Hydrogen Peroxide Toxicity and Detoxification in the Erythrocytes of Newborn Infants

By Ruth T. Gross, Rodolfo Bracci, Nathan Rudolph, Eleanor Schroeder and Joseph A. Kochen

Recent studies have emphasized the role of hydrogen peroxide (H₂O₂) as the toxic intermediate compound common to a variety of hemolytic agents.¹⁴ In the newborn, and particularly in the premature infant, there is increased susceptibility to hemolytic anemia associated with administration of compounds capable of acting as oxidizing agents.⁵,⁶ Further evidence that erythrocytes of young subjects are unduly susceptible to oxidative damage is provided by observations showing that in these cells there is an increased tendency toward methemoglobin formation,⁷,⁸ Heinz body production⁹ and in vitro hemolysis induced by H₂O₂.¹⁰ These phenomena have been explained in part by the demonstration that in the erythrocytes of newborn subjects there is decreased activity of methemoglobin reductase,¹¹,¹² catalase¹³ and low levels of vitamin E.¹⁴ However, recent studies have shown that at low concentrations, H₂O₂ may be detoxified in a glucose-dependent pathway in which the final reaction is catalyzed by the enzyme glutathione peroxidase (GSH-P).¹,²,⁴,¹⁵

The present study was undertaken to determine whether erythrocytes of newborn subjects showed increased susceptibility to toxicity from H₂O₂ at low steady-state concentrations and to examine the efficiency of the mechanisms for detoxification of H₂O₂ in these cells. The problem was investigated in three ways: (1) Toxic effects of perfusion with H₂O₂ at low concentrations in vitro were compared in erythrocytes from newborn infants and from adults. The effect of the addition of vitamin E to the incubation mixtures was also studied. (2) Fetal and adult hemoglobin samples were prepared free of protective enzymes, and the susceptibility of each to oxidation by low concentrations of H₂O₂ was compared. (3) In the various groups of subjects a comparison was made of the activities of the erythrocyte enzymes involved in the detoxification

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of \( \text{H}_2\text{O}_2 \). These included catalase and the enzymes and cofactors of the glucose-dependent pathway:\(^1\)

\[
\begin{align*}
\text{G6P}^* & \quad \text{TPN} \\
6\text{PG} & \quad \text{TPNH} \\
\text{G6PD} & \quad \text{GSSG-R} \\
\text{GSH} & \quad \text{GSH-P} \\
\text{H}_2\text{O}_2 & \quad \text{H}_2\text{O}
\end{align*}
\]

We have shown previously that there is increased activity of G-6-PD\(^1\) and adequate levels of TPN and TPNH\(^1\) in the erythrocytes of newborn and premature infants. In this report we are presenting results of measurements of activities of GSSG-R and GSH-P. To our knowledge this is the first assessment of the activity of GSH-P in the erythrocytes of young subjects.

**METHODS**

The studies to be described were performed on cord blood from normal newborn infants weighing 2800 to 3500 Gm., on venous blood from both full-term newborn infants 0 to 18 hours old and premature infants 1 to 3 days old weighing 1400 to 2500 Gm. None of these subjects showed signs of hemolysis, marked jaundice or other evidence of hematologic disease. Similar studies were made on venous blood of a group of healthy adults consisting either of hospital personnel or known blood donors and a group of mothers immediately postpartum. Observations were also included on samples from subjects with congenital nonspherocytic hemolytic anemia. All samples of blood were collected in acid citrate dextrose (ACD) solution, Type A, and refrigerated immediately at 0 to 4°C.

