The Reduction of Methemoglobin in Erythrocytes of a Patient with Congenital Methemoglobinemia, Subjects with Erythrocyte Glucose-6-Phosphate Dehydrogenase Deficiency, and Normal Individuals

By Ernst R. Jaffé

METHEMOGLOBIN, once formed within normal human erythrocytes, can be reduced to oxygen-carrying hemoglobin by mechanisms that require the generation of reduced pyridine nucleotides and an intact electron transport system. One mechanism, extensively studied by Kiese and their associates, involves the transfer of electrons from reduced triphosphopyridine nucleotide (TPNH) to methylene blue or an unknown natural cofactor and then to a heme enzyme. TPNH is the preferred reduced pyridine nucleotide in this system. The reduced enzyme can then reduce either methemoglobin, oxygen or cytochrome c. Another system, suggested by Gibson and demonstrated recently by Scott, involves the transfer of electrons from reduced diphosphopyridine nucleotide (DPNH) to methemoglobin, oxygen, cytochrome c or a dye, 2,6-dichlorobenzenone indophenol, in the presence of a diaphorase-like enzyme. The rate of this reaction with TPNH is only about 10 per cent of the rate with DPNH. The present report describes investigations of these two systems for the reduction of methemoglobin to hemoglobin in normal human erythrocytes, in erythrocytes deficient in glucose-6-phosphate dehydrogenase (G-6-PD) activity, and in erythrocytes of a patient with congenital methemoglobinemia of an enzymatic type.

Materials and Methods

Whole blood, anticoagulated with heparin, was obtained from six normal adults, from six adult Negro males whose erythrocytes were found to be deficient in G-6-PD activity by an enzymatic assay and from a 35 year old Puerto Rican woman with congenital methemoglobinemia of an enzymatic type. The Negro males were either healthy subjects or patients without anemia or evidence of hemolysis under treatment for pulmonary tuberculosis with isoniazid, paraaminosalicylic acid or streptomycin. One subject with G-6-PD deficient erythrocytes was found to have hemoglobin S trait on paper electrophoresis of his hemoglobin.

Erythrocytes were obtained from these blood samples by centrifugation and washing at 4°C three times with three to four volumes of isotonic sodium chloride-sodium phosphate buffer solution, pH 7.3 (nine parts 0.9 per cent sodium chloride solution and one

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part 0.11 M sodium phosphate buffer, pH 7.3). The washed erythrocytes were incubated at room temperature for 20 minutes after they were resuspended in an equal volume of 1.0 per cent sodium nitrite in the same isotonic solution. This procedure resulted in the conversion of essentially all of the hemoglobin to methemoglobin. The erythrocytes were then washed five times with eight to ten volumes of the isotonic sodium chloride-sodium phosphate buffer solution with centrifugation at 4 C. A 25 per cent suspension of the treated and washed erythrocytes in the same buffer solution was prepared and the actual volume of packed erythrocytes was determined. Four-ml. portions of the erythrocyte suspension were added to 25-ml. Erlenmeyer flasks containing 2 ml. of isotonic sodium chloride-sodium phosphate buffer solution (equal volumes of 0.9 per cent sodium chloride solution and 0.11 M sodium phosphate buffer, pH 7.3) in which were dissolved the desired substrates and 0.5 mg. per ml. of penicillin and of streptomycin. The flasks, loosely stoppered with cotton plugs, were incubated at 37 C. in a Dubnoff metabolic shaker-incubator oscillating 92 cycles per minute. At intervals during the incubation, aliquots of each suspension were removed for the determination of the concentration of methemoglobin by the method of Evelyn and Malloy12 as described previously.11 After 6 hours of incubation, a sufficient volume of a solution of methylene blue, 0.5 mg/ml. of isotonic buffer solution, was added to provide 0.12 tmoles of methylene blue per ml. of erythrocytes.

