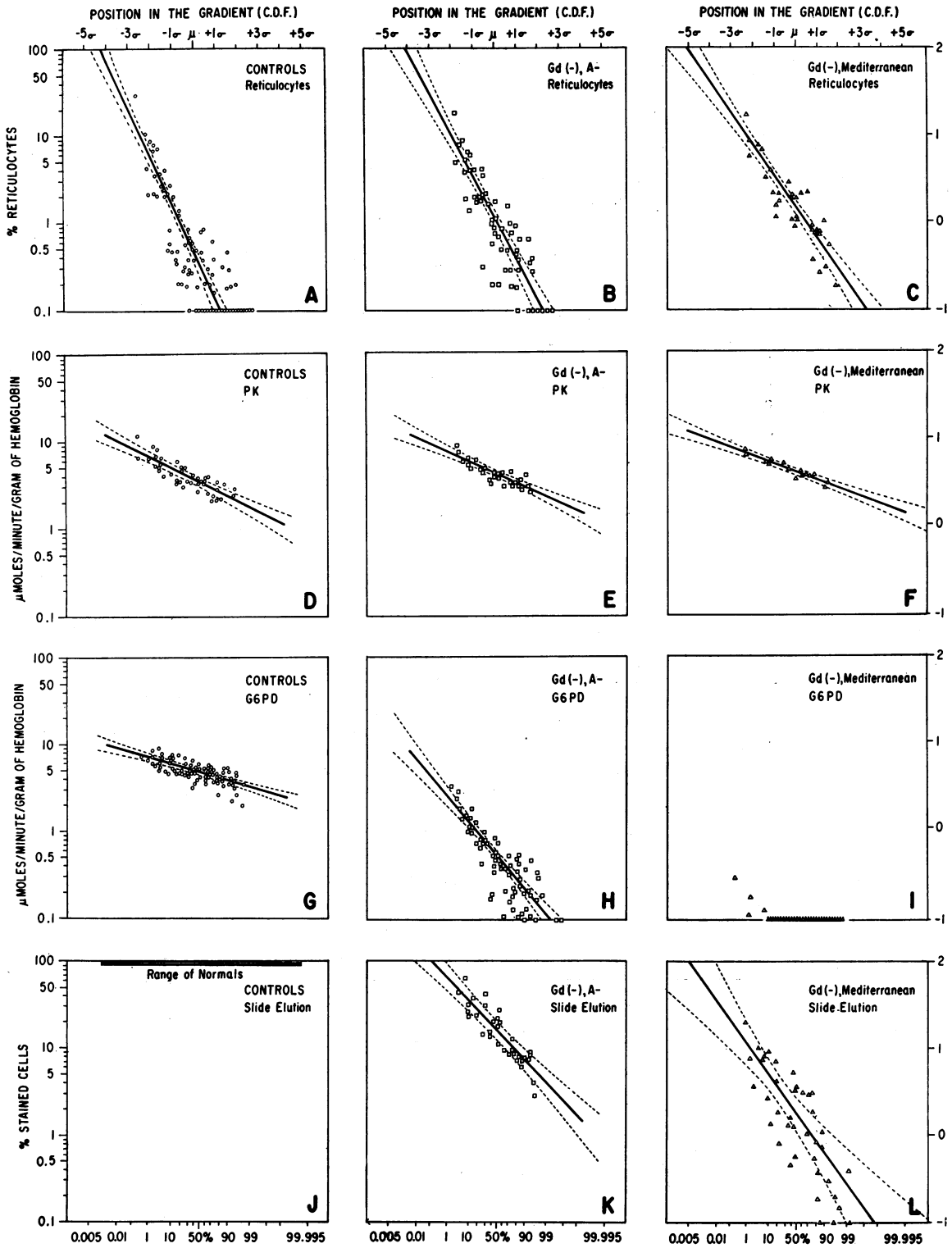

FIGURE 1 Rates of decline in the gradient. Horizontal axis: probability scale; vertical axis: logarithmic scale; heavy lines: slopes of regression; dotted lines: 95% confidence limits of the slopes. The upper end of the heavy line corresponds to the estimate for a "pure" fraction containing only reticulocytes; the lower end to the estimate for a "pure" fraction containing cells of age equal to the erythrocytes life span (for details, see text). The dotted lines have been extended to cover the outer 95% confidence limits of the levels where "pure" fractions are obtained. Points on the base line represent values equal or smaller than the threshold of the technique. *C.D.F.*, cumulative distribution function.

