Erythrocyte Glucose 6-Phosphate Dehydrogenase of Normal and Mutant Human Subjects

PROPERTIES OF THE PURIFIED ENZYMES*

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A deficiency in human erythrocyte glucose 6-phosphate dehydrogenase may be genetically determined (1-4) or occur in normal erythrocytes as they age in vivo (5, 6). The inherited glucose-6-P dehydrogenase deficiency, which is associated with hemolytic anemia upon exposure to a variety of agents, affords an opportunity in man to study further the mechanisms by which genes can lead to a decrease in the activity of an enzyme.

The diminution in enzyme activity with erythrocyte aging in vivo is relatively selective in that, of the several enzymes studied, only glyceraldehyde-3-phosphate dehydrogenase shows a marked change similar to that of glucose-6-P dehydrogenase (6). This suggests that some specific property of glucose-6-P dehydrogenase may make it peculiarly susceptible to destruction as red cells age.

The present investigation has been a study of the properties of glucose-6-P dehydrogenase purified from human erythrocytes. Elucidation of the factors determining the activity and stability of this enzyme is pertinent to the problems of (a) whether in mutant1 subjects, glucose-6-P dehydrogenase deficiency reflects a qualitatively or quantitatively altered protein and, (b) the mechanism of the decrease in enzyme activity as erythrocytes age.

Preliminary observations reported by Kirkman (7) and from this laboratory (8) have suggested that the red cell glucose-6-P dehydrogenase of normal and mutant subjects does not differ with regard to the properties of their catalytic site. In the present study, this dehydrogenase has been purified from erythrocytes of normal and Negro mutant subjects. Glucose-6-P, and particularly, triphosphopyridine nucleotide protect the enzyme against thermal inactivation.2 The preparations of enzyme from normal and mutant sources were found to be similar with respect to their stability properties, a wide variety of kinetic parameters, and in their electrophoretic mobility.

EXPERIMENTAL PROCEDURE

Materials—Glucose-6-P dehydrogenase purified from yeast (grade III), glucose-6-P, 6-P-gluconate, TPN, TPNH, DPN, and ATP were purchased from the Sigma Chemical Company. 3-Acetylpyridine-TPN and thioctic acid-TPN were kindly supplied to us by Dr. N. O. Kaplan. TPNH diaphorase was prepared by the method of Avron and Jagendorf (9).

Analytical Methods—Glucose-6-P dehydrogenase and 6-P-glucanonic dehydrogenase were assayed spectrophotometrically (10, 11). The glucose-6-P dehydrogenase assay was performed in a cuvette, with a light path of 1 cm, containing 0.5 ml of 0.25 M glycyglycine buffer, pH 7.6, 0.5 ml of 0.1 M MgCl2, 0.1 ml of 2.5 x 10-4 M TPN, 0.5 ml of 0.01 M glucose-6-P, enzyme, and water to a total volume of 2.5 ml. For 6-P-glucanonic dehydrogenase assay, 0.5 ml of 0.01 M 6-P-gluconate was added instead of glucose 6-P.

Hexokinase was assayed according to the method of Kornberg (12). Spectrophotometric measurements were made with a Cary model 14 spectrophotometer equipped with a slide wire with a range of 0 to 0.1 optical density units, or a Beckman model DU spectrophotometer. A unit of activity of these enzymes is defined as the amount of enzyme required to give an increase in optical density at 340 mμ of 0.001 per minute.

The fluorescence activation and emission spectra of the enzyme preparations were determined with a Farrand spectrophotofluorometer. Hemoglobin was determined spectrophotometrically in a solution of 0.04% NH4OH at 540 μμ. Protein was determined by the method of Lowry et al. (13).

Electrophoresis Studies—The electrophoretic mobility of purified glucose-6-P dehydrogenase in starch gel was carried out as described by Markert et al. (14). The electrophoresis was performed in 0.03 M borate buffer, pH 8.6, or 0.03 M Tris buffer, pH 7.4, at 4° for 16 to 24 hours. After this period, the dehydrogenase was identified directly on the starch gel. The enzyme preparations was determined with a Farrand spectrophotofluorometer. Hemoglobin was determined spectrophotometrically in a solution of 0.04% NH4OH at 540 μμ. Protein was determined by the method of Lowry et al. (13).

Source of Erythrocytes—The blood processed was either freshly drawn or obtained from the blood bank, where it had been stored at 4° for periods of about 1 month. Blood was prevented from clotting with a mixture containing per 100 ml, 1.32 g of sodium...
citrate, 0.44 g of citric acid, and 1.47 g of dextrose. Four parts of blood were mixed with one part of the anticoagulant mixture. No differences were noted in the enzyme purification starting with the freshly drawn or stored blood. Glucose-6-P dehydrogenase was purified from the erythrocytes of 17 subjects with normal levels of activity of this enzyme, as well as from seven Negroes with a marked deficiency in the activity of the enzyme.

