The Regeneration of Reduced Glutathione in Normal and Glucose-6-Phosphate Dehydrogenase Deficient Human Red Blood Cells

By Nechama S. Kosower, Grace A. Vanderhoff and Irving M. London

A rapid and marked decrease in the reduced glutathione (GSH) content of human erythrocytes can be produced by methyl phenylazoformate, C₆H₅N = NCOOCH₃ (azoester).¹ The mechanism proposed for its action could also explain the diminution in GSH content which occurs in erythrocytes treated with acetylphenylhydrazine (APH). In azoester-treated normal red blood cells, GSH can be regenerated by incubating the cells in the presence of glucose; APH, however, is unsuitable for studies on GSH regeneration because of the low rate of loss of GSH and because of the presence of excess active reagent. Thus, the azoester affords a useful tool for the study of various aspects of GSH regeneration.

This report is concerned with the rates of regeneration of GSH in normal human red blood cells and in erythrocytes of glucose-6-phosphate dehydrogenase (G-6-PD)-deficient males and heterozygote females.

Materials and Methods

Erythrocytes of the following human subjects were tested:

A. Normal G-6-PD individuals.
B. G-6-PD deficient males: One Caucasian (A. St.) and three Negro subjects.
C. G-6-PD deficient heterozygote females: Three Caucasian (R. I. Z.; P. R.; D. H.) and four Negro subjects.

All subjects were in good health and were taking no drugs at the time of the experiments.

Blood anticoagulated with heparin was centrifuged, the plasma anduffy coat were removed, and the erythrocytes were washed twice with 0.15 M NaCl. A red blood cell suspension was made in 0.1 M NaCl-0.1 M glycylglycine (GG) buffer, pH 7.4, containing glucose. Azoester* solution in 6 per cent Dimethyl Sulfoxide (DMSO)-NaCl-GG buffer was prepared as previously described.¹ The azoester solution was slowly added with constant mixing.

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* Methyl phenylazoformate was prepared from phenylhydrazine and methyl chloroformate followed by lead tetracacetate oxidation. We are grateful to Professor E. M. Kosower and Dr. F-K. C. Huang, Department of Chemistry, State University of New York, Stony Brook, for a supply of the compound.
mixing to the erythrocyte suspension. The final concentrations in the incubation mixture were: glucose, 11 μM/ml.; azoester, 2.17 μM/ml.; DMSO, 2.67 per cent; hematocrit, 32 per cent.

The mixtures were kept at 0-4 C. for 10 minutes and were then incubated at 37 C. in the presence of air with occasional mixing. The initial volume of the incubation mixture was 4.5 ml.; 0.5 ml. of the incubation mixture was removed for GSH determination before incubation, and at 10, 30, and 60 minutes of incubation. GSH was determined according to the method of Beutler.2 G-6-PD activity was measured by the procedure of Kornberg and Horecker as modified by Marks.3

RESULTS

Regeneration of GSH in Erythrocytes of Normal, G-6-PD Deficient Males, and G-6-PD Heterozygote Females

The GSH content of the erythrocytes of all subjects was diminished to 0-4 mg./100 ml. red blood cells when the incubation mixtures containing azoester were kept at 0-4 C. for 10 minutes.

The amounts of GSH regenerated in normal erythrocytes, calculated as per cent of control values found in untreated erythrocytes, and the G-6-PD activity of the untreated cells are shown in Table 1. The activity of G-6-PD in the erythrocytes of the normal individuals ranged between 13.7-18.1 O.D./Gm. Hb/min. About 40 per cent of the GSH is regenerated within 10 minutes, and 80 per cent in 30 minutes. In the following 30 minutes there is a slight additional regeneration to about 90 per cent of the control value.

G-6-PD activity in the erythrocytes of G-6-PD deficient males ranged between 1.5-2.7 O.D./Gm. Hb/min. In the red blood cells of the G-6-PD deficient males, less than 10 per cent of the control GSH values was regenerated in 30 minutes, with slight or no change during incubation for an additional 30 minutes (Fig. 1).

G-6-PD activity in erythrocytes of G-6-PD deficient heterozygote females ranged between 2.3-12.8 O.D./Gm. Hb/min. In the erythrocytes of six of the seven heterozygote females, the regeneration of GSH was significantly lower.