*In vitro perfusion of intact erythrocytes with \( \text{H}_2\text{O}_2 \)* was performed according to the procedure of Cohen and Hochstein.\(^15\) Erythrocytes were washed 3 times in isotonic saline buffered with phosphate at pH 7.4\(^18\) and resuspended to a hematocrit of 12 percent. Three ml. of erythrocyte suspension plus 50 μM of glucose were placed in the main chamber of a 25 ml. Warburg flask, and 0.25 ml. of 30 percent \( \text{H}_2\text{O}_2 \) was placed in the center well. The flasks were tightly stoppered and then incubated in a Dubnoff metabolic shaking incubator at 37°C at a shaking speed of 70 r.p.m. for the times noted, usually 13 to 14 hours. After incubation, the erythrocytes were washed 3 times and resuspended in buffered saline to a hematocrit of 12 percent. All determinations were then performed within 3 hours.

The perfusion experiments were performed only in calibrated Warburg flasks in which the diffusion rates of \( \text{H}_2\text{O}_2 \) were 6 to 9 μM per hour. These rates had been predetermined by substituting 3 ml. of 2N sulfuric acid for the erythrocyte suspension and then measuring the amount of \( \text{H}_2\text{O}_2 \) dissolved after various periods of incubation by titration with 0.01N potassium permanganate (KMnO\(_4\)).

*Methemoglobin and choleglobin* were measured and calculated by the method of Mills and Randall.\(^13\) Total hemoglobin was measured as cyanmethemoglobin.\(^19\) Heinz bodies were examined by one or both of two methods: (1) in wet preparations stained with crystal violet\(^20\) and examined by light microscopy; (2) in wet unstained preparations of erythrocyte ghosts prepared by lysis with distilled water and examined by phase contrast microscopy.

*Hemoglobin, free of protective enzymes,* was prepared according to Hennessey et al.\(^21\) with modifications as follows. Erythrocytes were washed twice and then hemolyzed by addition of an equal volume of deionized water followed by freezing and thawing once. Stroma was then removed by centrifugation at 6000 g at 4°C for 15 minutes in a Sorval refrigerated centrifuge. Cellulose \( N, N\)-diethylaminoethyl (DEAE) was prepared as described\(^21\) utilizing the extra washings recommended by Hill and co-workers.\(^22\) Hemolysate containing approximately 100 mg. of hemoglobin per ml. was mixed with DEAE in a ratio

\*G-6-P, Glucose-6-phosphate; 6-PG, 6-phosphogluconate; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; GSH, reduced glutathione; GSSG, oxidized glutathione; G-6-PD, glucose-6-phosphate dehydrogenase; GSSG-R, glutathione reductase; GSH-P, glutathione peroxidase.*
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of 2 Gm. DEAE per ml. hemolysate. An equal volume of deionized water was added and the mixture was allowed to stand for 10 minutes at 25 C. with occasional manual stirring. One ml. of 0.005 M phosphate buffer, pH 7, per Gm. DEAE was then added to the mixture. Hemoglobin was recovered in the supernatant fraction following centrifugation. For further recovery of hemoglobin, the resin was washed with another volume of phosphate buffer. The supernatant solutions were combined, and approximately one-sixth of the original amount of resin was added for further removal of nonhemoglobin protein. The absence of catalase activity was used as a criterion for absence of nonhemoglobin protein. This procedure usually sufficed; however, if residual catalase activity was detected, additional resin was added.

To obtain samples containing predominantly fetal and predominantly adult hemoglobin, respectively, cord blood was used for the former and venous blood from known healthy adult donors for the latter. Verification of the predominant type of hemoglobin in each case was obtained by starch gel electrophoresis.*

Perfusion of enzyme-free hemoglobin solutions with H2O2 was performed as described above for intact cells except that the main chamber of the Warburg flask contained 3 ml. of hemoglobin solution diluted in buffered saline to a concentration of 1.5 mg. hemoglobin per ml. Incubations were maintained for 15, 30 and 60 minutes, respectively.

Enzyme assays were performed on erythrocytes washed twice and resuspended to a hematocrit of 50 per cent in buffered saline and then lysed by freezing and thawing two times. All the enzymes were stable in ACD-blood at 4 C. for several days.