**DPNH-diaphorase assay:** Hemolysates of washed, nitrited erythrocytes were prepared by diluting packed erythrocytes 1:20 with distilled water and centrifuging for 10 minutes at 10,000 x g. The assay was performed by the method described by Scott8 after determining the total hemoglobin concentration in the supernatant hemolysate by the cyanmethemoglobin method. Reduction of the dye, 2,6-dichlorobenzenzenone indophenol, was determined by observing the decrease in absorbancy at 600 m, in a Zeiss PMQ II spectrophotometer at 25 to 27 C. The contents of the 1 cm. light path cuvettes, in a total volume of 3 ml., are indicated in figure 3. The change from the initial absorbancy reading was plotted against time for a blank cuvette without hemolysate and for the cuvette containing hemolysate equivalent to 3.25 mg. of methemoglobin. The latter curve was corrected for a small decline in absorbancy that was observed in a cuvette without DPNH but with hemolysate. DPNH was obtained commercially and its concentration in a freshly prepared solution was determined from the absorbancy at 340 m. Under these conditions, the rate of reduction of the dye with and without hemolysate was reproducible and nearly linear over a 20-minute period.

**TPNH-methemoglobin reductase assay:** Because of the lability of this system5 and because of interference in the assays resulting from the presence of methemoglobin in hemolysates of nitrited erythrocytes, untreated erythrocytes were employed for these determinations. Thrice-washed erythrocytes were suspended in an equal volume of 0.9 per cent sodium chloride solution and were hemolyzed by freezing in a dry-ice alcohol bath and thawing under tap water two times. After centrifugation at 10,000 x g for 10 minutes, the concentration of hemoglobin in the supernatant solutions was determined. Assays of these hemolysates for TPNH oxidase, cytochrome c reductase and methemoglobin reductase activity were performed as described by Huennekens et al.5 TPNH and cytochrome c were obtained commercially. Crystalline hemoglobin, prepared by the method of Drabkin,13 was treated with an approximately equal volume of 1.0 per cent sodium nitrite for 1 hour at room temperature. The material was dialyzed for 48 hours at 4 C. against 2.8 M phosphate buffer, pH 6.8, to prepare crystalline methemoglobin. TPNH oxidase activity was measured by observing the decline in absorbancy at 340 m, in a cuvette containing 110 ttmoles of sodium phosphate buffer, pH 7.4, 0.30 tmoles of TPNH, 0.027 tmoles of methylene blue and hemolysate containing 4.5 mg. of hemoglobin. The total volume of the reaction mixture was 3.0 ml. Cytochrome c reductase activity was determined by including 0.20 tmoles of cytochrome c in the cuvette and following the appearance of the reduced cytochrome c band at 550 m. Methemoglobin reductase activity was calculated from the increase in absorbancy at the 575 m band of oxyhemoglobin when 0.13 tmoles of crystalline methemoglobin was included in the cuvette. The observed changes in absorbancy were corrected for non-enzymatic oxidation of TPNH or...
Fig. 1.—Reduction of methemoglobin to hemoglobin during incubation of suspensions of normal erythrocytes and erythrocytes of a patient with congenital methemoglobinemia before and after addition of methylene blue.

Results

Data from a typical experiment performed with normal erythrocytes and with erythrocytes from the patient with congenital methemoglobinemia are presented in figure 1. In the presence of glucose or inosine, progressive reduction of methemoglobin to hemoglobin occurred in the normal erythrocytes, but failed to occur in the erythrocytes of the subject with congenital methemoglobinemia. After 6 hours of incubation, the addition of methylene blue produced an acceleration of methemoglobin reduction in normal erythrocytes during the subsequent hour of incubation and resulted in the rapid
reduction of methemoglobin in the erythrocytes of the patient to nearly the same level as in the normal cells. In figure 2 are shown the results of a similar experiment conducted with normal and with G-6-PD deficient erythrocytes. The rate of reduction of methemoglobin was essentially identical in the two types of cells during the first 6 hours. However, the marked acceleration of methemoglobin reduction produced in normal erythrocytes upon the addition of methylene blue did not occur in the G-6-PD deficient erythrocytes.

