Effects of Sulfhydryl Inhibition on Red Blood Cells

III. GLUTATHIONE IN THE REGULATION OF THE HEXOSE MONOPHOSPHATE PATHWAY*

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HARRY S. JACOB AND JAMES II. JANDL

From the Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02118

SUMMARY

Evidence is presented that the activity of the hexose monophosphate (HMP) pathway of red cells, assayed by $^{14}C$ production from glucose-$1-^{14}C$, is regulated primarily by glutathione. Methemoglobin had little, if any, effect on the activity of this pathway.

Increasing the ratio of oxidized to reduced glutathione, either by peroxidizing GSH to GSSG or by partially blocking GSH with NEM, increased the rate of HMP pathway metabolism. Complete blockage of cellular glutathione by NEM depressed the bulk of HMP pathway activity, despite little or no effect on the Embden-Meyerhof pathway.

Sustained low levels of hydrogen peroxide, whether generated by aerobic oxidases or by the coupled oxidation of ascorbic acid with oxyhemoglobin, stimulated the HMP pathway of cells. This stimulation was potentiated by blocking catalase and was prevented by blocking GSH. The oxidation of NADPH by $H_2O_2$ in hemolysates was specifically dependent upon the presence of GSH. These results substantiate the existence in human red cells of the glutathione peroxidase mechanism proposed by Mills, whereby GSH protects cellular constituents such as hemoglobin from oxidative damage induced by $H_2O_2$.

Oxidative denaturation of oxyhemoglobin to met- and sulf-hemoglobin by $H_2O_2$-generating mechanisms is markedly potentiated in cells lacking sufficient GSH, and commences only after GSH levels approach zero. In contrast, oxidation-reduction catalysts such as methylene blue and acetylphenylhydrazine catalyze the direct oxidation of NADPH and hemoglobin, as well as GSH, by molecular oxygen. Although GSH is partially protective against these agents, they appear to damage red cells by virtue of their ability to bypass the GSH peroxidase mechanism and to cause oxidative injury despite persisting GSH.

Approximately 10% of the glucose consumed by human red cells in serum in vitro is metabolized by way of the hexose monophosphate pathway (1, 2). In mature red cells, this HMP (pentose phosphate or phosphogluconate) pathway is the sole mechanism for glucose oxidation and is instrumental in the maintenance of NADPH. The importance of this metabolic pathway to the integrity of the red cell and the mechanism by which its rate is regulated are poorly understood. It is known that a severe restriction of the HMP pathway, as in red cells from patients having marked inherited deficiency or instability of the initial enzyme, glucose 6-phosphate dehydrogenase, is associated with a chronic, nonspherocytic hemolytic anemia (3, 4). The less complete restriction of potential HMP pathway activity encountered in red cells of patients with a less severe deficiency of glucose-6-P dehydrogenase is associated with only a slight reduction in red cell survival (5), yet produces marked vulnerability to hemolysis when the affected red cells are exposed to certain substances that act as oxidation-reduction catalysts (6). Such glucose-6-P dehydrogenase-deficient red cells that are hypersusceptible to oxidant drugs metabolize glucose through the HMP pathway at a normal rate under physiological resting conditions, but are unable to accelerate normally their HMP pathway activity in response to oxidants or oxidation-reduction catalysts (i.e. methylene blue).

The activity of the HMP pathway is believed to reflect the relative amount of nicotinamide adenine dinucleotide phosphate (NADP$^+$). If so, the rate of this metabolism is necessarily linked to reactions wherein NADPH is oxidized. These are few in the mature red cell since the tricarboxylic acid cycle and the fatty acid synthetic mechanisms, both importantly involved in NADPH metabolism in other tissues, are lacking in mature red cells (8). Since glutathione reductase specifically requires NADPH as its cofactor (9), and since glutathione is present in relatively large amounts in red cells, the reduction of oxidized glutathione to reduced glutathione presumably represents an important potential source of NADP$^+$ in red cells. This reaction is thought to be of crucial importance to the cell since reduced glutathione is believed to maintain other thiol groups of the cell in the reduced state through oxidation-reduction interaction (10, 11). Failure of maintenance of thiol activity in red cell membranes results in hemolysis in vitro (11) and in vivo (12), while oxidation or blockade of the reactive thiol groups of hemoglobin renders it highly vulnerable to oxidative denaturation and ultimate precipitation into so-called Heinz bodies (10, 13).