Glutathione peroxidase activity was measured in hemolysates by the method of Mills and Randall,24 with further minor revisions as follows. Hemolysate was diluted in buffered saline to a hemoglobin concentration of 25 to 35 mg/ml. To 12 parts of diluted hemolysate (1.2 ml.) were added 6 parts of a freshly prepared solution of GSH in buffered saline (2 mg. GSH/ml.) (0.6 ml.) and 30 parts of 0.002 M ethylenediamine tetraacetic acid (EDTA) and 0.005 M sodium azide in buffered saline (3.0 ml.). The entire procedure was performed at 25 C., and the reaction was initiated by the addition of 10 parts of 0.0018 M H2O2 (1.0 ml.) blown rapidly into the above mixture at zero time. Since the reaction followed first order kinetics for 3½ minutes, it was convenient to stop the reaction at 1 and 3 minutes. This was accomplished by transferring 2 ml. aliquots into tubes containing 5 ml. of 0.7 per cent metaphosphoric acid. The procedure for the measurement of GSH24 was then continued by the further addition to each tube of 2 ml. of 7.5 per cent metaphosphoric acid and 3 Gm. of sodium chloride. After vigorous shaking for 8 minutes followed by filtering, 2 ml. aliquots of the filtrate were used within 30 minutes for the determination of GSH. GSH peroxidase activity was calculated as K per Gm. hemoglobin per minute, where first order reaction rate constant K equals

$$\frac{2.3 \times \log \frac{\text{GSH}^{\dagger} \text{at } t_1 \text{ (1 min.)}}{\text{GSH}^{\dagger} \text{at } t_3 \text{ (3 min.)}}}{t_3 - t_1}.$$

The nonenzymatic oxidation of GSH was measured in an assay in which 1.2 ml. of buffered saline was substituted for hemolysate. This reaction, which was usually negligible, was subtracted from the enzymatic reaction before calculating K.

Catalase activity was measured by titration of residual H2O2 with 0.002 M KMnO4,25 modified as follows. One ml. of hemolysate containing 0.7 mg. of hemoglobin was incubated with 5 ml. of 0.006 M H2O2 in an ice bath for 20 seconds, at which time the reaction was stopped by the addition of 1 ml. of 6 NH4SO4. In a duplicate flask H2SO4 was added prior to the addition of hemolysate to obtain a zero time measurement. The zero time and 20 seconds measurements were used to obtain the first order reaction rate constant K in the manner noted above for GSH. Catalase activity was expressed as K per Gm. hemoglobin per second.

Glutathione reductase activity in red blood cell hemolysates was measured by the method

*Kindly performed by Dr. Ronald Nagel and Mr. Alan Jacobs in the laboratory of Dr. Helen M. Ranney, Albert Einstein College of Medicine, New York.
†Optical density reading minus blank at 520 mμ.
of Long and Carson modified by reducing the concentrations of Tris buffer to 0.1 M, EDTA to $5 \times 10^{-3}$ M, TPNH to $1 \times 10^{-3}$ M and GSSG to $1.5 \times 10^{-3}$ M, and by the addition of sodium chloride to a final concentration of 0.1 M. Enzyme activity at 25 C. (indicated by the disappearance of TPNH at 340 nm) was measured in a sample of hemolysate containing approximately 0.5 mg. hemoglobin diluted to a final volume of 1 ml. of assay mixture and was expressed as International Units per Gm. hemoglobin.

RESULTS

Perfusion with Hydrogen Peroxide

The possible deleterious effects of in vitro perfusion of red blood cell suspensions with low concentrations of hydrogen peroxide were assessed by the following parameters: presence of hemolysis; formation of methemoglobin, choleglobin and Heinz bodies; decrease in glutathione peroxidase activity and in the content of reduced glutathione. Identical experiments were performed on blood from adult subjects and from newborn infants. In both groups hemolysis and choleglobin formation were found to be negligible. In samples from 9 adults and 7 newborns, perfusion with H$_2$O$_2$ for 13 hours resulted in a lowering of the levels of GSH to comparable extents in the two groups. Mean adult levels before and after perfusion were 28.2 ± 4.7 mg. per cent and 16.4 ± 5.8 mg. per cent respectively. In the newborn infants' blood the comparable mean levels were 29.1 ± 5.1 mg. per cent and 18.0 ± 6.1 mg. per cent.