Since it was not possible to differentiate clearly between a deficient generation of reduced pyridine nucleotides and a deficiency in a methemoglobin reductase system in intact cells, it became necessary to study the two mechanisms in hemolysates. In figure 3 are presented the data from representative determinations of the DPNH-utilizing diaphorase-like system. Rapid and equal reduction of 2,6-dichlorobenzenone indophenol was observed with the nitrited hemolysates of normal and of G-6-PD deficient erythrocytes when compared to the non-enzymatic reaction between DPNH and the dye in the blank. In contrast, the nitrited hemolysate of the erythrocytes of the patient with congenital methemoglobinemia showed impaired reduction of the dye, even less than in the blank. This latter situation probably resulted from the
Fig. 3.—Assay of DPNH-diaphorase activity. Cuvettes contained indicated amounts of nitrited hemolysate, DPNH, 2,6-dichlorobenzene indophenol (2,6-DBI), trishydroxymethyl aminomethane buffer, pH 7.6 (TRIS), and sodium ethylene-diaminetetraacetate (EDTA) in a total volume of 3.0 ml., while the blank contained no hemolysate.

oxidation of the chemically reduced dye by the methemoglobin in the hemolysate in the absence of the activity of the enzyme present in the normal and the G-6-PD deficient hemolysates.

The results of experiments performed with untreated hemolysates to determine the activity of the TPNH-utilizing methemoglobin reductase system are summarized in table 1. It is apparent that the activity of this system was reduced by about 50 per cent in the hemolysates of G-6-PD deficient erythrocytes but was essentially normal in hemolysates of erythrocytes of the subject with congenital methemoglobinemia.

**DISCUSSION**

The experiments performed with intact human erythrocytes have confirmed the inability of erythrocytes from a subject with congenital methemoglobinemia to reduce methemoglobin to hemoglobin upon incubation with suitable substrates in the absence of an autoxidizable dye, such as methylene blue. Since the ability of the erythrocytes of the patient with congenital methemoglobinemia to metabolize glucose and inosine was shown to be normal,11
Table 1.—TPNH Oxidase, Methemoglobin Reductase and Cytochrome c Reductase Activity in Hemolysates of Human Erythrocytes

<table>
<thead>
<tr>
<th>Subjects*</th>
<th>TPNH Oxidase Activity†</th>
<th>Methemoglobin Reductase Activity†</th>
<th>Cytochrome c Reductase Activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal individuals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.F.</td>
<td>1.06</td>
<td>1.55</td>
<td>5.59</td>
</tr>
<tr>
<td>E.J.</td>
<td>1.15</td>
<td>2.68</td>
<td>9.86</td>
</tr>
<tr>
<td>E.J.</td>
<td>1.06</td>
<td>2.35</td>
<td>9.45</td>
</tr>
<tr>
<td>N.K.</td>
<td>1.15</td>
<td>1.51</td>
<td>7.16</td>
</tr>
<tr>
<td>M.R.</td>
<td>0.98</td>
<td>1.47</td>
<td>5.42</td>
</tr>
<tr>
<td>E.G.</td>
<td>1.20</td>
<td>1.68</td>
<td>7.46</td>
</tr>
<tr>
<td>B.L.</td>
<td>1.04</td>
<td>2.06</td>
<td>6.09</td>
</tr>
<tr>
<td>B.L.</td>
<td>1.08</td>
<td>1.87</td>
<td>6.84</td>
</tr>
<tr>
<td>Average ± S.D.§</td>
<td>1.09 ± 0.07</td>
<td>1.90 ± 0.44</td>
<td>7.23 ± 1.66</td>
</tr>
</tbody>
</table>

G-6-PD-deficient subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>TPNH Oxidase Activity†</th>
<th>Methemoglobin Reductase Activity†</th>
<th>Cytochrome c Reductase Activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.G.</td>
<td>0.51</td>
<td>1.02</td>
<td>2.89</td>
</tr>
<tr>
<td>C.W.</td>
<td>0.47</td>
<td>1.22</td>
<td>3.41</td>
</tr>
<tr>
<td>P.J.</td>
<td>0.45</td>
<td>1.02</td>
<td>3.71</td>
</tr>
<tr>
<td>J.C.</td>
<td>0.25</td>
<td>0.80</td>
<td>3.94</td>
</tr>
<tr>
<td>A.M.</td>
<td>0.49</td>
<td>0.96</td>
<td>2.71</td>
</tr>
<tr>
<td>D.G.</td>
<td>0.56</td>
<td>0.99</td>
<td>3.21</td>
</tr>
<tr>
<td>Average ± S.D.§</td>
<td>0.46 ± 0.11</td>
<td>1.00 ± 0.14</td>
<td>3.14 ± 0.37</td>
</tr>
</tbody>
</table>