Preparation of Hemolysates—The following procedure was carried out at 4°. Whole blood (generally 500 ml) was centrifuged at 2000 × g for 10 minutes and the supernatant plasma solution removed and discarded. The sedimented erythrocytes were washed three times with 4 volumes of isotonic KCl buffered at pH 7.4 and the washes discarded. The washed red cells were lyzed by addition of 4 volumes of cold distilled H2O. The hemoglobin concentration of the resulting lysate was adjusted to 0.035 g per ml by addition of water. This lysate was permitted to stand for 45 minutes to allow for more complete hemolysis.

First Ammonium Sulfate Precipitation—The hemolysate was made 35% saturated by the addition of 19.4 g of ammonium sulfate per 100 ml of solution and allowed to stand for 5 minutes. After centrifugation at 25,000 × g for 30 minutes, the supernatant solution was recovered and the precipitate discarded. This supernatant solution was made 55% saturated by addition of 11.8 g of ammonium sulfate per 100 ml of solution and allowed to stand for 5 minutes. The resulting precipitate was collected by centrifugation at 18,000 × g for 8 minutes and dissolved in about 25.0 ml of 0.25 M glycylglycine buffer, pH 7.6. This solution was centrifuged at 4000 × g for 8 minutes and the resultant supernatant recovered (ammonium sulfate Fraction I).

Calcium Phosphate Gel Adsorption and Elution—The calcium phosphate gel used in this procedure was prepared within 2 weeks of its use (15). The minimal amount of calcium phosphate gel required to adsorb a maximal amount of the enzyme activity was determined with small aliquots of the Ammonium Sulfate I preparation. Generally, 14 ml of calcium phosphate gel per 1.0 ml of Ammonium Sulfate I was found optimal. After addition of the gel to the Ammonium Sulfate I, the mixture was allowed to stand for 10 minutes before centrifugation at 18,000 × g. The precipitate was recovered and the enzyme eluted with 10 volumes of 0.1 M phosphate buffer, pH 7.6, per volume of Ammonium Sulfate I (gel eluate).

Second Ammonium Sulfate Precipitation—To the gel eluate, 16.4 g of ammonium sulfate per 100 ml of solution were added, and the mixture was allowed to stand for 5 minutes. After centrifugation at 18,000 × g for 10 minutes, the precipitate was discarded. The supernatant solution was made 50% saturated by the addition of 11.7 g of ammonium sulfate per 100 ml of solution and allowed to stand for 5 minutes. The precipitate was collected by centrifugation at 18,000 × g for 10 minutes and dissolved in about 3.0 ml of 0.25 M glycylglycine buffer, pH 7.5 (ammonium sulfate Fraction II).

As shown in Table I, this procedure resulted in an approximately 500-fold purification with a recovery of about 50% of the glucose-6-P dehydrogenase activity of the erythrocytes of both normal subjects and mutant Negroes. Although the degree of purification is about the same for the enzyme from normal and mutant red cells, glucose-6-P dehydrogenase from normal subjects had a significantly higher specific activity, presumably the result of the marked difference in the enzyme activity of the starting material.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Normal</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude hemolysate</td>
<td>340</td>
<td>6.5</td>
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<tr>
<td>Ammonium sulfate</td>
<td>282</td>
<td>249</td>
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<tr>
<td>Fraction I</td>
<td>211</td>
<td>1930</td>
</tr>
<tr>
<td>CaPO₄ gel eluate</td>
<td>182</td>
<td>3320</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction II</td>
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</table>
| * The normal subject had a red cell glucose-6-P dehydrogenase of 15,300 units per g of Hb. The mutant subject was a Negro male with red cell glucose-6-P dehydrogenase activity of 2100 units per g of Hb.

The purification of the enzyme from erythrocytes of two affected Caucasian males was attempted. In general, mutant Caucasian males compared with mutant Negro males have a significantly lower glucose-6-P dehydrogenase activity in their erythrocytes (16). The red cell enzyme activities of affected Caucasian males average less than 3%, whereas those of affected Negro males average about 15% of the mean value for normal subjects. The two Caucasian males had very low levels of erythrocyte enzyme activities, i.e. below 0.5 units per g of Hb. Glucose-6-P dehydrogenase of the erythrocytes of these subjects could be purified only 10-fold with a yield of about 30%. These preparations were inadequate for studies comparable to those with enzyme purified from Negro mutant or normal subjects.

