Reduction of Oxidized Glutathione in Normal and Glucose-6-Phosphate Dehydrogenase Deficient Erythrocytes and Their Hemolysates

By Egmond E. Rieber and Ernst R. Jaffé

DEFICIENCY IN D-GLUCOSE-6-PHOSPHATE: NADP oxidoreductase (E.C. 1.1.1.49) (G-6-PD) activity in human erythrocytes is manifested by a decreased capacity to generate NADPH in vitro and an increased susceptibility to hemolysis in vivo. This enzyme activity is measured routinely by the reduction of NADP to NADPH in very dilute hemolysates because of excessive light absorption at higher hemoglobin concentrations. By measuring G-6-PD activity in a reaction coupled with the reduction of oxidized glutathione (GSSG), a much higher hemolysate and, thus, enzyme concentration can be used.

Although reticulocytes and young erythrocytes of G-6-PD deficient subjects may have demonstrable or even normal activity of G-6-PD, a rapid decrease in enzyme activity occurs with aging of the cells. Approximately the same serological and enzymatic activity have been demonstrated for the deficient enzyme (A−) as for the variants with normal activity (A+ and B+) with the aid of quantitative immunologic neutralization tests. The deficiency in activity, as demonstrated by generation of NADPH or by reduction of GSSG, may be considered to be due to increased lability, inactivation or denaturation of the mutant enzyme either within the cell or during the preparations required for the determination of activity.

In the present study, the reduction of both endogenous and exogenous GSSG to reduced glutathione (GSH) in intact erythrocytes and in hemolysates of normal and G-6-PD deficient red cells was investigated. This procedure avoided the dilution required for the spectrophotometric determination of the reduction of NADP to NADPH.

MATERIALS AND METHODS

Blood samples, anticoagulated with heparin, were obtained from normal subjects, four enzyme-deficient Negro males, two enzyme-deficient Caucasian males, and one man of Italian descent with moderately severe hereditary nonspherocytic hemolytic anemia (HNSHA) associated with severe deficiency in C-6-PD activity. None of the subjects was
Fig. 1.—Regeneration of endogenous glutathione. Incubation 60 minutes at 37° C. Incubation volume 3.0 ml. MgCl₂ 3.3 × 10⁻³ M, glucose-6-phosphate 1.0 × 10⁻³ M, NADP 2.0 × 10⁻⁷ M, azoester 1.7 × 10⁻³ M, hemoglobin 3.8 Gm. per 100 ml., pH 7.30. A—Without azoester (control). B—All reagents. C—Without glucose-6-phosphate. Per cent values calculated from original GSH concentrations.

Anemic and none had a reticulocytosis, except for the patient with HNSHA. Erythrocytes were used within two hours after collection and after thrice washing with 0.15 M sodium chloride solution with centrifugation and removal of the Buffy coat. Lysates were prepared by freezing in dry ice-ethanol and thawing in warm water three times. The erythrocytes or lysates were diluted to the desired packed cell volume or hemoglobin concentration with a pH 7.4 buffer composed of 0.03 M phosphate, 0.05 M sodium chloride and 0.1 M glycylglycine.

Methyl-phenylazoformate (azoester) which rapidly oxidizes GSH on a two-to-one molar basis was used as the oxidizing agent.⁶ After oxidation of GSH with azoester at 4° C, the erythrocyte suspensions or hemolysates were incubated in air at 37° C for 60–120 minutes. The composition of the incubation medium and the concentrations of the additives which were all of reagent grade have been detailed in the legends of the figures.

The concentrations of GSH were determined by the method of Beutler et al.⁸ with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB).

**RESULTS**

The extent of regeneration of endogenous GSH oxidized with azoester in intact erythrocytes confirmed previous observations.⁷ In normal red cells, complete recovery of GSH was observed, whereas only 10–15 per cent recovery could be obtained with G-6-PD deficient cells. With the erythrocytes from the patient with HNSHA, no regeneration of GSH was observed.

Figure 1 illustrates that the regeneration of endogenous GSH in normal and
G-6-PD deficient hemolysates is similar. The initial lower GSH concentration of G-6-PD deficient erythrocytes is well known. No difference in the per cent regeneration of GSH between hemolysates of erythrocytes from normal subjects or Caucasian (Ashkenasi Jews, probably of Sephardic descent) and Negro G-6-PD deficient subjects has been observed. Omission of NADP from the incubation medium does not alter the extent of reduction in hemolysates of normal red cells, but allows only 85 per cent regeneration, instead of 95–100 per cent, in G-6-PD deficient hemolysates. This slightly lower capacity for reduction can be corrected by the addition of $2.0 \times 10^{-7}$ M NADP. The results are not influenced by removal of red cell stroma.

The dependency of GSH regeneration upon substrate concentration is shown in Fig. 2. The optimal concentration of glucose-6-phosphate is $1.0 \times 10^{-3}$ M under the conditions of these experiments. Higher substrate concentrations do not enhance the rate of reduction. Regeneration of GSH with hemolysates of the red cells from the patient with HNSHA does not occur under any of the experimental conditions employed in these studies.