Congenital methemoglobinemia subject

<table>
<thead>
<tr>
<th>Subjects</th>
<th>TPNH Oxidase Activity†</th>
<th>Methemoglobin Reductase Activity†</th>
<th>Cytochrome c Reductase Activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.D.</td>
<td>1.03</td>
<td>1.86</td>
<td>7.80</td>
</tr>
<tr>
<td>E.D.</td>
<td>1.01</td>
<td>2.24</td>
<td>7.43</td>
</tr>
</tbody>
</table>

*Each pair of initials represents a different individual. Where initials appear twice, the determinations were performed on erythrocytes from the same subject on different days.
†Activity expressed as change in absorbancy per minute per Gm. of hemoglobin, corrected for a blank, at 340 mµ for TPNH oxidase, at 575 mµ for methemoglobin reductase and at 550 mµ for cytochrome c reductase.
‡Average of all determinations in the group ± standard deviation.

These observations are consistent with the concept that in at least one type of congenital methemoglobinemia of the enzymatic type there is a defect in electron transport from reduced pyridine nucleotides to methemoglobin. Further support for this conclusion is derived from the observed defect in the DPNH-utilizing diaphorase-like methemoglobin reductase system (fig. 3) and the normal TPNH-methemoglobin reductase system (table 1) demonstrated in hemolysates. It is apparent, therefore, that even in the presence of adequate supplies of reduced pyridine nucleotides, these erythrocytes are unable to reduce methemoglobin to hemoglobin without the assistance of a substitute electron transport system that is provided by methylene blue.

Under the conditions of the experiments described here, where only the reduction of methemoglobin to hemoglobin was studied, erythrocytes deficient in G-6-PD activity were capable of reducing methemoglobin as rapidly as were normal erythrocytes upon incubation with glucose or inosine. Similar results were reported by Dawson et al.14 However, the marked acceleration of methemoglobin reduction that resulted from the addition of methylene blue to normal erythrocytes did not occur in the G-6-PD deficient erythro-
cytes. The methemoglobin reductase system that is accelerated by methylene blue is known to utilize TPNH preferentially, so the limited acceleration observed in erythrocytes incapable of reducing TPN to TPNH as readily as normal cells would be expected. The limited enhancement of methemoglobin reduction by methylene blue in such enzyme-deficient erythrocytes has been reported before. A test for detecting erythrocyte G-6-PD deficiency based on this phenomenon has been described and studied by Brewer et al. The normal reduction of methemoglobin to hemoglobin in intact G-6-PD deficient erythrocytes in the absence of methylene blue observed in the present studies and previously is compatible with the finding that the DPNH-utilizing diaphorase-like methemoglobin reductase system was normal in nitrited hemolysates (fig. 3).

The studies recently reported by Brewer et al. are at variance with the finding of normal reduction of methemoglobin in G-6-PD deficient erythrocytes. These investigators followed the formation and conversion of methemoglobin after the addition of varying concentrations of sodium nitrite to samples of whole blood mixed with acid-citrate-dextrose (ACD) solution. They observed a less rapid fall in the concentration of methemoglobin in G-6-PD deficient erythrocytes than in normal erythrocytes that was statistically significant when the highest concentration of sodium nitrite (9 x 10^{-3} M) was employed. However, since these experiments involved both the formation and the reduction of methemoglobin, they did not permit the evaluation of mechanisms that may protect against the formation of methemoglobin. For example, the decreased concentration and the instability of reduced glutathione (GSH) in G-6-PD deficient erythrocytes may limit the ability of glutathione peroxidase to protect against oxidation of hemoglobin to methemoglobin. A direct effect of GSH in protecting hemoglobin against oxidation has been implied. However, the presence of glutathione peroxidase and its protective effect in hemolysates of human erythrocytes and a direct protective effect of GSH in the oxidation of hemoglobin to methemoglobin have been questioned. The deficiency of catalase activity reported to exist in G-6-PD deficient erythrocytes might also impair protection against methemoglobin formation, although the importance of this mechanism has been questioned recently. It must be emphasized, therefore, that the studies reported in this paper were designed to evaluate the reduction of methemoglobin to hemoglobin within erythrocytes in the absence of residual oxidizing agents and in hemolysates under optimal conditions.