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1 The abbreviations used are: HMP, hexose monophosphate; NEM, N-ethylmaleimide.
It has been suggested that $\text{H}_2\text{O}_2$ generated continuously during red cell metabolism (14), is an important oxidant of GSH in red cells. Mills (15) and Mills and Randall (16) have isolated a glutathione peroxidase from bovine erythrocytes which, when coupled with glutathione reductase, provides a means of detoxifying $\text{H}_2\text{O}_2$ through the reversible oxidation of GSH. The following reactions pertain.

$$\text{H}_2\text{O}_2 + 2 \text{GSH} \underset{\text{glutathione peroxidase}}{\rightarrow} \text{GSSG} + 2 \text{H}_2\text{O} \quad (1)$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \underset{\text{glutathione reductase}}{\rightarrow} 2 \text{GSH} + \text{NADP}^+ \quad (2)$$

The NADPH required to regenerate GSH and maintain this mechanism is generated exclusively in the HMP pathway, the rate of which, in turn, would be expected to be accelerated by the NADP$^+$ produced in Reaction 2. Since red cells contain abundant quantities of catalase and glutathione peroxidase, since in the complete absence of catalase, as in hereditary acatalasia, there is an increase, apparently compensatory, in the rate of GSH oxidation and of HMP pathway activity (14).

The following studies were undertaken to clarify the role of glutathione oxidation, especially by peroxidation, in the regulation of HMP pathway activity in the mature red cell. In addition, observations on the relative roles of the HMP pathway and of catalase in detoxifying $\text{H}_2\text{O}_2$ and on the importance of GSH in protecting red cells from damage by oxidant hemolytic drugs have been made. The findings have been partially presented in preliminary form elsewhere (19).

**Experimental Procedure**

Fresh blood from normal subjects was drawn into heparin and centrifuged at 1000 $\times$ g; the buffy coat was removed and the cells were suspended in isotonie, buffered bicarbonate-phosphate medium of pH 7.4 containing 200 mg/100 ml of glucose. Following recentrifugation and a second removal of any remaining buffy coat, the red cells were suspended in the buffered medium to a concentration of 50%. The suspension medium was as follows: Na$^+$, 145 mm; K$^+$, 5 mm; HCO$_3^-$, 25 mm; PO$_4^{3-}$, 1.5 mm; Cl$^-$, balance of anions. The medium was equilibrated to a pH of 7.4 with 95% air-5% CO$_2$ prior to use. Leukocytes in the red blood cell buffy coat, the red cells were suspended in the buffered medium at a concentration of about 250 red cells per mm$^3$.

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For measurements of NADPH oxidation, it was necessary to determine the rate of [NADPH]$^+$ oxidation at room temperature was followed at 340 $\mu$m in a Beckman DU spectrophotometer with the use of quartz cuvettes with light paths of 1 cm. For these studies, stroma-containing aqueous hemolysates (representing 3 $\times$ 10$^7$ red cells per ml), NADPH (0.08 $\mu$moles per ml), and nicotineamide (10 $\mu$moles per ml) were used.

GSH was measured by the method of Grunnert and Phillips (25), as modified by Beutler (26). Methemoglobin, sulfhemoglobin, and total hemoglobin were determined by the Evelyn and Malloy method (27). The appearance of incubated cells was examined microscopically in wet preparations after resuspension in autologous plasma diluted with an equal volume of saline containing crystal violet.

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Fig. 1. Effect of H$_2$O$_2$ on the HMP pathway of red cells. Progressive increments in the H$_2$O$_2$-generating substrates, ascorbic acid (left), and D-leucine in the presence of excess D-amino acid oxidase (right), produced progressive increments in HMP pathway activity in red cells. HMP pathway metabolism is expressed as a percentage of the over-all glucose consumption (top) and as the absolute quantity of glucose consumed via the pathway (bottom).