Heinz body formation (Fig. 1), methemoglobin formation and loss of glutathione peroxidase activity were noted in both groups; however, these effects were of significantly greater magnitude in the blood samples from the newborn infants than in those from the adult controls (Table 1).

A small number of in vitro experiments were performed to determine whether the addition of tocopherol in the perfusion mixtures would result in protecting the red cells of either the adults or the newborns from hydrogen peroxide toxicity. No evidence of protection was noted in either the adult or newborn samples (Table 2).

Perfusion of Solutions of Purified Hemoglobin with Hydrogen Peroxide

Solutions of fetal hemoglobin free of protective enzymes were prepared from 10 samples of cord blood, and each was perfused with H$_2$O$_2$ for periods up to 60 minutes. Hemoglobin solutions were similarly prepared from 7 samples of blood from adults and were treated identically. Failure to prevent the oxidative effects of H$_2$O$_2$ by the addition of glucose to the perfusion mixtures, as well as lack of demonstrable catalase activity, was used as evidence for the absence of protective enzyme activity in these preparations. Table 3 shows the extent of formation of methemoglobin and choleglobin in these samples. In the absence of protective enzyme activity the toxic effects of low steady-state levels of hydrogen peroxide are similar for solutions of adult and fetal hemoglobin.

Enzyme Activities

Glutathione Peroxidase. Mean levels of activity in the erythrocytes of 29 full-
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**Table 1.—Effects of in Vitro Perfusion of Erythrocytes with \( \text{H}_2\text{O}_2 \)^* **

<table>
<thead>
<tr>
<th></th>
<th>Newborn Infants</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methemoglobin formation—per cent of total hemoglobin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>32.6 ± 20.3</td>
<td>15.6 ± 11.8</td>
</tr>
<tr>
<td>No. of samples</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>“p value”</td>
<td>0.025 &gt; p &gt; 0.01</td>
<td></td>
</tr>
<tr>
<td><strong>Presence of Heinz bodies†</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. positive</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>No. of samples</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>“p value”</td>
<td>p &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td><strong>Glutathione peroxidase activity—per cent remaining†</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>72.5 ± 7.9</td>
<td>85.5 ± 6.8</td>
</tr>
<tr>
<td>No. of samples</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>“p value”</td>
<td>p &lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

*Details of perfusion are given in text. Duration of perfusion was 13 to 16 hours.
†One hundred red blood cells were examined in each sample. Positive samples were defined as those in which at least 10 per cent of the cells contained 5 or more Heinz bodies.
†Glutathione peroxidase activity was measured in control samples incubated without \( \text{H}_2\text{O}_2 \), as well as in the perfused samples. The activity in the perfused sample divided by activity in control sample \( \times 100 \) = per cent remaining.

**Table 2.—Effect of in Vitro Addition of Tocopherol on Oxidative Changes in Erythrocytes Perfused with \( \text{H}_2\text{O}_2 \)^* **