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Table 1.—Regeneration of GSH in Normal Erythrocytes

<table>
<thead>
<tr>
<th>Subject</th>
<th>GSH mg./100 ml. RBC</th>
<th>% of Control GSH Regenerated in:</th>
<th>G-6-PD OD/1 Gm. Hb/min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Cells</td>
<td>Azoester Treated Cells</td>
<td>10 min.</td>
</tr>
<tr>
<td>1</td>
<td>47</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>67</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>3</td>
<td>77</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>92</td>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td>5</td>
<td>64</td>
<td>2</td>
<td>52</td>
</tr>
<tr>
<td>6</td>
<td>70</td>
<td>2</td>
<td>39</td>
</tr>
<tr>
<td>7</td>
<td>68</td>
<td>4</td>
<td>46</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>4</td>
<td>34</td>
</tr>
<tr>
<td>9</td>
<td>65</td>
<td>3</td>
<td>45</td>
</tr>
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</table>

Mean ± S.D.

<table>
<thead>
<tr>
<th>Controls</th>
<th>Azoester Treated Cells</th>
<th>10 min.</th>
<th>30 min.</th>
<th>60 min.</th>
<th>G-6-PD OD/1 Gm. Hb/min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>81</td>
<td>87</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>±6.8</td>
<td>±5.1</td>
<td>±6.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>G-6-PD Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>13.7-18.1</td>
</tr>
<tr>
<td>R. L.</td>
<td>12.8</td>
</tr>
<tr>
<td>C. D.</td>
<td>12.0</td>
</tr>
<tr>
<td>S. M.</td>
<td>8.5</td>
</tr>
<tr>
<td>A. G.</td>
<td>10.8</td>
</tr>
<tr>
<td>F. R.</td>
<td>8.8</td>
</tr>
<tr>
<td>F. R.</td>
<td>9.0</td>
</tr>
<tr>
<td>J. B.</td>
<td>5.9</td>
</tr>
<tr>
<td>D. H.</td>
<td>2.3</td>
</tr>
</tbody>
</table>

### Fig. 1.—Regeneration of GSH in erythrocytes of G-6-PD deficient males and heterozygote females.

Range of normal values is presented for comparison. Subjects are indicated by their initials. The values for G-6-PD activity in the erythrocytes of each subject are expressed as O.D. Gm. Hb min. Shaded area: normal. Solid lines: G-6-PD deficient heterozygote females. Broken lines: G-6-PD deficient males.

than normal. GSH regeneration in erythrocytes of one heterozygote female was in the low normal range. The rate of regeneration was most rapid in the first 30 minutes; in the subsequent 30 minutes GSH regeneration was usually slight (Fig. 1).

### Rate of GSH Regeneration in Mixtures of Erythrocytes

Artificial mixtures of erythrocytes were prepared as follows: (a) 75 per cent normal erythrocytes and 25 per cent erythrocytes of G-6-PD deficient males, and (b) 50 per cent normal erythrocytes and 50 per cent erythrocytes of G-6-PD deficient males. These mixtures, as well as normal erythrocytes and the erythrocytes of G-6-PD deficient males, were treated with azoester and incubated under the conditions described above. The results of two experiments, as shown in Figures 2A and 2B demonstrate that the pattern of GSH regeneration in the artificial mixtures is similar to that observed in the cells of heterozygote females.

### Availability of GSSG as Substrate for Regeneration of GSH

The availability of oxidized glutathione (GSSG) in azoester-treated erythrocytes was demonstrated as follows: lysates of azoester-treated erythrocytes
Fig. 2A.—Regeneration of GSH in mixtures of normal and G-6-PD deficient erythrocytes.

Fig. 2B.—See legend under Fig. 2A.
were incubated with added TPNH; the lysates of normal and of G-6-PD deficient cells regenerated their GSH to 100 per cent of their initial values within 10 minutes.

DISCUSSION

The results of the present experiments demonstrate that GSH is rapidly regenerated in normal human erythrocytes treated with the GSH oxidizing agent, methyl phenylazoformate. The G-6-PD deficient erythrocytes, on the other hand, regenerate little, if any, GSH under the same experimental conditions. The mechanism previously proposed for the oxidation of GSH involves formation of free radicals in the red blood cell; these radicals react with GSH leading to GS radicals with subsequent formation of GSSG. The rapid rate at which the normal red blood cell can regenerate its GSH might render it capable of continuously absorbing free radicals derived from drugs without harmful consequences to the cell, whereas the deficient cell will be affected by them.