μmoles per ml, were utilized, and H$_2$O$_2$ was generated by a mixture of 1 mg/100 ml of glucose oxidase, obtained from Nutritional Biochemicals, and 20 mM glucose. Blank solutions contained all additives except NADPH.

RESULTS
Stimulation of HMP Pathway in Red Cells by H$_2$O$_2$—Comparable quantities of H$_2$O$_2$ were provided to red cells either by generation through the coupled oxidation of ascorbic acid with oxyhemoglobin (28) or by the oxidative deamination of D-leucine with D-amino acid oxidase. As shown in Fig. 1, red cell HMP pathway activity increased progressively with increments in H$_2$O$_2$-generating substrate. There was an increase both in the percentage contribution of the pathway to total glucose consumption (Fig. 1, top) and in the absolute amount of glucose metabolized thereby (Fig. 1, bottom). H$_2$O$_2$ added to red cell suspensions as a vapor by diffusion in Warburg flasks (24) also stimulated HMP pathway activity (not shown).

The stimulation of HMP pathway metabolism by H$_2$O$_2$ was potentiated when red cell catalase was inhibited. As shown in Table I, addition of the catalase inhibitors sodium cyanide or sodium azide to red cells which were exposed to ascorbic acid or D-amino acid oxidase plus D-leucine potentiated the increment in HMP pathway activity. Of note also was the marked stimulation of the HMP pathway when azide alone was added to normal red cells. In previous studies of the role of H$_2$O$_2$ in oxidative hemolysis, this substance has been extensively utilized as an inhibitor of catalase. That in addition to its catalase-binding properties it has an oxidative effect as well has been suggested.

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of catalase inhibition on HMP pathway in red cells</td>
</tr>
<tr>
<td>Duplicate aliquots of washed and buffered 35% red cell suspensions containing 11 mM glucose were incubated for 4 hours at 37⁰. When appropriate, additions were as follows: ascorbic acid, pre-neutralized with NaOH, was added to a final concentration of 0.012 mM; a mixture yielding final concentrations of 0.2% D-amino acid oxidase and 0.015 mM D-leucine was utilized; catalase inhibition was with sodium cyanide or sodium azide, both pre-neutralized with HCl, and present in final concentrations of 0.010 mM. The experiment depicted is representative of three performed.</td>
</tr>
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<table>
<thead>
<tr>
<th>H$_2$O$_2$-generating system</th>
<th>Catalase inhibitor</th>
<th>Glucose metabolized via HMP pathway</th>
<th>Effect of catalase inhibition: change in glucose transit through HMP shunt</th>
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<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>164</td>
<td>-5</td>
</tr>
<tr>
<td></td>
<td>Cyanide</td>
<td>91</td>
<td>+34</td>
</tr>
<tr>
<td></td>
<td>Azide</td>
<td>125</td>
<td>+95</td>
</tr>
<tr>
<td></td>
<td>Cyanide</td>
<td>395</td>
<td>+61</td>
</tr>
<tr>
<td></td>
<td>Azide</td>
<td>637</td>
<td>+128</td>
</tr>
<tr>
<td>Ascorbic acid</td>
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<td>395</td>
<td>+61</td>
</tr>
<tr>
<td></td>
<td>Cyanide</td>
<td>637</td>
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<tr>
<td></td>
<td>Azide</td>
<td>901</td>
<td>+128</td>
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<tr>
<td>D-Amino acid oxidase + D-leucine</td>
<td>None</td>
<td>525</td>
<td>+34</td>
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<tr>
<td></td>
<td>Cyanide</td>
<td>701</td>
<td>+34</td>
</tr>
<tr>
<td></td>
<td>Azide</td>
<td>654</td>
<td>+25</td>
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</table>
TABLE II