The meaning of the apparent decrease in the activity of the TPNH-utilizing methemoglobin reductase observed in hemolysates of G-6-PD deficient erythrocytes remains uncertain. An even more striking deficiency in TPNH-methemoglobin reductase activity was reported by Bonsignore et al. in erythrocytes from seven Sardinian subjects of a family with favism. Lühr and Waller found that the TPNH-methemoglobin reductase system in G-6-PD deficient erythrocytes was nearly normal when measured by the oxygen uptake in a hemolysate in which TPNH was generated by exogenous glucose-6-phosphate dehydrogenase. In contrast, in the present studies and
in the ones reported by Bonsignore et al., the methemoglobin reductase was assayed with exogenous TPNH and was determined as TPNH oxidase, methemoglobin reductase and cytochrome c reductase activity. An apparent deficiency in TPNH-methemoglobin reductase activity in cord blood has been reported. It will be necessary to isolate the TPNH-methemoglobin reductase system from normal and G-6-PD deficient erythrocytes in order to confirm or refute the apparent diminished activity of this system in the enzyme-deficient erythrocytes, to evaluate the role of possible inhibitors and to attempt to resolve the present discrepancies.

The studies reported here have provided further evidence that in intact normal human erythrocytes, as well as in intact G-6-PD deficient erythrocytes, the major pathway for the reduction of methemoglobin to hemoglobin probably proceeds by way of a DPNH-utilizing diaphorase-like system (Fig. 4). The required DPNH is produced during glycolysis by way of the Embden-Meyerhof pathway upon conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate through the activity of triosephosphate dehydrogenase. The lactic dehydrogenase reaction can also generate DPNH, but only when lactate is present at a high concentration. The pathways for the generation of DPNH do not appear to be impaired in G-6-PD deficient erythrocytes in the absence of hemolytic agents, such as acetylphenylhydrazine. An increase in the concentration of methemoglobin in G-6-PD deficient erythrocytes has not been found, although the concentration of DPNH in these enzyme-deficient erythrocytes was reported to be decreased. Normal reduction of methemoglobin, therefore, would be anticipated in erythrocytes deficient in G-6-PD activity. In one type of congenital methemoglobinemia, the DPNH-utilizing diaphorase-like system appears to be deficient in the erythrocytes, despite adequate sources of DPNH. A recently reported case of congenital methemoglobinemia may be the result of deficient generation of DPNH in erythrocytes with normal DPNH-diaphorase activity, but a strikingly low
The importance of GSH for the reduction of methemoglobin remains uncertain. Patients with hereditary absence of GSH in their erythrocytes apparently do not have methemoglobinemia. If the TPNH-methemoglobin reductase were a physiologically important pathway for the reduction of methemoglobin, normal reduction of methemoglobin in G-6-PD deficient erythrocytes would not have occurred. Conversely, a deficiency of DPNH-diaphorase activity in the erythrocytes of the patient with congenital methemoglobinemia should not result in complete inability to reduce methemoglobin to hemoglobin. The TPNH-utilizing methemoglobin reductase may be an auxiliary or reserve system for the reduction of methemoglobin. Although it was suggested that ionic iron might act as the natural cofactor, this system appears to require another artificial electron carrier, such as methylene blue, to become fully effective in reducing methemoglobin to hemoglobin. In G-6-PD deficient erythrocytes, where generation of TPNH by way of the hexosemonophosphate shunt pathway is decreased, the activity of the TPNH-methemoglobin reductase is impaired. This decreased capacity to reduce methemoglobin, however, may not only be due to a deficient source of TPNH, but, perhaps, may also result from a defect in the methemoglobin reductase system itself.