<table>
<thead>
<tr>
<th>Sample</th>
<th>( \mu \text{g} ) of Tocopherol† Added to Flask</th>
<th>Per Cent Methemoglobin</th>
<th>Per Cent Choleglobin</th>
<th>Per Cent Red Blood Cells with &gt; 5 Heinz Bodies‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult #1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood perfused</td>
<td>0</td>
<td>11.9</td>
<td>2.8</td>
<td>23</td>
</tr>
<tr>
<td>16 hours</td>
<td>5</td>
<td>11.5</td>
<td>4.1</td>
<td>37</td>
</tr>
<tr>
<td>Adult #2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood perfused</td>
<td>0</td>
<td>16.1</td>
<td>2.9</td>
<td>14</td>
</tr>
<tr>
<td>16 hours</td>
<td>5</td>
<td>14.4</td>
<td>2.3</td>
<td>14</td>
</tr>
<tr>
<td>Newborn #1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood perfused</td>
<td>0</td>
<td>76.7</td>
<td>3.3</td>
<td>100</td>
</tr>
<tr>
<td>16 hours</td>
<td>5</td>
<td>73.4</td>
<td>1.3</td>
<td>100</td>
</tr>
<tr>
<td>Newborn #2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood perfused</td>
<td>0</td>
<td>22.6</td>
<td>0.2</td>
<td>71</td>
</tr>
<tr>
<td>5 hours</td>
<td>10</td>
<td>21.8</td>
<td>0.7</td>
<td>80</td>
</tr>
<tr>
<td>Cord blood #1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood perfused</td>
<td>0</td>
<td>11.0</td>
<td>0.2</td>
<td>84</td>
</tr>
<tr>
<td>3 hours</td>
<td>25</td>
<td>8.7</td>
<td>0.1</td>
<td>86</td>
</tr>
<tr>
<td>Cord blood #2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood perfused</td>
<td>0</td>
<td>10.6</td>
<td>0.9</td>
<td>87</td>
</tr>
<tr>
<td>3 hours</td>
<td>50</td>
<td>14.7</td>
<td>0.3</td>
<td>85</td>
</tr>
</tbody>
</table>

*Details of perfusion are given in text. Duration is noted under each sample.
†Tocopherol was solubilized with 4 parts of tween 80 at a concentration of 200 mg. per ml. It was then diluted for this study with saline to a concentration of 500 \( \mu \text{g} \). per ml.
‡Two hundred red blood cells were counted for each determination.
Fig. 1A.

Fig. 1A and B.—Heinz bodies in the erythrocytes of a newborn infant (A) and an adult (B) after perfusion with H₂O₂ for 13 hours. Details of perfusion procedure are described in the text. The erythrocytes pictured here were lysed with distilled water and examined and unstained in wet preparations by phase contrast microscopy.
Fig. 1B.—See legend under Figure 1A.

term and 13 premature newborn infants, measured either in cord blood or in venous blood obtained from infants 0 to 3 days of age, were significantly lower than mean levels in a group of 17 mothers immediately postpartum or 17 healthy adult controls (Fig. 2 and Table 4). A significant increase in level of activity was demonstrated in the red cells of 10 patients with congenital
Table 3.—Perfusion of Purified Hemoglobin with H$_2$O$_2$*

<table>
<thead>
<tr>
<th>Duration of Perfusion</th>
<th>Per Cent Methemoglobin†</th>
<th>Per Cent Choleglobin†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cord Blood</td>
<td>Adult Controls</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero time</td>
<td>4.3 ± 3.1</td>
<td>0.7 ± 1.0</td>
</tr>
<tr>
<td>(9)</td>
<td>(7)</td>
<td>(10)</td>
</tr>
<tr>
<td>15 minutes</td>
<td>43.4 ± 9.8</td>
<td>34.2 ± 11.7</td>
</tr>
<tr>
<td>(7)</td>
<td>(5)</td>
<td>(8)</td>
</tr>
<tr>
<td>30 minutes</td>
<td>31.3 ± 19.9</td>
<td>20.7 ± 4.8</td>
</tr>
<tr>
<td>(5)</td>
<td>(6)</td>
<td>(5)</td>
</tr>
<tr>
<td>60 minutes</td>
<td>28.4 ± 11.1</td>
<td>22.5 ± 7.4</td>
</tr>
<tr>
<td>(10)</td>
<td>(7)</td>
<td>(10)</td>
</tr>
</tbody>
</table>

**Purified hemoglobin** refers to hemoglobin separated from protective enzymes and dissolved in buffered saline as described in the text. The technic for perfusion with H$_2$O$_2$ is also described in the text.