RESULTS

Stability and Purity—The purified enzyme preparation can be stored in the frozen state for at least 4 months without significant loss of activity. The ammonium sulfate Fraction II is contaminated with less than 0.1% 6-P-gluconic dehydrogenase and less than 0.2% hexokinase activity relative to glucose-6-P dehydrogenase activity.

pH Optimum—Variation in pH between 5.5 and 10.0 had a similar effect on the activity of the normal and mutant enzymes under the conditions of the standard assay procedure. The pH was varied with the following buffers: 0.05 M trismaleate, pH 5.5 to 7.8, 0.05 M glycylglycine, pH 7.0 to 9.0, and 0.05 M glycine, pH 9.0 to 10.4. The maximal activity was obtained over a pH range between 8.0 and 9.0.

Comparison of Substrate Affinity Constants and Inhibitor Effects—The normal and mutant enzymes do not differ with respect to the Michaelis constants, Kₘ, for glucose-6-P and TPN. The Kₘ of the normal enzyme for glucose-6-P is 3.5 × 10⁻⁴ M and that of the mutant enzyme, 6.0 × 10⁻⁴ M. The Kₘ for TPN is 4.2 × 10⁻⁴ M for the normal enzyme and 6.5 × 10⁻⁴ M for the mutant enzyme.

The TPN analogues, 3-acyetylpyridine.*TPN or thionicotinamide.*TPN, do not replace TPN as cofactors for the purified normal or mutant red cell glucose-6-P dehydrogenase. When 3-acyetylpyridine.*TPN was substituted for TPN in the standard assay procedure, a rate less than 5% of that observed with comparable concentrations of TPN was found. No change in optical
density was observed when $5 \times 10^{-4}$ M thionicotinamide-*TPN was substituted for TPN in the assay system. Both 3-acetylpyridine-*TPN and thionicotinamide-TPN are competitive inhibitors of the mutant and normal enzymes (Fig. 1). The $K_i$ for 3-acetylpyridine-*TPN is $3.0 \times 10^{-4}$ M for the normal enzyme and $5.0 \times 10^{-3}$ M for the mutant enzyme. The $K_i$ values for thionicotinamide-*TPN are $1.7 \times 10^{-5}$ M and $1.3 \times 10^{-5}$ M, respectively.

Both the normal and mutant enzymes are inhibited by nicotinamide. This inhibition is competitive with TPN, and the $K_i$ of the normal enzyme is $3.8 \times 10^{-2}$ M and that of the mutant enzyme is $6.5 \times 10^{-2}$ M. This difference in $K_i$ was not significant.

Dehydroisoandrosterone has been shown to be a potent inhibitor of mammalian glucose-6-P dehydrogenase (17, 18). This inhibition is noncompetitive with respect to either TPN or glucose-6-P (17). Dehydroisoandrosterone inhibited the activity of the enzyme prepared from normal subjects and that from mutant subjects to a similar extent. Thus, at $5 \times 10^{-3}$ M dehydroisoandrosterone, the activity of both enzyme preparations was inhibited 70%, and at $5 \times 10^{-7}$ M, both were inhibited 10%.

It has been reported (19, 20) that glucose-6-P dehydrogenase purified from yeast required Mg$^{+2}$ for its optimal activity. The purest preparations of red cell enzyme showed, in the absence of added Mg$^{+2}$, approximately 70% of the maximal activity. The concentration of Mg$^{+2}$ required to obtain maximal enzyme activity was 0.01 M for both the normal and mutant enzymes. In the absence of added Mg$^{+2}$, the addition of sodium EDTA in concentrations as high as 0.043 M to preparations of purified enzyme did not reduce the activity of the enzyme (Fig. 2). This suggests that these preparations of glucose-6-P dehydrogenase do not have an absolute requirement for Mg$^{+2}$. Addition of sodium EDTA in the presence of 0.01 M MgCl$_2$ resulted in inhibition of the activity of the purified enzyme. As indicated in Fig. 2, at an EDTA concentration of 0.017 M in the presence of 0.01 M MgCl$_2$, an EDTA-Mg$^{+2}$ enzyme complex is formed which is markedly inhibitory to erythrocyte glucose-6-P dehydrogenase activity, and at higher and lower EDTA concentrations the complex formed is less inhibitory. The concentration-inhibition curve for EDTA was similar for the normal and mutant enzyme (Fig. 2).