Summary and Conclusions

The pathways for the reduction of methemoglobin to hemoglobin that are dependent upon the generation of reduced pyridine nucleotides were studied in normal human erythrocytes, in erythrocytes deficient in G-6-PD activity and in erythrocytes of a subject with congenital methemoglobinemia. Reduction of methemoglobin, produced by treatment with nitrite, occurred at equivalent rates in normal and G-6-PD deficient erythrocytes, but failed to occur in the erythrocytes of the patient with congenital methemoglobinemia upon incubation with glucose or inosine. The DPNH-utilizing diaphorase-like system was normal in hemolysates of G-6-PD deficient erythrocytes, but was markedly deficient in hemolysates of erythrocytes of the subject with congenital methemoglobinemia. The marked acceleration of methemoglobin reduction that occurred upon the addition of methylene blue to normal erythrocytes and to the erythrocytes of the woman with congenital methemoglobinemia did not occur with G-6-PD deficient erythrocytes. The TPNH-utilizing methemoglobin reductase system was normal in hemolysates of erythrocytes of the patient with congenital methemoglobinemia, but was reduced to about 50 per cent of normal activity in hemolysates of G-6-PD deficient erythrocytes.

The reduction of methemoglobin to hemoglobin in intact normal and G-6-PD deficient human erythrocytes probably proceeds by way of a DPNH-utilizing, diaphorase-like system that is deficient in the erythrocytes of one type of congenital methemoglobinemia. The TPNH-utilizing methemoglobin reductase appears to be a reserve system that requires an artificial electron carrier, such as methylene blue, to become fully effective in reducing methemoglobin to hemoglobin. The TPNH-methemoglobin reductase system is
impaired in G-6-PD deficient erythrocytes not only because of a deficient source of TPNH, but, perhaps, also because of a defect in this methemoglobin reductase system itself.

**SUMMARIO IN INTERLINGUA**

Le sequentia del reactiones in le reduction de methemoglobina ad in hemoglobina, dependente del generation de reduceite nucleotidos de pyridina, esseva studiate in normal erythrocytos human, in erythrocytos a carentia de activitate de dishydrogenase de glucosa-6-phosphato, e in erythrocytos ab un patiente con congenite methemoglobinemia. Le reduction de methemoglobina sub le effecto de un tractamento con nitrito occurreva con equivalentes intensitates in erythrocytos normal e a carentia de dishydrogenase de glucosa-6-phosphato. Illa non occurreva in le erythrocytos ab le patiente con congenite methemoglobinemia post incubation con glucosa o inosina. Le sistema diaphorase-simile utilisante reduceite nucleotido de diphosphopyridina (DPNH) esseva normal in hemolysatos de erythrocytos a carentia de dishydrogenase de glucosa-6-phosphato sed esseva marcatemente deficiente in hemolysatos de erythrocytos ab le subjecto con congenite methemoglobinemia. Le marcate acceleration del reduction de methemoglobina que occurreva post le addition de blau methylene a erythrocytos normal e etiam a erythrocytos ab le patiente con congenite methemoglobinemia non occurreva in erythrocytos a carentia de dishydrogenase de glucosa-6-phosphato. Le sistema de reductase de methemoglobina utilisante reduceite nucleotido de triphosphopyridina (TPNH) esseva normal in hemolysatos de erythrocytos ab le patiente con congenite methemoglobinemia sed esseva reduceite per circa 50 pro cento ab le nivello normal in hemolysatos de erythrocytos a carentia de dishydrogenase de glucosa-6-phosphato.

Le reduction de methemoglobina ad in hemoglobina in intacte normal erythrocytos human e in intacte erythrocytos human a carentia de dishydrogenase de glucosa-6-phosphato progredie probablemente via un sistema diaphorase-simile utilisante DPNH, le qual es defective in le erythrocytos de un typo de congenite methemoglobinemia. Le reductase de methemoglobina utilisante TPNH pare esser un sistema de reserva que require un portator artificial de electrones—como blau methylene—pro devenir plenmente efficace in reducer methemoglobina a hemoglobina. Le sistema de reductase de methemoglobina utilisante TPNH es defective in erythrocytos a carentia de dishydrogenase de glucosa-6-phosphato, non solmente a causa de un defective fonte de TPNH sed probablemente etiam a causa de un defecto in iste sistema de reductase de methemoglobina mesme.

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The Reduction of Methemoglobin in Erythrocytes of a Patient with Congenital Methemoglobinemia, Subjects with Erythrocyte Glucose-6-Phosphate Dehydrogenase Deficiency, and Normal Individuals

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