†Per cent methemoglobin and per cent choleglobin are expressed as mean values for each group ± 1 S.D. The only significant difference between any of the means was that for methemoglobin formation at zero time (0.01 > p > 0.005).

Numbers in parentheses refer to numbers of samples.

Table 4.—Activity of Glutathione Peroxidase and of Glutathione Reductase in Erythrocytes

<table>
<thead>
<tr>
<th>Glutathione Peroxidase*</th>
<th>Full-term Newborn Infants</th>
<th>Premature Newborn Infants</th>
<th>Mothers Postpartum</th>
<th>Adult Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± S.D.</td>
<td>41.1 ± 12.3</td>
<td>37.2 ± 7.9</td>
<td>60.4 ± 11.7</td>
<td>57.3 ± 13.3</td>
</tr>
<tr>
<td>No. of subjects</td>
<td>29</td>
<td>13</td>
<td>17</td>
<td>17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glutathione Reductase†</th>
<th>Full-term Newborn Infants</th>
<th>Premature Newborn Infants</th>
<th>Mothers Postpartum</th>
<th>Adult Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± S.D.</td>
<td>6.3 ± 1.4</td>
<td>5.6 ± 1.1</td>
<td>4.1 ± 0.9</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td>No. of subjects</td>
<td>27</td>
<td>12</td>
<td>10</td>
<td>26</td>
</tr>
</tbody>
</table>

*Activity is expressed as first order reaction constant K per Gm. hemoglobin per minute. p values for significance of differences between means are given in Figure 1.

†Activity is expressed as International Units per Gm. hemoglobin. p values for significance of difference between means are given in text.

nonspherocytic hemolytic anemia, all of whom exhibited reticulocytosis of varying degrees (Fig. 2).

Glutathione reductase activity was found to be significantly increased (p < 0.001) (Table 4) in erythrocytes of full-term newborn infants and premature newborn infants compared either to mothers immediately postpartum or healthy adult controls.

Catalase activity was measured in the red blood cells of 20 full-term newborn infants in the first 3 days of life and in 24 adult controls. The infants had significantly lower enzyme activity with a mean of 46.4 ± 16 units per gram of hemoglobin compared to the adult mean of 66.2 ± 12.2 (p < 0.001).

**DISCUSSION**

The present study provides evidence for increased susceptibility of the red blood cells of newborn subjects to the oxidative toxicity of low steady-state
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Fig. 2.—Glutathione peroxidase activity in erythrocytes. Individual observations are indicated by dots and mean values by horizontal bars. Significance between the means is indicated by p values in the lower part of figure. The groups compared (from left to right) are premature vs. full-term infants, premature and full-term infants vs. normal adults, normal adults vs. mothers immediately postpartum, and normal adults vs. subjects with congenital nonspherocytic hemolytic anemia.

concentrations of H$_2$O$_2$ in vitro. The formation of methemoglobin and Heinz bodies in the presence of H$_2$O$_2$ is similar to that observed when blood of newborn infants is incubated with potential oxidizing agents which in turn may produce H$_2$O$_2$ as their active intermediate compound—namely, menadione, ascorbic acid and acetylphenylhydrazine. Several mechanisms may be postulated to explain these observations. Among them are increased susceptibility of fetal hemoglobin to oxidation, increased permeability of erythrocytes of the newborn to oxidizing agents, and decreased efficiency of the mechanisms for detoxification of these agents.

The present data indicate that fetal hemoglobin in the absence of protective enzyme activity is no more susceptible to oxidation by perfusion with H$_2$O$_2$ than is adult hemoglobin under the same conditions. Although Betke et al.²
reported that nitrite produced greater oxidation of purified hemoglobin F than purified hemoglobin A, the mechanism of oxidation by nitrite may differ from that by H$_2$O$_2$ or the other agents cited above.

