The demonstration of abnormality of red-cell glutathione metabolism in vitro in cases of primaquine induced hemolytic anemia and the observations of its correlation with red cell aging have stimulated investigations into mechanisms of hemolysis and normal red cell aging. Current knowledge suggests that this drug-induced hemolysis is closely related to a deficiency of erythrocyte glucose-6-phosphate dehydrogenase with its consequent metabolic defects. In the glutathione stability test, incubation of red cells from primaquine sensitive patients with appropriate drugs causes a drop in the level of reduced glutathione (GSH). This is probably the result of an inadequate supply of reduced triphosphopyridine nucleotide (TPNH), normally provided by the action of glucose-6-phosphate dehydrogenase, which is required by glutathione reductase to maintain glutathione in a reduced state. Since this phenomenon of hemolysis in vivo in the presence of one of these oxidant drugs has been demonstrated in a non-sensitive patient, erythrocyte glutathione stability was studied under varying circumstances to determine whether normal red cells might be made to appear "sensitive" in the in vitro tests.

Materials and Methods

Blood was drawn from donors known to have normal glucose-6-phosphate dehydrogenase by assay. This was collected in a heparinized syringe, and experiments were begun within 30 minutes after venesection. When studies were done with whole blood, 2 mg. of glucose were added per ml. unless otherwise noted. In experiments in which plasma and buffy coats were removed, red cells were suspended in a buffered Krebs-Ringers solution prepared by adding 30 ml. of 0.35 M Tris buffer, pH 7.4, to 105 ml. of Krebs-Ringers solution. Two mg. of glucose per ml. were added to the red-cell suspension. The experiments were carried out at 37.5 C. in a water bath or in a Dubnoff shaker according to the conditions of the given experiment. The arm of the shaker rotated at a speed of 100 times per minute unless otherwise noted. The glutathione stability test was carried out as previously described, using one ml. volumes of blood in unstoppered test tubes to which 5 mg. of acetylphenylhydrazine had been added. These were agitated briefly at one hour's time, and the GSH was measured at zero hour and at 2 hours in triplicate samples according to the method of Gruenert and Phillips as modified by Beutler.

Methemoglobin and sulphemoglobin were measured according to Evelyn and Malloy. Factors were calculated for readings on the Coleman Jr. Spectrophotometer. The measurement at 620 mμ, which is proportional to the optical density remaining after the addition of cyanide has converted methemoglobin to cyanmethemoglobin, was corrected for absorption of methemoglobin cyanide and oxyhemoglobin at this wave length and is termed "sulphemoglobin." This is expressed only as optical density, rather than as a per cent of...
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Fig. 1.—The value of erythrocyte reduced glutathione is plotted at 0 and 2 hours. (- - -) indicates that the samples were left standing in the water bath and (———) indicates that the samples were placed in a Dubnoff shaker during this period. Acetyldihydrazine was added to those samples marked with a closed circle (●) and the controls are those with a cross (x).

total pigment, because of the inaccuracies involved in using a reference curve for what is probably multiple pigments. This is further complicated by the necessity of filtering before measuring these samples for heme pigments because of turbidity in the acetyldihydrazine treated samples. This eliminates unknown amounts of precipitated denatured hemoglobin from the calculation.

Glucose was measured by the Somogyi-Nelson procedure.

RESULTS

Four ml. aliquots of whole blood were incubated in 50 ml. Erlenmeyer flasks and placed in the Dubnoff shaker in room air. At 30-minute intervals, a flask was removed and GSH measured in triplicate. Under these circumstances, it was evident that incubation with acetyldihydrazine at a concentration of five mg. per ml. caused a drop in GSH in normal red cells (fig. 1). No change was noted in the usual glutathione stability test carried out at 37 C. without continued agitation, nor was there any drop in blood incubated in the shaker without drug.

To determine whether this effect might be due to variations in pH under these circumstances, an aliquot of red cells was suspended in buffered Krebs-Ringers solution and acetyldihydrazine was added to this. The results were compared to those experiments with whole blood. Comparison of the
Table 1.—The Effect of Adding Increasing Amounts of Acetylphenylhydrazine on Erythrocyte Glutathione Levels

<table>
<thead>
<tr>
<th>Acetylphenylhydrazine mg. per cent</th>
<th>GSH mg./100 cc. of red cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>62</td>
</tr>
<tr>
<td>300</td>
<td>32</td>
</tr>
<tr>
<td>500</td>
<td>7</td>
</tr>
</tbody>
</table>

*Acetylphenylhydrazine was added at zero hour to make a final concentration in mg. per cent as indicated. The erythrocyte glutathione was measured after 2 hours’ incubation in the shaker.

pH values and the GSH measurements at 2 hours revealed that the level of GSH in the shaker was similarly depressed to 22 and 24 mg. per cent despite differing pH values of 7.92 and 7.47, while the whole blood samples in the shaker and in the water bath had significantly different GSH values despite a similar pH range of 7.92 and 8.00.