Effect of GSH inhibition by NEM on HMP pathway in red cells

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Conversion of glucose-1-¹⁴C to HCO₃⁻</th>
<th>Glucose metabolized via HMP pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No NEM</td>
<td>NEM</td>
</tr>
<tr>
<td>None</td>
<td>7.2 ± 1.2</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>41.5 ± 1.6</td>
<td>11.6 ± 1.9</td>
</tr>
<tr>
<td>n-Amino acid oxidase + n-Leucine</td>
<td>28.7 ± 2.6</td>
<td>11.2 ± 0.9</td>
</tr>
<tr>
<td>Acetylphenylhydrazine</td>
<td>24.8 ± 1.4</td>
<td>19 ± 1.9</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>41.1 ± 1.3</td>
<td>42.5 ± 1.5</td>
</tr>
</tbody>
</table>

* Range.

b In control experiments, the enzymatic activity of n-amino acid oxidase was shown to be unaffected by exposure to washed NEM-treated red cells. Its ability to stimulate the HMP pathway in fresh untreated red cells persisted unabated.

Fig. 2. Effect of GSH blockade on the HMP pathway of red cells. HMP pathway activity was regularly stimulated when red cell GSH was partly blocked by NEM (left). With complete blockade, HMP pathway activity was inhibited, despite normal over-all glucose consumption (not shown). These phenomena are accentuated in GSH-blocked red cells exposed to HzO₂ generated by 6 mM ascorbic acid (right). The paired experiments depicted are representative of four performed.

by previous studies with acatalasric red cells (14), and is confirmed by the present results.

Role of Glutathione in Regulation of HMP Pathway Activity in Red Cells—As reported previously, red cell GSH can be blocked by small doses of NEM without reducing over-all utilization of glucose (11). However, despite the maintenance of over-all glucose consumption, the metabolism of glucose through the HMP pathway in red cells was reduced by more than half when GSH was completely blocked by NEM, as shown in Table II. In addition, as also shown in Table II, the marked acceleration of HMP pathway activity in red cells exposed to H₂O₂ by treatment with ascorbic acid or n-amino acid oxidase and n-leucine was strikingly inhibited by blockade of GSH. In parallel studies, HMP pathway activity was stimulated to a comparable extent by the addition of an appropriate concentration of the oxidation-reduction catalyst methylene blue; in sharp contrast to the H₂O₂-generating systems, HMP pathway activity in the methylene blue-stimulated system was unaffected by GSH blockade (Table II). In previous studies, we have found that acetylphenylhydrazine, a potent hemolytic oxidant compound, does not generate appreciable amounts of H₂O₂ (14). Blockage of cellular GSH inhibited only moderately the capacity of acetylphenylhydrazine to accelerate HMP pathway metabolism (Table II).

As shown in Fig. 2 (left), when red cell GSH was blocked to various extents by NEM, stimulation, rather than inhibition, of HMP pathway activity was regularly seen when the GSH block-
ade was partial. This stimulatory effect was exaggerated when the NEM-treated red cells were simultaneously exposed to H$_2$O$_2$ by treatment with ascorbic acid (Fig. 2, right). Stimulation was greatest when 70 to 80% of cellular GSH had been inhibited. With complete GSH blockade, HMP pathway activity diminished sharply as noted above (Table II). It may be noted that NEM reacts to form a stable addition product with GSH, thereby lowering its concentration, but does not react with GSSG. Thus, NEM increases the ratio of oxidized to reduced glutathione by decreasing its denominator, and, until GSH is almost completely blocked, it increases HMP pathway activity.