"pure" erythrocyte suspensions the activity was extremely low (average 0.02 U). The "pure" erythrocyte suspensions from 5 Gd(−),Mediterranean males were also prepared for assay by hemolysis in presence of NADP and mercaptoethanol as described by Kirkman, Rosenthal, Simon, Carson, and Brinson (22). Although this procedure resulted in increased activity in the erythrocytes of a subject with the chronic nonspherocytic hemolytic anemia variant of G6PD deficiency, no increase in activity could be detected in the Gd(−),Mediterranean erythrocytes.

Pattern of fractionation

The pattern of age-related fractionation in the three groups studied was compared by the use of two parameters independent of G6PD: the distribution in the gradient of reticulocytes and the rate of decline of another age-dependent enzyme, such as PK (23).

The results of the fractionation experiments are shown in Fig. 1 for the three experimental groups.

Reticulocyte counts (Fig. 1; A, B, C). The concentration of reticulocytes in the gradient rapidly declined from top to bottom in all groups. The rate of decline was less rapid for Gd(−),A− than for control reticulocytes, but the difference did not attain significance for the sample size ($P = 0.1$). The rate of decline for Gd(−),Mediterranean reticulocytes was significantly slower than that for control reticulocytes ($P = < 0.001$).

PK (Fig. 1; D, E, F). The rate of decline for control and Gd(−),A −erythrocytes did not differ significantly, but it was significantly slower in the case of the Gd(−),Mediterranean group ($P = < 0.01$).

These results indicated that the pattern of age-related fractionation of Gd(−),A −erythrocytes is similar to that of control erythrocytes, but it is considerably different in the case of Gd(−), Mediterranean erythrocytes.

Rate of decline of G6PD

This rate was measured by two different methods: the enzymatic assay that measures the average activity of a group of cells and the methemoglobin slide elution test that examines the activity of the individual cells.

Enzymatic assay (Fig. 1; G, H, I). The rate of decline of G6PD for the Gd(−),A −erythrocytes was much faster than for the control erythrocytes ($P < 0.001$). In the case of the Gd(−), Mediterranean erythrocytes, G6PD activity was measurable only in the four lightest fractions. Therefore, it was not possible to calculate a slope.

In the control erythrocytes, the rate of decline of G6PD was significantly slower than the rate of decline of PK ($P < 0.001$).

Slide elution test (Fig. 1; J. K. L). In the control group, over 95% of the cells was stained in all fractions, without correlation with position in the gradient. The rate of decline of stained cells in the Gd(−),A −group was significantly different from the rate of decline of reticulocytes ($P < 0.001$) but did not differ from the rate of decline of G6PD. In the case of Gd(−),Mediterranean group, however, the rate of decline of stained cells did not differ significantly from the rate of decline of reticulocytes.

DISCUSSION

The life span of the erythrocytes from G6PD deficient males has been found to be shortened, with averages estimated at 96 days for Negro (24) and 100 days for Mediterranean erythrocytes (25). This degree of shortening of the life span does not clinically result in anemia, because of compensation from the bone marrow: a small decrease in mean hemoglobin level is only detectable when large series are studied (26). In the present experiments, the lower mean age of Gd(−),A− erythrocytes was reflected by a higher percentage of reticulocytes and a slight increase in PK level, but the rates of decline in the gradient of both were not different from that of the controls. In Gd(−), Mediterranean erythrocytes, increase in reticulocytes and in PK activity were of the same order of magnitude as in Gd(−),A− erythrocytes, but the rates of decline in the gradient of reticulocytes and PK were considerably slower than for the controls. This discrepancy could be explained only if in the case of Gd(−), Mediterranean erythrocytes, besides the over-all reduction in life span, some degree of random destruction contributed to a further decrease in the mean cell age. The ^{51}Cr technique used to estimate the life span of Gd(−),Mediterranean erythrocytes does not permit a clear test of this hypothesis (25), An alternative explanation would require a direct effect of G6PD deficiency

on the increase in specific gravity with age of the cell.