Since the constant agitation might lead to trauma of the red cells, this was evaluated by noting the effect of the addition of glass beads to provide increased trauma. This had no effect on the results. Moreover, variations in the speed of shaking did not cause significant differences in the final GSH readings within a range of 60 to 130 rotations per minute.

To determine whether the amount of glucose supplied was a critical factor in lowering the reduced glutathione in these experiments, measurements were made before and after incubation in the shaker. Since the acetylphenylhydrazine interferes with measurement of glucose by the Somogyi-Nelson procedure, 0.7 mg. of menadione sodium bisulfite was added per ml. of red-cell suspension. The cells were suspended in buffered Krebs-Ringers and 4 ml. volumes were added to each of four flasks. Two hundred and fifty mg. of glucose were added per ml. of suspension, and a comparison was made between flasks containing glucose with and without menadione sodium bisulfite and between flasks containing the latter with and without glucose. The lack of added glucose did not result in a loss of GSH, and the addition of glucose did not prevent a depression of GSH in the presence of oxidant drug after 2 hours’ incubation. The 2-hour glucose values of 16 and 292 mg. per cent were associated with GSH levels of 69 and 58 mg. per cent when acetylphenylhydrazine was not added. With the latter drug, GSH dropped to levels of 20 and 16 mg. per cent with respective glucose values of 223 and 22 mg. per cent.

Effects of the addition of varying amounts of acetylphenylhydrazine were also noted, and it was evident that there is a definite dose effect under the conditions of these experiments. Using dry acetylphenylhydrazine, when a final concentration of 100 mg. per cent was used, there was no effect. At 300 mg. per cent, there was a significant decrease in GSH which was increased with 500 mg. per cent (table 1). When the drug was first placed in a solution of buffered Krebs-Ringers and molar concentration ratios of the drug to hemoglobin varying from 1:1 to 25:1 were used, a significant decrease was noted at the highest concentration of drug.

To determine whether maintenance of agitation of the blood is necessary,
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Fig. 2.—Values of erythrocyte reduced glutathione are plotted against hours of incubation. The solid line (———) and the broken line (•••••••) indicate incubation in Dubnoff shaker and water bath respectively. Acetylphenylhydrazine was added to all samples in a concentration of 5 mg. per ml. "A" refers to an experiment done with normal blood and "B" to one done with blood from a "primaquine-sensitive" patient.

Blood was removed from the shaker after one hour and one aliquot was taken for measurement while the other was placed in a water bath at 37.5°C. and left undisturbed. Observations of GSH levels revealed a gradual increase in GSH over the next few hours, following an initial drop in the shaker (fig. 2). No experiments were carried beyond 5 hours, and during this period GSH was always noted to rise in normal blood, though it did not return to zero-hour values. For comparison, a patient with a history of drug-induced hemolytic anemia and low levels of glucose-6-phosphate dehydrogenase was studied. In figure 2, the results of the routine glutathione stability test are plotted along with the results of incubation of the patient's blood in the shaker followed by the water bath. It is evident that in the routine stability test as well as in the Dubnoff shaker, the glutathione levels dropped to extremely low values in 2 hours. Removal of this sample from the shaker to undisturbed incubation failed to allow any recovery of GSH.

Since the major difference between incubation in shaker and in water bath is constant aeration in the former, experiments were carried out to evaluate the effects of a nitrogen atmosphere. Experiments under these circumstances failed to demonstrate any drop in reduced glutathione in normal samples. Similar experiments were also carried out with the blood of a patient with a known positive glutathione stability test (zero hour value, 36; 2-hour value,
Fig. 3.—Reduced glutathione of erythrocytes suspended in buffered Krebs-Ringers solution with glucose is plotted against time of incubation in hours. Blood samples are from a "primaquine-sensitive" patient, one experiment being carried out in a nitrogen atmosphere (N₂) and one in an atmosphere of 95 per cent O₂/5 per cent CO₂ (O₂). The solid and broken lines refer to incubation in the Dubnoff shaker and in the water bath as in figures 1 and 2. Acetylphenylhydrazine was added after one hour of shaking the blood under nitrogen and was added at zero hour in the experiment in oxygen.

11). Red cells suspended in buffered Krebs-Ringers were placed in the Dubnoff shaker in a nitrogen atmosphere for one hour before acetylphenylhydrazine was added. Measurements of glutathione were made before adding the drug and again at one hour's time. The blood was then removed and allowed to sit undisturbed in a water bath of 37 C. The results are plotted in figure 3 and demonstrate a markedly diminished response to acetylphenylhydrazine in the absence of oxygen.