Thermostability and Activation of Normal and Mutant Glucose-6-P Dehydrogenase—Preincubation of undiluted preparations of glucose-6-P dehydrogenase purified from normal subjects at 37° resulted in an increase in enzyme activity (Fig. 3). The preincubation period required to observe maximal activation varied, but was generally between 1 and 4 hours. Preparations of purified mutant enzyme showed similar, but smaller, increments in activity after incubation at 37°. Thus, the increase in activity of the preparations of normal enzyme ranged between 1.5- and 3.0-fold, and that of the preparations of mutant enzyme, between 1.2- and 2.0-fold. This difference was not dependent on the protein concentration. The activation of the enzyme was temperature-dependent. Maximal stimulation was obtained at 90°, and smaller effects were noted at 25° and 37°. Generally, no activation of purified normal or mutant preparations was observed during incubation at 0°. Fifty per cent of either the

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**Fig. 1.** Inhibition by 3-acetylpyridine-*TPN (left) and by thionicotinamide-*TPN (right) of the activity of glucose-6-P dehydrogenase purified from normal and mutant subjects at various TPN concentrations. The reaction mixture contained 0.5 ml of 0.25 M glycylglycine buffer, pH 7.6, 0.5 ml of 0.1 M MgCl$_2$, 0.5 ml of 0.01 M glucose-6-P, enzyme with (O) or without (□) the addition of the TPN analogue. 3-Acetylpyridine-*TPN was present in a final concentration $5 \times 10^{-4}$ M. Thionicotinamide-*TPN was present in a final concentration of $5 \times 10^{-5}$ M.

**Fig. 2.** Effect of EDTA in the presence and absence of added MgCl$_2$ (0.01 M) on glucose-6-P dehydrogenase activity. EDTA was added to the reaction mixture as a 50 mg per ml solution, pH 7.4.

**Fig. 3.** Effect of preincubation at 37° of glucose-6-P dehydrogenase purified from red cells of normal (left) and mutant (right) subjects. The enzyme activity is plotted on a logarithmic scale. The normal enzyme "undiluted" was incubated in a concentration of 5.5 mg of protein per ml with a specific activity of 2930 units per mg of protein. The mutant enzyme "undiluted" was incubated in a concentration of 5.7 mg of protein per ml with a specific activity of 300 units per mg of protein. All incubations were in 0.25 M glycylglycine buffer pH 7.6, and all dilutions indicated in the graphs were made with this buffer.

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The following abbreviation is used: EDTA, ethylenediaminetetraacetate, disodium salt.
normal or mutant enzyme activity was lost after incubation at 45° for 12 minutes.

The noted activation of glucose-6-P dehydrogenase after preincubation does not involve any alteration in the affinity of the enzyme for its substrates, glucose-6-P or TPN, but is associated with an increase in the observed $v_{\text{max}}$. Thus, the $K_m$ of normal enzyme for glucose-6-P is $3.5 \times 10^{-5}$ M and for TPN is $4.2 \times 10^{-4}$ M. After preincubation at 37°, which resulted in a 260% increase in activity, the $K_m$ for glucose-6-P is $3.8 \times 10^{-5}$ M and for TPN is $4.9 \times 10^{-4}$ M. The $K_m$ of the mutant enzyme for glucose-6-P is $3.0 \times 10^{-5}$ M and for TPN is $6.5 \times 10^{-4}$ M. After preincubation at 37°, which yielded a 190% increase in activity, the $K_m$ for glucose-6-P is $4.0 \times 10^{-5}$ M and for TPN is $0.7 \times 10^{-4}$ M.

Effect of Dilution on Stability of Glucose-6-P Dehydrogenase—It was observed (Fig. 3) that dilution of normal or mutant enzyme preparations was associated with a loss of activation. Further, the stability of both enzymes decreased with dilution. At the higher dilutions, the kinetics of the loss in activity of the enzymes was first order (Fig. 3). With both the normal and mutant enzyme preparations, a point was reached beyond which additional dilution did not further decrease their stability. At these dilutions, the time required for the loss of 50% of the initial activity was determined for normal and mutant enzymes. The average $t_{1/2}$ for normal enzyme preparations was 40 minutes and for mutant enzyme preparations, 44 minutes. This difference was not statistically significant. The effect of dilution on preparations of glucose-6-P dehydrogenase could reflect the dissociation of the enzyme from a factor necessary for its activation and stability. No definitive evidence for this possibility has been obtained.

Effect of TPN on Glucose-6-P Dehydrogenase Thermostability—It was found that the thermostability of glucose-6-P dehydrogenase activity in crude hemolysates and in leukocyte lysates was enhanced by addition of TPN in final concentrations as low as $1 \times 10^{-5}$ M (21). In addition, it has previously been reported that TPN stabilized the enzyme partially purified from erythrocytes (7, 8). A study has been made of the relative effectiveness of TPN in stabilizing glucose-6-P dehydrogenase purified from normal and from mutant subjects. The enzyme preparations were diluted with the minimal amount of 0.25 M glycylglycine buffer, pH 7.6, to a concentration at which the $t_{1/2}$ for the enzyme was minimal. Incubation was carried out at 37°. The addition of this amount of TPN to aliquots of the enzyme solution at subsequent times during the incubation caused a stabilization of the remaining enzyme activity, but was not associated with any reactivation of the enzyme. These studies suggest that the loss in enzyme activity during incubation at 37° involves an irreversible inactivation of the enzyme.