The reducing capacity of hemolysates of G-6-PD deficient and normal erythrocytes has been further evaluated by the addition of exogenous GSSG. Increased increments of added GSSG promote the accumulation of proportionally greater quantities of GSH (Fig. 3). At submaximal levels of exogenous GSSG, for a given hemoglobin concentration, the ratio of GSSG to GSH is maintained at about unity, and increases only when the concentration of GSSG exceeds $6.0 \times 10^{-3}$ M. Reduction of exogenous GSSG is dependent upon the presence of added NADP. With GSSG added at a concentration of $1.43-11.4 \times 10^{-3}$ M and a hemoglobin concentration of 3.5–4.0 Gm. per 100 ml., the NADP concentration employed has been $3.2 \times 10^{-7}$ M. Higher concentrations of NADP do not increase the amount of GSSG reduced. The requirement for added NADP is not altered by the removal of stroma or the addition of nicotinamide. An
increase in the concentration of glucose-6-phosphate also does not alter the rate of reduction or the ratio of GSSG to GSH. Although no striking difference in the ability of normal and G-6-PD deficient hemolysates of erythrocytes from Negro and Caucasian subjects is seen with added GSSG, the hemolysate of the red cells of the patient with HNSHA is unable to reduce exogenous GSSG at any hemoglobin concentration.

The consequences of G-6-PD deficiency for reduction of GSSG can be demonstrated in hemolysates only at hemoglobin concentrations below 2.0 Gm. per 100 ml. (Fig. 4). At these lower hemoglobin and, therefore, lower enzyme concentrations, Negro G-6-PD deficient hemolysates show only about 50 per cent reduction of exogenous GSSG when compared to normal red cell hemolysates, and Caucasian G-6-PD deficient hemolysates can accomplish only about 10 per cent reduction. These results are not affected by the removal of stroma, the addition of NADP, or increasing the concentration of glucose-6-phosphate.

DISCUSSION

The present observations have emphasized a clear difference in the pattern of reduction of GSSG between intact erythrocytes and their respective hemolysates. Although intact G-6-PD deficient red cells could regenerate only 10-15 per cent of their endogenous GSH, the corresponding hemolysates could accomplish almost 100 per cent regeneration. With the addition of excess GSSG, the maximal level of reduction attained was about 15-20 times the normal concentration of GSH, when expressed as mg. per Gm. of hemoglobin. An impaired ability to reduce GSSG to GSH could be demonstrated only with G-6-PD deficient hemolysates when the concentration of hemoglobin was below 2 Gm. per 100 ml. or with the hemolysate of erythrocytes from a patient with life-long HNSHA and a severe deficiency of G-6-PD activity.
Fig. 4.—Reduction of exogenous glutathione at low hemoglobin concentration. Incubation 120 minutes at 37° C. Incubation volume 3.0 ml. GSSG $2.86 \times 10^{-3}$ M, other additives as Fig. 3, pH 7.28. A—Normal red cell hemolysate. B—Negro G-6-PD deficient hemolysate. C—Caucasian G-6-PD deficient hemolysate.

The variables in the experimental conditions with hemolysates versus intact cells included the process of hemolysis and the higher concentration of glucose-6-phosphate. These factors may have promoted a process of activation of the G-6-PD in the deficient hemolysates. Due probably to the effect of dilution upon enzymatic activity, activation could not be demonstrated with dilute hemolysates. Defects in phosphorylation or intracellular inhibitors in G-6-PD deficient erythrocytes have never been documented, despite extensive attempts to demonstrate such abnormalities. Kirkman and Hendrickson have pointed out an apparent increased reactivation effect of temperature on concentrated preparations of normal G-6-PD which did not conform to kinetic formulae. Since the temperature was the same in experiments with intact erythrocytes and hemolysates, this factor probably was not involved. The increased GSSG-reductase activity which has been described as being present in G-6-PD deficient hemolysates also could not explain the quantitative results obtained in the present experiments. The progressive increase in the formation of GSH with increasing concentrations of GSSG supported the contention of Jacob and Jandl that hexose monophosphate shunt pathway activity was proportional to the level of GSSG. Whether GSSG exerted a direct stimulatory effect or promoted increased shunt activity as a consequence of the lower NADPH concentration which resulted from GSSG-reductase activity remains to be determined.

A protective effect against the oxidative action of acetylphenylhydrazine on GSH in the presence of added glucose-6-phosphate and other substrates in G-6-PD deficient hemolysates was reported by Beutler et al. These studies,
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however, were not concerned with the capacity to generate GSH from endogeneous or exogenous GSSG.

The present observations do not define the mechanism of the enhanced G-6-PD activity demonstrated in deficient hemolysates as compared to intact cells when assessed by the reduction of GSSG to GSH. They do, however, indicate that the greatly decreased activity of G-6-PD observed with a population of G-6-PD deficient erythrocytes of average age may not involve molecular destruction and can be recovered or revealed.

SUMMARY

When assayed by the ability to reduce oxidized glutathione to reduced glutathione, glucose-6-phosphate dehydrogenase deficiency of either the Negro or Caucasian mutant variety could be demonstrated in hemolysates only with hemoglobin concentrations below 2.0 Gm. per 100 ml. In intact erythrocytes, the inability to regenerate reduced glutathione was apparent regardless of the concentration of red cells. The process of hemolysis, therefore, appeared to permit the demonstration of higher levels of activity in G-6-PD deficient human erythrocytes than was possible in intact cells. A markedly deficient capacity to regenerate endogenous reduced glutathione or to reduce exogenous oxidized glutathione, however, could be demonstrated with the hemolysate of erythrocytes from a patient with hereditary nonspherocytic hemolytic anemia associated with a deficiency of glucose-6-phosphate dehydrogenase activity. These studies have emphasized the hazards involved in extrapolating the results of studies performed with hemolysates to metabolic processes within intact erythrocytes.

REFERENCES

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