An attempt was made to study populations of young and old cells separated by differential centrifugation. In a normal patient in whom a population of red cells had been tagged with Fe⁵⁹ to given an age span of approximately 10 to 20 days, it was possible to document a relative increase in younger red cells in the top layer of the red-cell column. When this layer and the bottom layer of the column were separated and each resuspended in buffered Krebs-Ringers solution, no difference could be demonstrated in the degree of loss of GSH at one hour, nor in the degree of recovery after 2 hours of undisturbed incubation (fig. 4). However, it should be noted that separation was not
Fig. 4.—Erythrocyte reduced glutathione is plotted against time of incubation in hours. Acetylphenylhydrazine was added to the suspended samples of red cells taken from the top and bottom layers of a red-cell column. The top (T) contained 690 counts per minute of Fe⁶⁹ and the bottom (B) contained 440. The solid and broken lines have the same significance as in the first three figures.

Complete since some radioactivity was present on the so-called aged or bottom sample. Further attempts were made to evaluate glutathione instability under these circumstances in patients with reticulocytosis. In a patient with acquired hemolytic anemia associated with disseminated lupus, separation of the younger cells from the older by this technic failed to show any difference in the level of reduced glutathione at 2 hours. Both populations showed the expected drop. Since the patient had a very rapid rate of hemolysis, however, there was probably little difference in age between the two samples. Patients with pernicious anemia responding to treatment were also studied in a similar manner. Although reticulocyte counts of the top layer sample documented a striking increase in these younger cells, there was no significant difference between the two samples.

Heme pigments were also studied during the course of these experiments. Methemoglobin was noted to rise to the range of 40 to 50 per cent at one hour in the shaker and to decline at a variable rate when the samples were placed undisturbed in the water bath. This was seen both in young and in old cells in the presence of oxygen or air. Sulfhemoglobin formation was also noted in most experiments. When the blood was removed from the shaker and placed undisturbed in the water bath, this pigment was found either to
Fig. 5.—Acetylphenylhydrazine was added to whole blood at zero hour and observations were made of reduced glutathione levels and methemoglobin values during incubation under nitrogen. The former is plotted as mg. per 100 ml. of red cells and the latter as per cent of total heme pigment.

increase or to remain at approximately the same level. Since, however, the process of filtering may have removed some of the "sulphhemoglobin," which could be in a precipitated state, an accurate determination was not possible. It is interesting to note that methemoglobin appeared even in the experiments which were carried out under a nitrogen atmosphere. In this situation, glutathione was not observed to drop (fig. 5). In experiments in nitrogen atmosphere, no sulphhemoglobin was observed. In the experiments in which the blood was incubated with varying amounts of acetylphenylhydrazine, increasing amounts of methemoglobin were formed (table 2). Again, there was a difference in degree of methemoglobin production and GSH destruction in that changes in the former appeared with less amounts of acetylphenylhydrazine than were necessary to produce a drop in the latter.

**DISCUSSION**

Drug-induced hemolytic anemia occurring in "primaquine-sensitive" individuals is now a well-recognized entity. There is evidence, however, that the hemolytic action of some of these drugs in vivo is not confined to those cases in which such a sensitivity can be demonstrated. In sulfoxone therapy, hemolysis is an expected phenomenon with high doses, and the dynamics of
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Table 2.—The Effect of Increasing Concentrations of Acetylphenylhydrazine *

<table>
<thead>
<tr>
<th>Molar Ratio</th>
<th>GSH</th>
<th>MHb</th>
<th>GSH</th>
<th>MHb</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:1</td>
<td>84</td>
<td>1.4</td>
<td>87</td>
<td>13.6</td>
</tr>
<tr>
<td>15:1</td>
<td>85</td>
<td>2.8</td>
<td>66</td>
<td>43.6</td>
</tr>
<tr>
<td>25:1</td>
<td>83</td>
<td>2.8</td>
<td>53</td>
<td>55.0</td>
</tr>
</tbody>
</table>

*Acetylphenylhydrazine was added to give the molar ratio to hemoglobin as indicated. Samples were placed in the shaker and measurements made at zero and 2 hours. GSH refers to reduced glutathione in mg. per 100 ml. of red cells. MHb refers to methemoglobin expressed as per cent of total heme pigment.

the hemolytic mechanism with selection of the more aged erythrocyte population are similar to those described in the "primaquine-sensitive" patient. Moreover, phenylhydrazine is recognized as an hemolytic agent both in man and animals regardless of the erythrocyte glutathione stability. It has also been demonstrated that patients with primaquine sensitivity demonstrate a mild hemolytic state, even in the absence of exogenous drug.