Further evidence that the HMP pathway in red cells is regulated importantly by the relative levels of reduced and oxidized glutathione is shown in Fig. 3. Following preincubation in the presence of an H$_2$O$_2$-generating system, red cells were washed free of H$_2$O$_2$ and resuspended in buffered medium containing glucose-1-14C to measure HMP pathway activity. As shown in the lower portion of Fig. 3, HMP pathway metabolism was inversely proportional to the cellular level of GSH. It is assumed that GSH had been converted quantitatively to GSSG during preincubation with H$_2$O$_2$, through the influence of GSH peroxidase (15, 18), disputed by others (29), for the existence of GSH peroxidase in human red cells. In ancillary studies (not shown), the oxidation of NADPH by H$_2$O$_2$ in the presence of GSH does indeed exist in human red cells is shown in Fig. 4. Control hemolysates containing endogenous GSH (Fig. 4, left) facilitated the oxidation of NADPH when H$_2$O$_2$ was added (O --- O). Supplementation with exogenous GSH (additional to that originally present in the hemolysate) may have further accelerated this reaction. In contrast, hemolysates, which were pretreated with NEM and therefore devoid of free GSH (Fig. 4, right), did not catalyze the oxidation of NADPH even with H$_2$O$_2$ present; they did so, however, when GSH was added. These studies support previous evidence (15-18), disputed by others (29), for the existence of GSH peroxidase in human red cells. In ancillary studies (not shown), the oxidation of NADPH by molecular oxygen in the presence of methylene blue or acetylphenylhydrazine occurred at undiminished rates with GSH blockaded.

**Role of GSH in Protection of Hemoglobin from Oxidation by H$_2$O$_2$**—Previous studies in genetically acatalasic red cells (14) have shown that on exposure to H$_2$O$_2$ the absence of catalase is accompanied by an increased rate of oxidation of glutathione, presumably through the influence of glutathione peroxidase (15, 16). Oxidation of hemoglobin is thereby avoided. This se-
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Fig. 5. The effect of GSH blockade on methemoglobin production by H₂O₂ in red cells. Diaphorase-deficient red cells from a patient with congenital methemoglobinemia were unable to reduce methemoglobin upon incubation with glucose (0.02 M) alone (○—○). Methemoglobin increased only slightly in these cells with GSH intact (left) even during their exposure to H₂O₂, which was generated by 1 mg per 100 ml of glucose oxidase (O— □). After GSH blockade by NEM alone (right, ●—●), methemoglobin levels remained constant. When H₂O₂ was added to NEM-treated cells, however, methemoglobin levels increased rapidly. Methylene blue (A—A) catalyzed the rapid reduction of methemoglobin, regardless of cellular GSH content.

Fig. 6. Relationship of GSH oxidation to sulfhemoglobin accumulation in red cells exposed to oxidant compounds. In glucose-free red cells exposed to 18 mM ascorbic acid (left), GSH was progressively oxidized (○—○). Following its complete oxidation, sulfhemoglobin (O—□) accumulated very rapidly. In contrast, oxidation of GSH and formation of sulfhemoglobin began earlier and occurred concomitantly in cells exposed to 12 mM acetylphenylhydrazine (right). The experiment depicted is representative of two performed.

The effect of GSH blockade on methemoglobin production by H₂O₂ in red cells. Diaphorase-deficient red cells from a patient with congenital methemoglobinemia were unable to reduce methemoglobin upon incubation with glucose (0.02 M) alone (●—●). Methemoglobin increased only slightly in these cells with GSH intact (left) even during their exposure to H₂O₂, which was generated by 1 mg per 100 ml of glucose oxidase (O—□). After GSH blockade by NEM alone (right, ●—●), methemoglobin levels remained constant. When H₂O₂ was added to NEM-treated cells, however, methemoglobin levels increased rapidly. Methylene blue (A—A) catalyzed the rapid reduction of methemoglobin, regardless of cellular GSH content.

Further evidence for the role of GSH in protecting hemoglobin from oxidative damage by H₂O₂ is presented in Fig. 6. When glucose-free red cells were exposed to H₂O₂ by treatment with 10 mM ascorbic acid and sodium cyanide (Fig. 6, left), GSH levels fell steadily (○—○). In the absence of GSH, following its complete oxidation, did hemoglobin begin to undergo conversion to irreversibly denatured hemochromes, which, when measured at 620 nm in the presence of cyanide, are usually termed sulfhemoglobin (□—□). In contrast, with acetylphenylhydrazine (Fig. 6, right), shown in previous studies (14) to cause oxidation of hemoglobin without involving H₂O₂, GSH was oxidized somewhat more slowly, whereas sulfhemoglobin content formed steadily even while considerable amounts of GSH were still present in the cells. During oxidation by H₂O₂, GSH and NADPH linked reductase was provided. Such cells were used in these studies to allow direct assessment of the oxidative effect of H₂O₂ on hemoglobin without the complicating influence of mechanisms favoring methemoglobin reduction. As shown in Fig. 5 (left), 22% of the hemoglobin of these cells was present as methemoglobin at the beginning of the experiment. No methemoglobin reduction occurred during 4 hours of incubation with glucose alone (○—○). When these cells were exposed to low levels of H₂O₂ which were steadily generated by enzymatic means (Fig. 5, left, O—□), defense of hemoglobin from further oxidation was apparent, methemoglobin levels increasing only slightly. However, when H₂O₂ was added to these red cells after their GSH had been blocked by preincubation with NEM (Fig. 5, right), the methemoglobin concentration increased markedly (□—□). When methylene blue was supplied to these cells, methemoglobin was rapidly reduced whether or not GSH had been blocked (A—A).