The specificity of TPN stabilization of glucose-6-P dehydrogenase was investigated by determining the effect of TPNH, DPN, and glucose-6-P on the stability of the enzyme. In studies similar to that illustrated in Fig. 4, TPNH, DPN, or glucose-6-P had no stabilizing action on glucose-6-P dehydrogenase. DPN in concentrations as high as $1.4 \times 10^{-4}$ M had no stabilizing action on glucose-6-P dehydrogenase preparations. Glucose-6-P could protect the enzyme against inactivation at 37°. However, the $K_m$ for glucose-6-P was considerably higher than that for TPN. The $K_m$ values for glucose-6-P for the normal and mutant enzyme preparations were $3.5 \times 10^{-5}$ M and $3.1 \times 10^{-4}$ M, respectively.

Other substances tested, including glucose, reduced glutathione, ribose, and inosine, in concentrations as high as $1 \times 10^{-4}$ M, had no stabilizing action on glucose-6-P dehydrogenase preparations.
Fluorescence activation during preincubation at 37°.

To illustrate the fluorescence intensity, which is scaled in arbitrary units. These units depend upon the amplification of the signal which is employed in a particular study.

During purification of glucose-6-P dehydrogenase from red cells, it was noted that the thermostability of the enzyme appeared to be enhanced by ammonium sulfate. Various other salts were tested for their stabilizing effect on the enzyme. Thus, preparations of glucose-6-P dehydrogenase purified from normal or from mutant subjects were diluted with water, 0.44 M (NH₄)₂SO₄, 0.44 M Na₂SO₄, 0.88 M NH₄Cl, or 0.88 M NaCl to a protein concentration of about 0.4 mg per ml. The percentage of enzyme activity at zero time remaining after incubation at 37° for 2 hours was 60% in (NH₄)₂SO₄, but only 10 to 18% in water or Na₂SO₄ solutions, and none in NH₄Cl or NaCl solutions.

Effect of Nicotinamide on Glucose-6-P Dehydrogenase Thermostability—Nicotinamide, in concentrations of 0.005 M or greater, when preincubated with normal or mutant enzyme preparations at 37° could prevent the activation of the enzyme. Increasing the concentration of nicotinamide between 0.005 M and 0.2 M was associated with a progressive decrease in the thermostability of the normal and mutant enzyme. For example, incubation of the enzyme preparations with 0.1 M nicotinamide in 0.25 M glycylglycine buffer pH 7.4, for 1 hour at 37° was associated with about 40% decrease in enzyme activity, in contrast to a 20 to 50% increase in activity observed under identical conditions of incubation in the absence of nicotinamide. The effect of 0.1 M nicotinamide in decreasing the stability of the glucose-6-P dehydrogenase could be prevented by addition of 1 × 10⁻⁴ M TPN.

Fluorescence Activation and Emission Spectra of Glucose-6-P Dehydrogenase—Preparations of the enzyme purified from normal and mutant subjects show identical fluorescence activation and emission spectra (Fig. 5) characteristic of reduced pyridine nucleotide-enzyme complex (25, 26). Thus, the fluorescence activation spectrum of preparations of both the normal and mutant enzyme had a maximum at 350 mλ which corresponded to that of free TPNH. A second peak in the activation spectrum of the enzyme preparations was present at 290 mλ, corresponding to the 280 mλ absorption band which is characteristic for proteins. The emission spectrum of the preparations of glucose-6-P dehydrogenase revealed a maximum at 440 mλ, compared to the free TPNH which has a maximal emission intensity at 460 mλ (Fig. 5). Boyer and Theorell (25) first reported that enzyme-bound DPNH was characterized by a fluorescence emission spectrum which has a maximum shifted towards the ultraviolet compared to that of free DPNH. As Velick (26) has shown, an enzyme-DPNH complex may or may not reveal a shift in activation maximum relative to the unbound reduced pyridine nucleotide. Attempts to remove glucose-6-P dehydrogenase-bound pyridine nucleotide by charcoal treatment and to recover active enzyme were unsuccessful. Thus, mixing acid-washed Norit A suspended in 0.1 M Tris buffer, pH 7.6, in proportions which varied between 2.1 to 21 mg of Norit per mg of protein was associated with a loss in enzyme activity which ranged between 30 and 75% with the increasing amounts of Norit. This Norit treatment of the enzyme was not associated with a disappearance of the fluorescence absorption and emission spectra illustrated in Fig. 5. Preincubation of preparations of glucose-6-P dehydrogenase with 0.1 M nicotinamide or 5 × 10⁻⁶ M p-chloromercuribenzoate for 15 minutes at 24°, which yielded inactive enzyme preparations, did not result in an alteration in the fluorescence activation or emission maxima at 350 mλ and 440 mλ, respectively. Heat inactivation of the enzyme was associated with a loss in these fluorescence properties. In view of the fact that attempts to remove the pyridine nucleotide from the purified enzyme were unsuccessful, these data can only be considered suggestive of a glucose-6-P dehydrogenase-bound TPNH complex.