Although erythrocytes of infants and adults may differ in their respective permeability to H$_2$O$_2$ or in the presence of detoxifying mechanisms on their membranes, the present study offers no means of comparing the role of the membrane in the two groups. Reports that tocopherol can reverse the hydrogen peroxide hemolysis test in newborn infants$^{14}$ as well as the anemia seen in certain premature infants$^{29}$ suggest the possibility that the red cell membrane in the young infant may be subject to increased lipid peroxidation as a result of tocopherol deficiency. In the present studies, using low concentration of hydrogen peroxide, no evidence was obtained for a protective action by tocopherol in vitro.

The importance of GSH-P and the glucose-dependent pathway in the detoxification of H$_2$O$_2$ has been demonstrated in normal$^{1,2}$ G-6-PD-deficient$^2$ and acatalasic$^4$ erythrocytes. No similar study has been reported in newborn infants. The present findings of decreased activity of GSH-P in the erythrocytes of both newborn and premature infants are of particular interest in view of the susceptibility of these cells to oxidative toxicity. Despite the increased levels of activity of the two other enzymatic steps in the metabolic pathway, G-6-PD and GSSG-R, the overall efficiency of the pathway would be limited by the activity of the final enzymatic step, GSH-P. Since it is unlikely that the decreased levels of catalase activity and of tocopherol are important at low levels of H$_2$O$_2$, it remains to be determined under what circumstances they might also be disadvantageous to the newborn.

Although GSH-P activity was decreased in the erythrocytes of young infants, it was significantly increased in samples from subjects with congenital non-spherocytic hemolytic anemia. The latter subjects all had varying degrees of reticulocytosis. Thus their increased levels of enzyme activity suggest that GSH-P is increased in activity in young erythrocytes and decreases with the in vivo aging of the cell. In the present study the observed toxic effects of H$_2$O$_2$ in the intact cell were limited to methemoglobin and Heinz body formation and were significantly more marked in the erythrocytes of newborns than in those of adults. Nitowski et al.$^{14}$ also found that H$_2$O$_2$ was more toxic to the erythrocytes of newborn infants than to those of adults, but in contrast to the present observations they encountered hemolysis in vitro. This discrepancy may be explained by the fact that the latter group not only employed much greater concentrations of H$_2$O$_2$, but also worked in a glucose-deprived system. The present studies, using low steady-state levels of H$_2$O$_2$ and providing glucose, are more comparable to the situation observed in vivo in which administration of oxidizing agents leads to the denaturation of hemoglobin and the formation of Heinz bodies. The damaged erythrocytes apparently do not rupture spontaneously in the blood stream but are first trapped in the spleen where they then undergo phagocytosis and lysis.$^{29}$
It is likely that the mechanisms responsible for the increased susceptibility of newborn infants to drug-induced hemolytic anemia in vivo are similar to those which permit denaturation of the hemoglobin of their erythrocytes by \( \text{H}_2\text{O}_2 \) in vitro. Among the possible causes which have been considered, the present study has provided positive evidence for decreased efficiency of the mechanisms for detoxification of \( \text{H}_2\text{O}_2 \).

**Summary**

The deleterious effects on erythrocytes of low steady-state concentrations of \( \text{H}_2\text{O}_2 \) in vitro have been compared in samples from full-term and premature infants and adults. Methemoglobin and Heinz bodies were formed to a greater degree in the intact erythrocytes of the young subjects. In the absence of protective enzymes, however, the extent of oxidation was similar in hemoglobin prepared from cord blood and from adult blood, respectively.

Activity of the enzymes involved in the detoxification of \( \text{H}_2\text{O}_2 \) has been measured in the red blood cells of the aforementioned groups of subjects. Of note was the finding of significantly decreased activity of glutathione peroxidase in the full-term newborn and premature infants.

These findings of increased toxicity from \( \text{H}_2\text{O}_2 \) and decreased efficiency of the detoxification mechanisms are considered to have bearing on the susceptibility of young subjects to drug-induced hemolytic anemia.

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REFERENCES


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