Effect of methemoglobin on HMP pathway in red cells

Duplicate incubations of normal and methemoglobinemic red cells were made in parallel with glucose-1-¹⁴C. Congenitally methemoglobinemic red cells, which lacked the NADH-linked pathway of methemoglobin reduction, were used in Experiment 1. In Experiments 2 and 3, 1 volume of normal red cells was incubated for 1 hour at 37° with 3 volumes either of 1% NaN₃ or of 0.90% NaCl alone. Both aliquots were washed four times in buffered suspension medium (see "Experimental Procedure") containing 150 mg/100 ml of glucose prior to subsequent incubation with glucose-1-¹⁴C.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Red cells</th>
<th>Methemoglobin</th>
<th>Total glucose consumed</th>
<th>Glucose metabolized via HMP pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% total hemoglobin</td>
<td>μmoles/ml cells/hr</td>
<td>μmoles/ml cells/hr</td>
</tr>
<tr>
<td>1</td>
<td>Congenital methemoglobin</td>
<td>20</td>
<td>1.2</td>
<td>76 ± 1.0*</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>0</td>
<td>1.0</td>
<td>78 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>Nitrited normal</td>
<td>76</td>
<td>1.4</td>
<td>167 ± 1.2*</td>
</tr>
<tr>
<td></td>
<td>Normal control</td>
<td>0</td>
<td>1.5</td>
<td>147 ± 8.5</td>
</tr>
<tr>
<td>3</td>
<td>Nitrited normal</td>
<td>82</td>
<td>1.3</td>
<td>176 ± 10.5*</td>
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<tr>
<td></td>
<td>Normal control</td>
<td>0</td>
<td>1.3</td>
<td>124 ± 1.0</td>
</tr>
</tbody>
</table>

* Range.
† The increased number of washings of red cells required in Experiments 2 and 3 may explain the slightly increased HMP pathway activity in these experiments (33).
sulfhemoglobin did not appreciably coexist but, with extinction of GSH, sulfhemoglobin began to accumulate very rapidly. During oxidation by the hemolytic compound, acetylphenylhydrazine, however, GSH and sulfhemoglobin did coexist or overlap to various extents and extinction of GSH had comparatively much less effect on the rate of accumulation of sulfhemoglobin.

Effect of Methemoglobin on HMP Pathway Activity in Red Cells—The possibility that the level of methemoglobin is an important determinant of HMP pathway activity is implied by the existence of an NADPH-linked methemoglobin reductase in red cells (31, 32). Evidence on this point was sought by studying red cells from the patient described above with congenital methemoglobinemia and in normal red cells made methemoglobinemic by appropriate pretreatment with sodium nitrite. As shown in Table III, red cells from the patient with congenital methemoglobinemia and normal cells that had been preincubated with nitrite manifested little if any acceleration of HMP pathway activity and no abnormal overall utilization of glucose despite high levels of methemoglobin. In parallel studies with various concentrations of methylene blue, the HMP pathway of methemoglobinemic red cells was stimulated to the same degree as in control cells having low levels of methemoglobin (not shown).