Electrophoretic Mobility of Glucose-6-P Dehydrogenase Preparations—The electrophoretic mobilities of glucose-6-P dehydrogenase purified from red cells of normal and mutant Negro subjects were compared in two different buffer systems: 0.03 M borate buffer at pH 8.6 and 0.03 M Tris buffer at pH 7.4. No electrophoretic differences were observed between the normal and mutant enzyme preparations. The electrophoretic mobility of glucose-6-P dehydrogenase purified from yeast was distinctly faster in both buffer systems than that of the red cell dehydrogenase. The glucose-6-P dehydrogenase of human erythrocytes and of yeast showed one band with enzyme activity, in both buffer systems.

Effect on Enzyme Activity of Mixing Normal and Mutant Preparations of Glucose-6-P Dehydrogenase—The possibility that the genetically determined deficiency in glucose-6-P dehydrogenase reflects the lack of an activator or presence of an inhibitor has been examined by determining the activity of mixtures of the enzyme purified from normal and from mutant subjects. If differences between the two preparations of enzyme exist with respect to the concentrations of an activator or of an inhibitor, the extraction is increased 10-fold to counter nicotinamide inhibition of glucose-6-P dehydrogenase activity in the assay system.

In studies of the effect of nicotinamide on glucose-6-P dehydrogenase thermostability, all assays for enzyme activity were performed in the standard mixture save that the TPN concentration was increased 10-fold to counter nicotinamide inhibition of glucose-6-P dehydrogenase activity in the assay system.

5 No corrections have been applied to the activation or emission spectra. The results cited for the preparations of enzymes were compared to those obtained with free TPNH during the same experiment.
the activity of a mixture of the two enzymes might reflect an interaction effect and not a simple summation of the individual rates.

Aliquots of normal and mutant enzyme preparations were mixed in varying proportions and allowed to stand for 5 minutes before assay for the activity of the mixture. The activity of the separate aliquots of normal and mutant enzyme preparations was determined simultaneously with that of the mixture. The enzyme activity of the mixture was found to exceed the expected activity (based on a summation of the enzyme activities of the aliquots) by 10 to 30% in 16 of 27 experiments and was lower than the expected activity in two studies. Similar results were obtained whether the preparations of normal or mutant enzyme employed were purified about 500-fold or only 50-fold, relative to the crude hemolysate. Although activation of glucose-6-P dehydrogenase may have occurred in certain experiments upon mixing normal and mutant enzyme preparations, the effects were small and too variable to permit conclusions.

**Effect of Red Cell Stroma on Glucose-6-P Dehydrogenase Activity**—Recently, Rimon et al. (27) reported that stroma prepared from normal red cells hemolyzed by gradual osmotic lysis could increase the glucose-6-P dehydrogenase activity of mutant, but not normal, hemolysates. No increase in dehydrogenase activity of mutant hemolysates was noted upon addition of red cell stroma prepared from mutant subjects. These data were interpreted as indicating that an activator of glucose-6-P dehydrogenase was lacking in mutants. An attempt has been made to repeat these observations. The addition of normal stroma to mutant hemolysates was associated with an increment in dehydrogenase activity which was somewhat greater than that observed upon addition of normal stroma to normal hemolysate (Table II). However, upon addition of normal stroma to 1.0 M NaCl or to boiled hemolysate, an amount of glucose-6-P dehydrogenase activity was recovered comparable in amount to the observed increment in enzyme activity upon addition of normal stroma to hemolysate. This suggests that the increase in enzyme activity observed upon addition of normal stroma to hemolysate may result from glucose-6-P dehydrogenase lysed or "clutered" from the stroma, rather than from the presence of an activator of the enzyme. In the study of Rimon et al. and, to a less striking extent, in the present study, the addition of normal stroma to normal hemolysate did not give a similar increase in glucose-6-P dehydrogenase activity as when added to mutant hemolysate. Further studies are required to clarify the significance of this observation.