The percentile position in the gradient where the probability of contamination with more mature erythrocytes becomes negligible can be estimated for each group by the intercept of the slope of decline of the reticulocytes with the 100% line. If the distribution is symmetrical, in a corresponding percentile at the other extreme the probability of contamination by erythrocytes younger than the maximum age becomes similarly negligible. These "pure" fractions consist of less than 0.01% of the total number of control and Gd(−),A− erythrocytes and less than 0.001% of Gd(−),Mediterranean erythrocytes. Such minute groups of cells cannot be isolated experimentally, but from the slope of regression it becomes possible to estimate their average value for any age-dependent parameter of the erythrocytes (and thus the life span of the parameter). Accordingly, for the PK (an enzyme dependent on age, but genetically independent from G6PD) the estimates obtained for the average value in the reticulocytes were 12.4 U for the control group, 12.3 U for the Gd(−),A− group, and 12.3 U for the Gd(−), Mediterranean group. The average values at the 50% level were 3.65, 4.24, and 4.00 U respectively, in close agreement with the mean activity obtained in corresponding unfractionated samples. From these data the life span of PK in the erythrocytes was estimated as an exponential rate of decline with a half-life of 33, 31, and 32 days in the three groups, respectively. The close agreement between the estimates obtained in the three groups for the PK supports the validity of the method for estimation of the G6PD (a variable different in the three experimental groups).

The average value of G6PD in the reticulocytes was estimated at 9.7 U for control and at 8.8 U for Gd(−),A− erythrocytes. The average values at the 50% level were 4.95 and 0.55 U respectively, in close agreement with the mean activity obtained in the corresponding unfractionated samples. The average half-life of G6PD in the erythrocytes was estimated at 62 days for the control and 13 days for the Gd(−),A− group. The estimated half-life of the G6PD in the control group is similar to that obtained by Lohr and Waller with a completely different approach (6).

In order to experimentally verify these estimates, fractions containing over 95% reticulocytes were isolated from peripheral blood of patients with extreme reticulocytosis and their G6PD activities were measured. In fractions from two Gd(+),B individuals (one patient with sickle cell anemia crises and one patient with hemolytic uremic syndrome) the values were 10.3 and 10.8 U respectively. In fractions from two Gd(−),A− males (one patient with sickle cell anemia crises and one child with naphthalene-induced hemolysis) the values were 8.8 and 9.6 U. The values measured are very close to those expected.

The activity in the Gd(−),Mediterranean erythrocytes is too low to permit similar estimates. In this group, however, the percentage of stained cells in the slide elution test, as well as their rate of decline is not different from that of the reticulocytes. At the level in the gradient where 100% reticulocytes are expected, similarly the percentage of stained cells is estimated at 100%. These findings suggest that among the Gd(−),Mediterranean erythrocytes the only cells stained in the slide elution test are the reticulocytes.

The slide elution test is an indirect measurement of G6PD. The $NADPH_2$ generated by the G6PD serves as electron donor for the methemoglobin reductase ($NADPH_2$: cytochrome oxidoreductase, EC1.6.2.1.), in presence of Nile blue as an electron carrier (15). Methemoglobin reductase is not an age-dependent enzyme (27) and its activity in individual erythrocytes thus varies according to a normal distribution pattern. Methemoglobin reductase activity has been found to be significantly decreased in erythrocytes of both Negro and Mediterranean deficient males (28, 29). In the Gd(−),A− erythrocytes, the rate of decline of the percentage of cells stained in the slide elution test is parallel to the rate of decline of G6PD. The slope of regression shows that a small percentage of the oldest erythrocytes is still stained, although their estimated value of G6PD is well below 0.1 U. These findings suggest the existence of an upper threshold (above which all cells are stained) and a lower threshold (below which no cell is stained). Above and below the two thresholds, the percentages of stained cells are 100% and 0 respectively, independently of the individual cell methemoglobin reductase activity. At levels of G6PD activity in between the two thresholds, the probability of a cell to stain depends both on G6PD and methemoglobin reductase. At levels of G6PD closer to the upper threshold, the major-