**DISCUSSION**

The present studies show that cellular glutathione and its oxidation state are importantly involved in the regulation of HMP pathway metabolism in red cells. The data depicted in Fig. 3 are consistent with the proposition that the rate of metabolism of the HMP pathway is directly proportional to the cellular concentration of oxidized glutathione. Presumably this follows from the specificity of NADP+ generation in the glutathione reductase reaction. Since only levels of reduced, but not oxidized, glutathione were measured in these experiments, the conclusion that HMP pathway activity was proportional to GSSG levels must be based on the assumption that in red cells exposed to H2O2 (Fig. 3) the levels of GSH are reciprocal with those of GSSG. The unique specificity with which H2O2 oxidizes GSH to GSSG in red cells (15–18), as well as the rapid and complete regeneration of GSH following removal of H2O2 in the experiments depicted in Fig. 3, presumably by means of glutathione reductase, corroborates the validity of this assumption. It is acknowledged that a part of the glutathione may have become reversibly linked in mixed disulfide bonds with other sulfhydryl compounds of the cell. Indeed, the formation of mixed disulfides involving glutathione and globin thiols during nonspecific oxidation was shown (10). However, there is no evidence that this linkage is metabolically reversible or that it occurs during peroxidation. Thus, although it cannot be concluded unequivocally that GSSG per se is the primary stimulant of HMP pathway metabolism in our experiments, such an interpretation seems most reasonable. The present studies also show that the rate of HMP pathway metabolism is inversely proportional to the level of reduced glutathione (Fig. 2). Under conditions in which GSSG was not being generated, selective partial blockade of reduced glutathione by NEM also accelerated HMP pathway metabolism. The combined data of Figs. 2 and 3 thus indicate that the ratio of oxidized to reduced glutathione, rather than either moiety alone, correlates with the rate of glucose-1-14C oxidation in red cells.

Complete (and reversible) oxidation of GSH by H2O2 did not impair glycolysis, and was in fact markedly stimulatory to glucose oxidation by way of the HMP pathway (Fig. 3). On the other hand, blocking more than about 80% of GSH with NEM caused a decline in the stimulated activity of this pathway (Fig. 2), and the addition of NEM in amounts equal to, or slightly exceeding, that required to block GSH fully caused a sharp decline below resting normal levels (Fig. 2; Table II). This occurred prior to any inhibition of the Embden-Meyerhof pathway and is attributable in part to inhibition of glucose-6-P dehydrogenase (unpublished observations), possibly by a direct action of NEM, as free GSH becomes limiting. That GSH is oxidized more rapidly than hemoglobin in red cells exposed to a variety of oxidant compounds (10, 14) and the present data showing that methemoglobin and sulfhemoglobin appear only after depletion of GSH during peroxidation (Figs. 5 and 6) indicate that GSH per se serves to protect hemoglobin and possibly other cellular constituents from oxidative injury. As a corollary, the importance of the HMP pathway to red cell integrity is validated. Recently, patients have been described (33, 34) whose red cells possess less than 10% of the normal amount of GSH, as the result of a specific inherited deficiency of glutathione synthetase (34). These cells have a moderately short life span in vivo and are highly susceptible to oxidant compounds in vitro and in vivo, although quite normal with respect to glycolytic activity, ATP levels, and the levels of a number of critical enzymes measured, including glucose-6-P dehydrogenase and glutathione reductase.

Recent evidence has suggested that glutathione and the HMP pathway in red cells might function importantly to dissipate hydrogen peroxide. This has been observed in normal cells exposed for long periods to H2O2 (14, 17, 24, 35), in catalase-inhibited cells (14–17, 35), and in genetically acatalasic cells (14). The present studies directly show that the mature human red cell, when challenged by H2O2, accelerates the rate of glucose metabolism through the HMP pathway. This response requires the presence of glutathione and may occur despite an abundance of catalase within these cells. If catalase is also inhibited, however, a further, usually marked, potentiation of HMP pathway activity occurs, whether the H2O2 level is high or low. It has been suggested by others (17) that glutathione peroxidation, with presumed HMP pathway stimulation, is solely responsible for dissipating the low, steady levels of H2O2 shown to be involved when red cells are exposed to certain oxidant compounds (14, 36). However, the potentiation of oxidative damage to hemoglobin by H2O2, which occurs when neither the HMP pathway nor catalase is functioning (14), and the results of the present studies would suggest rather that both systems act together to defend red cells from H2O2.