Adequate amounts of GSSG are available in normal and in G-6-PD deficient erythrocytes treated with azoester, as demonstrated by the rapid and full recovery of GSH in lysates of these cells on addition of TPNH. Essentially all of the regenerated GSH arises from GSSG, since de novo synthesis is too slow to account for any but a minute fraction of the observed recovery rates. Variable amounts of GSH were regenerated in red blood cells of G-6-PD heterozygote females: Erythrocytes of one out of seven heterozygote females tested showed GSH regeneration in the low normal range. In erythrocytes of six heterozygote females, the amount of GSH regenerated was less than the mean of the normal by at least 3 S.D. The finding of a variable capacity of the erythrocytes of heterozygote females to regenerate GSH is in agreement with previous studies on the variable expression of G-6-PD deficiency in affected females as reflected in in vitro assays and in clinical manifestations.

The shape of the curve of GSH regeneration in erythrocytes of G-6-PD deficient heterozygote females was found to be similar to that of mixtures of normal red blood cells and erythrocytes of G-6-PD deficient males. The rate of regeneration of GSH in erythrocytes of heterozygote females is consistent with the assumption of the existence of two populations of red blood cells in heterozygote females: One population deficient in G-6-PD activity, and the other with normal activity of this enzyme.

The amounts of GSH regenerated in the erythrocytes of G-6-PD deficient heterozygote females do not correspond precisely to the enzymatic activity measured in the lysate. Since the determination of any enzyme activity in a lysate may not measure the true rate of that enzymatic step within the cell, tests based on reactions occurring in the intact erythrocyte, such as the regeneration of GSH, may reflect better the capacity of the cell to withstand injury by hemolytic drugs.

The GSH regeneration test, performed under the conditions described here, would, of course, reflect not only the rate of TPNH regeneration in the G-6-PD step, but would also measure the rate of conversion of glucose to glucose-6-phosphate, as well as the efficiency of the reduction of GSSG to GSH by GSSG.
reductase in the intact erythrocyte. It could, therefore, serve as a screening procedure for detecting deficiencies in those reactions.

**Summary**

Reduced glutathione (GSH) was rapidly regenerated in normal human red blood cells treated with the GSH oxidizing agent, methyl phenylazoformate. Erythrocytes of G-6-PD deficient males regenerated little, if any, GSH under the same conditions. The rate of regeneration of GSH in erythrocytes of G-6-PD deficient heterozygote females was similar to that of a mixture of normal red blood cells and erythrocytes of G-6-PD deficient males. It was compatible with the assumption of mosaicism of the erythrocytes in the heterozygote females. The rapid rate at which the normal erythrocyte can regenerate its GSH may render it capable of continuously absorbing free radicals derived from drugs without harmful consequences to the cell.

Study of the rate of regeneration of GSH in erythrocytes treated with methyl phenylazoformate may be useful in the detection of deficiencies of G-6-PD, GSSG reductase, and hexokinase.

**Summartio in Interlingua**

Reducte glutathiona (GSH) esseva regenerate rapidemente in human erythrocytos normal tractate con le agente de oxydation de GSH, phenylazoformato methylic. Erythrocytos ab masculos carente in dehydrogenase de glucosa-6-phosphato (DG-6-P) regenerava pauc o nulle GSH sub le mesme conditiones. Le intensitate del regeneration de GSH in erythrocytos de heterozygoticos feminin carente in G-6-P esseva simile a illo de un mixtura de erythrocytos ab masculos carente in G-6-P. Illo esseva de accordo con le supposition de mosaicismo del erythrocytos in le feminina heterozygotic. Le rapiditate con le qual le erythrocyto normal pote regenerar su GSH rende lo possibilemente capace a absorber continuemente radicales libere derivate ab pharmacos sin adverse effectos pro le cellula.

Un studio del intensitate regeneratori de GSH in erythrocytos tractate con phenylazoformato methylic es possibilemente utile in le detection de carentias de DG-6-P, de reductase de glutathiona oxydate, e de hexokinase.

**REFERENCES**


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