ity of the cells are stained, except for those with the lowest individual level of methemoglobin reductase. At levels of G6PD closer to the lower threshold, conversely, the majority of the cells are unstained, except for those with the highest individual level of methemoglobin reductase. Thus the slide elution test appears a sensitive tool, directly correlated to G6PD activity. The exact value of the upper threshold is unknown, but it cannot be more than 2 U for normal cells, as practically all control cells are stained, including the oldest; in the case of the Gd(−),A− and Gd(−),Mediterranean the threshold is probably higher, as in these cells the level of methemoglobin reductase is reduced. The lower threshold is similarly unknown, but it must be below 0.1 U from the findings in the Gd(−),A− erythrocytes. Therefore, in the Gd(−),Mediterranean erythrocytes, some G6PD activity is present in the reticulocytes, but this rapidly disappears as the rest of the erythrocytes are completely unstained. The average level of G6PD in the Gd(−),Mediterranean reticulocytes was estimated by their percentage in the four fractions with detectable activity as 1.4 U. These indirect estimates suggest an extreme in vivo instability of the $Gd^{\text{Mediterranean}}$ enzyme in the erythrocytes if its activity declines at least from 1.4 U to 0.1 U in the short time interval required for a reticulocyte to become a mature erythrocyte.

The result of these experiments therefore support the hypothesis that, both in the Gd^{A-} and the $Gd^{\text{Mediterranean}}$, the rate of synthesis in the bone marrow is normal and the deficiency in the erythrocytes results from in vivo inactivation of the variant enzymes.

The findings of an increased rate of decline in the Gd(−),A− erythrocytes confirm the previously reported data of Powell et al. (3) and the conclusions of Yoshida et al. (5) and provide a more quantitative estimate. The studies of the Gd(−),Mediterranean erythrocytes indicate that in this variant as well, a similar mechanism operates to produce an even greater rate of decay.

The rate of inactivation of G6PD in the Gd(−),A− cells is faster than in the normal cells, but not extreme, thus the activity of these reticulocytes is near that of normal reticulocytes. Hyperactive erythropoiesis increases the level of G6PD of the circulating erythrocytes in Gd(−),A− individuals more than in normal individuals. Also, G6PD activity in leukocytes is normal.

The rate of inactivation of the $Gd^{\text{Mediterranean}}$ enzyme is so rapid that it is only barely exceeded by the rate of synthesis. Thus, the reticulocytes emerge from the bone marrow of deficient individuals with G6PD activity already greatly reduced and the increase in level during hyperactive erythropoiesis is too small to be detectable. Similarly, the leukocytes have a greatly reduced, but present, G6PD level.

Several variants of G6PD are presently known, some resulting in decreased activity, some normal in this regard (30). The recent work of Yoshida (31) has shown that at least in the case of one common variant with normal activity (Gd^{A}) the substitution of a single amino acid is the only difference from the most common "normal" type (Gd^{B}). In the case of the human hemoglobins, single amino acid substitutions in different positions result in completely different abnormalities of the final product, associated or not with decreased rate of synthesis or with lability. If a single amino acid substitution in different positions is responsible for the variants of G6PD, these will also have different abnormalities. Some variant enzymes (as the Gd^{A}) will be different only in electrophoretic mobility, some might not even show an altered electrophoretic mobility, others (as the Gd^{A-} and the $Gd^{\text{Mediterranean}}$) will show varying degrees of lability.

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