A study was made of the effect of red cell stroma on preparations of glucose-6-P dehydrogenase purified from normal and mutant subjects. Normal and mutant stroma preparations had similar effects on the activity of the purified enzymes (Table II). The supernatant fluid recovered from the mixtures of stroma and purified enzymes had higher enzyme activity than that of the mixtures of boiled stroma and purified glucose-6-P dehydrogenase. It is unlikely that this difference in activity reflects glucose-6-P dehydrogenase which was present in the stroma, since the normal and mutant stroma had the same effect. It is possible that fresh stroma prepared from normal or from mu-

<table>
<thead>
<tr>
<th>Preparation added to Stroma</th>
<th>Boiled</th>
<th>Normal</th>
<th>Mutant</th>
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<tbody>
<tr>
<td>0.05 M NaCl</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.0 M NaCl</td>
<td>0</td>
<td>7.3</td>
<td>0.9</td>
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<td>Normal hemolysate</td>
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<td>23.1</td>
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<tr>
<td>Mutant hemolysate</td>
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<tr>
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<tr>
<td>Purified mutant enzyme</td>
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<td>11.2</td>
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</tr>
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* Stroma was prepared by a modification of the method of Danon et al. (28). Erythrocytes, washed three times with 0.15 M NaCl and dialyzed against 0.015 M NaCl for 15 hours at 4°. The stroma was recovered by centrifugation of the contents of the dialysis bag at 28,000 X g for 30 minutes and washed three times with 0.015 M NaCl. Glucose-6-P dehydrogenase activity is expressed as units of enzyme activity per ml of the incubation mixture. The figures indicated are the mean values for 10 experiments.

* Hemolysates were prepared from erythrocytes by the method previously described (27). Boiled hemolysates and boiled stroma were prepared by heating the preparations at 100° for 5 minutes.

* The stroma (dry weight, 15 mg per ml) and hemolysate, purified enzyme preparation, or NaCl solution were mixed in equal volumes, allowed to stand for 5 minutes at 24°, and then centrifuged at 28,000 X g for 3 minutes. An aliquot of the clear supernatant fluid was taken for determination of glucose-6-P dehydrogenase activity. Boiled stroma was found to have no effect on glucose-6-P dehydrogenase activity of hemolysates and was used as a control. Incubation of the mixtures for longer than 5 minutes at 24° or 37° was not associated with any increase in enzyme activity in the supernatant fluid. Similar results were obtained whether the hemolysates employed were prepared from normal Negroes or mutant Caucasians.

* The preparations of purified enzyme were diluted in 0.25 M glycylglycine buffer pH 7.4.

Glucose-6-P dehydrogenase purified from red cells of normal subjects and Negroes with a genetically determined deficiency of this enzyme have been found not to differ with respect to the properties of their catalytic site, stability characteristics, fluorescence spectra, or electrophoretic mobility. Thus, no evidence has been obtained to indicate that the deficiency in glucose-6-P dehydrogenase activity in these mutant subjects reflects the production of an altered enzyme.

In the present study, glucose-6-P dehydrogenase was shown to be activated upon incubation at 37°, suggesting that the enzyme may exist in an inactive, as well as in an active form. Preparations of normal glucose-6-P dehydrogenase were generally activated to a greater degree than preparations of mutant glucose-6-P dehydrogenase. This finding could reflect the presence of larger amounts of an inactive form of the enzyme in the nor-

**TABLE II**

**Effect of red cell stroma on glucose-6-P dehydrogenase activity in purified preparations and crude hemolysates from normal and mutant subjects**

<table>
<thead>
<tr>
<th>Preparation added to Stroma</th>
<th>Boiled*</th>
<th>Normal</th>
<th>Mutant</th>
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<tr>
<td>0.05 M NaCl</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.0 M NaCl</td>
<td>0</td>
<td>7.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Normal hemolysate</td>
<td>23.5</td>
<td>26.1</td>
<td>23.1</td>
</tr>
<tr>
<td>Boiled normal hemolysate</td>
<td>0</td>
<td>3.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Mutant hemolysate</td>
<td>6.6</td>
<td>11.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Boiled mutant hemolysate</td>
<td>0</td>
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<td>0.4</td>
</tr>
<tr>
<td>Purified normal enzyme</td>
<td>12.9</td>
<td>14.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Purified mutant enzyme</td>
<td>9.9</td>
<td>11.2</td>
<td>12.0</td>
</tr>
</tbody>
</table>

* Stroma was prepared by a modification of the method of Danon et al. (28). Erythrocytes, washed three times with 0.15 M NaCl and dialyzed against 0.015 M NaCl for 15 hours at 4°. The stroma was recovered by centrifugation of the contents of the dialysis bag at 28,000 X g for 30 minutes and washed three times with 0.015 M NaCl. Glucose-6-P dehydrogenase activity is expressed as units of enzyme activity per ml of the incubation mixture. The figures indicated are the mean values for 10 experiments.

**Discussion**

Glucose-6-P dehydrogenase purified from red cells of normal subjects and Negroes with a genetically determined deficiency of this enzyme have been found not to differ with respect to the properties of their catalytic site, stability characteristics, fluorescence spectra, or electrophoretic mobility. Thus, no evidence has been obtained to indicate that the deficiency in glucose-6-P dehydrogenase activity in these mutant subjects reflects the production of an altered enzyme.