One of the striking biochemical features of the red cell in primaquine sensitivity is the rapid drop in reduced glutathione levels in vitro on exposure to acetylphenylhydrazine, which finding has been used as a test for hemolytic sensitivity to drugs. The technic of this glutathione stability test, which limits aeration of the blood to a single period of agitation during incubation, allows differentiation of patients who have diminished erythrocyte glucose-6-phosphate dehydrogenase activity, and, therefore, a limited supply of TPNH.

The present experiments demonstrate that a similar change can be brought about in normal cells in the presence of continuous aeration. That the change depends on the presence of oxygen is demonstrated by its absence under nitrogen and its presence in air and in 95 per cent O₂/5 per cent CO₂. That it is not related to pH change in the presence of constant aeration is also evident in these experiments. Since incubation with constant aeration mimics the in vivo state more closely than the routine glutathione stability test, the results suggest that in the normal individual, critical levels of oxidant drugs may also exist above which biochemical trauma may be sufficient to produce hemolysis.

Addition of adequate glucose does not prevent the drop in reduced glutathione, demonstrating that the limiting step is not substrate supply, but instead a reaction further along the pathway of hydrogen donation from glucose to oxidized glutathione. The amount of drug and the degree of oxygenation appear to determine whether glutathione is maintained in the reduced state in the erythrocytes. It should be noted, however, that in the neonatal period, erythrocyte glutathione instability is common and is prevented by the addition of glucose. The significance of these findings is not clear, but they may be related to the findings of Heinz body anemia in this age group.

The present experiments failed to demonstrate differences in in vitro susceptibility in older cells. However, since it was not possible to separate young and old cells completely, one cannot conclude that aged cells are not more susceptible. It is at least evident that in the preparations with high reticulocyte counts, the drug effect was still evident.

It is significant that under the conditions of the experiment, hemoglobin was
more susceptible to the effects of the drug than glutathione. This is the reverse of observations in isolated systems, where reduced glutathione was noted to be oxidized more rapidly than hemoglobin. In the intact red cell, however, other mechanisms of equilibrium are brought into play. Moreover, since the molar concentration of hemoglobin in the red cell is approximately twice that of GSH, the reduction mechanisms for the former may be in a more critical state. Thus, in this environment, glutathione appears to be reduced more efficiently or oxidized less effectively than hemoglobin. This raises doubt as to the application of a protective function for hemoglobin to glutathione. Marks has been unable to document the presence of glutathione peroxidase in human red cells which has been postulated as playing a role in this protective function, and he has pointed out that triphosphopyridine nucleotide appears to be the most critical substance in maintaining hemoglobin in the reduced state. It may well be that glutathione affords protection in a more indirect manner by preventing damage to the glycolytic sulfhydryl enzymes as well as preventing the irreversible oxidation of methemoglobin to sulfhemoglobin as suggested by Jandl.

Thus, there are several mechanisms postulated for drug-induced hemolysis, all of which could be affected in the normal erythrocyte, given enough toxic exposure. The present studies demonstrate that normal cells can be made to appear “glutathione unstable” by introducing a constant supply of oxygen to the preparation. This is consistent with the fact that some of these drugs may cause hemolytic anemia in normal patients.

Summary

Studies have been carried out in vitro to determine whether normal erythrocytes may be made to appear glutathione unstable. Results demonstrate that constant agitation in air or oxygen will cause a drop in reduced glutathione upon incubation of normal blood with an oxidant drug in concentrations which have no significant effect during quiet incubation.

The results are consistent with the fact that drug-induced hemolysis may be seen in nonsensitive individuals and that sensitive individuals may have a mild hemolytic state without exogenous drug.

Summario in Interlingua

Studios esseva conducite in vitro pro determinar si on pote causar in erythrocytos normal un apparentia de instabilitate de glutathiona. Le resultatos demonstra que constante agitation in aere o in oxygeno pote causar un declino in le nivello de reducite glutathiona post incubation de sanguine normal con un droga oxydante in concentrationes que produce nulle effecto significative durante incubation quieta.

Le resultatos es congruente con le facto que hemolyse inducite per drogas pote ocurrer in individuos non-sensibile e que individuos sensibile pote haber un leve forma del stato hemolytic sin le intervention de un droga exogene.

Acknowledgments

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REFERENCES


Jane F. Desforges, M.D., Associate Professor of Medicine, Tufts Medical School; Associate Director, Tufts Hematology Laboratory; and Assistant Director, First and Third (Tufts) Medical Services at Boston City Hospital, Boston, Mass.
Glutathione Instability in Normal Blood

JANE F. DESFORGES

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