Acceleration of HMP pathway activity by H2O2-generating substances requires the presence of intact GSH. By contrast, agents such as methylene blue and acetylphenylhydrazine that catalyze the direct transfer of electrons from NADPH to molecular oxygen can stimulate the HMP pathway independently of GSH. The implication that, in contrast to methylene blue and acetylphenylhydrazine, H2O2 does not readily oxidize NADPH directly, but does so indirectly by oxidizing GSH is supported by the experiments in hemolysate systems (Fig. 4). With H2O2, the presence of both GSH and H2O2, but neither alone, was required for NADPH oxidation, whereas with methylene blue or acetylphenylhydrazine oxidation was affected little or not at all by the presence of GSH.

From these data, it is concluded that a major proportion of HMP pathway activity in normal red cells is regulated by the
demand for NADPH required for the maintenance of cellular thiol groups in the reduced state. That thiol metabolism might also significantly regulate HMP pathway activity throughout the entire body is suggested by the studies of Carson et al. (37) in patients with inherited deficiency in glutathione reductase. Following parenteral administration of glucose-1-14C, these patients expired abnormally low quantities of 14CO2 (37).

It is also likely that H2O2 generated continuously within tissues is responsible for much of the resting HMP pathway activity of red cells through peroxidation of GSH. That the bombardment of these cells with H2O2 is significant even in the absence of added peroxide-generating substances is suggested by the finding of 3 times the normal HMP pathway activities in genetically acatalasemic red cells (14). In addition, normal plasma constituents which are capable of generating H2O2, such as ascorbic acid, uric acid, and thyroxine, might well affect the rate of red cell HMP pathway metabolism at any given time. Indeed, the production of 14CO2 by red cells in vitro is enhanced slightly (+20%) in artificial media to which are added physiological amounts of ascorbic acid (unpublished observations).

The present studies support previous evidence as to the importance of the HMP pathway in red cells in defending against oxidative damage. The regulation of its rate is primarily related to alterations in the oxidation state of glutathione. The oxidation state of the hemes of hemoglobin, on the other hand, has little if any effect on the velocity of this pathway (Table III).

The failure of methemoglobin to have an appreciable effect on the activity of the HMP pathway was evident both in red cells with endogenous methemoglobin, because of a defect in the NADH-linked reductase, and in red cells that had been exposed to nitrite. (It may be noted that nitrite selectively oxidizes hemoglobin to methemoglobin but does not oxidize GSH under physiological conditions (38).) When an oxidation-reduction catalyst such as methylene blue is present, however, the GSH-regulated mechanism is bypassed. Hemolytic compounds such as acetylphenylhydrazine and primaquine appear to possess both properties in some degree, for they show some selectivity in oxidizing GSH but at the same time can also activate the HMP pathway by directly catalyzing the interaction between NADPH and molecular oxygen. Consequently, there is a difference in the kinetics of hemoglobin oxidation in red cells exposed to H2O2-generating systems as compared to other oxidant systems. Because of the specificity conferred by glutathione peroxidase, H2O2 oxidizes and denatures hemoglobin only after GSH has been fully destroyed. On the other hand, oxidation-reduction catalysts oxidize hemoglobin coincident with the oxidation of GSH (10) (Fig. 6). Although the enzymatic reduction of GSSG would act to oppose such oxidative injury, and this reductive process thereby provide a protective mechanism, the nonenzymatic activity of oxidant action can lead to oxidative damage to hemoglobin and other cellular components despite the persistence of some GSH. Presumably, for this reason, oxidation-reduction catalysts such as acetylphenylhydrazine are potent hemolytic agents.

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Effects of Sulphydryl Inhibition on Red Blood Cells: III. GLUTATHIONE IN THE REGULATION OF THE HEXOSE MONOPHOSPHATE PATHWAY

Harry S. Jacob and James H. Jandl


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