In the present study, glucose-6-P dehydrogenase was shown to be activated upon incubation at 37°, suggesting that the enzyme may exist in an inactive, as well as in an active form. Preparations of normal glucose-6-P dehydrogenase were generally activated to a greater degree than preparations of mutant glucose-6-P dehydrogenase. This finding could reflect the presence of larger amounts of an inactive form of the enzyme in the nor-

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7 It has been demonstrated previously that stroma prepared from human erythrocytes by gradual osmotic lysis may retain hemoglobin and other constituents, and that such stroma preparations can be further lysed (29).
Attempts to demonstrate the presence of an activator of glucose-6-P dehydrogenase either in preparations of the enzyme purified from normal subjects, or in red cell stroma prepared from normal subjects, have been inconclusive.

Although the present study has provided no evidence that hereditary glucose-6-P dehydrogenase deficiency reflects the formation of an altered protein, the possibility has not been excluded that an inactive enzyme-molecule, analogous to that described in certain mutant microorganisms (30), is produced in mutant subjects.

Several laboratories (21, 31–33) have reported that glucose-6-P dehydrogenase in crude hemolysates of mutant subjects is less stable than the enzyme in hemolysates of normal persons during incubation at 37°. However, this difference in glucose-6-P dehydrogenase stability between normal and mutant subjects is not observed in hemolysates from which the stroma has been removed (31). In lysates of leukocytes of mutant Negroes and those of normal persons, the thermostability of glucose-6-P dehydrogenase was found to be similar (21). On the basis of the present study, the decreased thermostability of the enzyme in mutant hemolysates appears to reflect an alteration in the mutant which is not detected upon purification of the enzyme.

There are several reports of the stabilization of an enzyme by its substrate or coenzyme (34–37). The protection of glucose-6-P dehydrogenase by TPN and glucose-6-P against inactivation by heat or upon dilution suggests that the substrate and coenzyme are important to the maintenance of a stable structure of this protein. There is another aspect of this stabilization which is of possible physiological significance. A factor is present in the stroma of red cells which can specifically bring about the inactivation of glucose-6-P dehydrogenase (8, 38, 39). This factor appears to be TPNase. The destruction by TPNase of pyridine nucleotide necessary to the stability of the dehydrogenase may be a determinant of the decrease in the activity of glucose 6-P dehydrogenase as erythrocytes age in vivo. Such action by TPNase may also play a role in the expression of the genetically determined deficiency of this enzyme. Mutant subjects are known to have a greater decrease in glucose-6-P dehydrogenase activity in erythrocytes than in other tissues (16, 40). This variation in expression of the enzyme deficiency might reflect the peculiar susceptibility of glucose-6-P dehydrogenase to inactivation. In red cells, which synthesize little or no protein, the decline in enzyme activity with aging in vivo could lead to a more marked deficiency of this enzyme than in tissues which can synthesize proteins.

Glucose-6-P dehydrogenase of human erythrocytes differs in several respects from that of yeast and other sources. The $K_m$ of red cell glucose-6-P dehydrogenase for TPN is about one order of magnitude less than that of the yeast enzyme. The enzyme of erythrocytes and of yeast have different electrophoretic mobilities. In addition, as previously demonstrated (17), mammalian glucose-6-P dehydrogenase, but not that of yeast or spinach, is strongly inhibited by certain steroids. These data provide further evidence for the heterogeneity that may exist among proteins which serve the same function in different metabolic environments (41).

**Summary**

1. Glucose-6-phosphate dehydrogenase has been purified from red cells of normal subjects and Negroes with a genetically determined deficiency of this enzyme.
2. The preparations of purified glucose-6-phosphate dehydrogenase of normal and of mutant subjects are similar with respect to their affinities for triphosphopyridine nucleotide, glucose-6-phosphate, and several competitive and noncompetitive inhibitors; their pH optimum curves; their electrophoretic mobility; and various aspects of their stability properties.
3. Triphosphopyridine nucleotide, its reduced form, and, in higher concentrations, glucose-6-phosphate, but not diphosphopyridine nucleotide or nicotinamide, can protect the preparations of normal and mutant enzyme against inactivation by heat or upon dilution.
4. The fluorescence activation and emission spectra of the preparations of purified glucose-6-phosphate dehydrogenase of normal and of mutant subjects were similar and suggestive of enzyme-bound reduced pyridine nucleotide complex.
5. The present results are discussed in relation to the problems of the mechanism of the genetically determined glucose-6-phosphate dehydrogenase deficiency and of the selective decrease in the activity of this enzyme that occurs in normal red cells as they age in vivo.

**References**

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