Glucose 6-Phosphate Dehydrogenase of Human Erythrocytes

I. PURIFICATION AND CHARACTERIZATION OF NORMAL (B+) ENZYME*

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SUMMARY

Human erythrocyte glucose 6-phosphate dehydrogenase (β-glucose 6-phosphate:nicotinamide adenine dinucleotide phosphate oxidoreductase, EC 1.1.1.49) was purified by column chromatography with diethylaminoethyl cellulose, calcium phosphate gel, carboxymethyl cellulose, diethylaminoethyl Sephadex, and carboxymethyl Sephadex. A homogeneous preparation was obtained in over-all yield of about 50%. The specific activity (750) and the yield were significantly greater than those so far reported for the enzyme.

The sedimentation patterns of analytical ultracentrifugation and interference pattern of sedimentation equilibrium indicated a homogeneous preparation. The sedimentation constant (s20, w) was 10.0 S at a protein concentration of 0.3%. The molecular weight was estimated as 240,000.

The molecular weight was about 43,000 in 8 M guanidine-HCl, indicating that the enzyme consisted of six similar size subunits.

Removal of nicotinamide adenine dinucleotide phosphate from the enzyme caused dissociation into inactive (or weakly active) subunits with a molecular weight of about one-half of the native protein.

The enzyme was partially inactivated by dilution without dissociating into subunits.

Optimal pH and Michaelis constants for the primary substrates were determined. Analogues of β-glucose 6-phosphate accepted as substrates were 2-deoxy-D-glucose 6-phosphate and D-galactose 6-phosphate. Nicotinamide adenine dinucleotide was a weak substrate.

Amino acid composition of the enzyme was determined.

Because of the existence of many genetic variants in man, glucose 6-phosphate dehydrogenase (β-glucose 6-phosphate:nicotinamide adenine dinucleotide phosphate oxidoreductase, EC 1.1.1.49) from erythrocytes is of particular interest in studies of human biochemical genetics. About 20 types of glucose 6-phosphate dehydrogenase, which are distinguishable by electrophoretic mobility in starch gel, or by enzymic characteristics, or by both methods, have been reported (1-3). Thus, about 20% of American Negroes have an electrophoretically rapid variant of enzyme with normal enzyme activity, while another 10 to 15% have an electrophoretically similar variant with deficient activity (1). The deficient glucose 6-phosphate dehydrogenase variant found in many Mediterranean populations has normal electrophoretic migration and a high relative rate of utilization of substrate analogues (i.e., galactose 6-phosphate or 2-deoxyglucose 6-phosphate) (4). Deficiencies of the enzyme have been related to drug- and food-induced anemia and to chronic hemolytic diseases (2, 5, 6).

In spite of the efforts of several investigators (7-9), the enzyme has not yet been sufficiently purified to elucidate genetic alterations on the basis of molecular structure. In an attempt to work out hereditary alterations and defects of the enzyme on the molecular level and to understand the nature of alterations of enzyme specificity introduced by mutational change, glucose 6-phosphate dehydrogenases from blood of normal and mutant human individuals is being purified and characterized with respect to chemical, serological, and enzymic properties. This paper provides data on isolation and purification of glucose 6-phosphate dehydrogenase from normal human blood. Molecular weight, amino acid composition, and some enzymic characteristics of a homogeneous preparation are described. It has been known that the activity of glucose 6-phosphate dehydrogenase from human erythrocytes is markedly reduced when bound NADP is removed (10, 11). This reversible inactivation has been attributed to dissociation of the enzyme into subunits. Contradictory results regarding molecular size of the active enzyme and its inactive subunits have been presented with the use of partially purified preparations (12, 13). In this paper, the molecular weight of the enzyme has been determined by a sedimentation equilibrium method under various conditions, and the relationships between enzyme activity and structural changes are discussed.

MATERIALS AND METHODS

Human blood was obtained from the King County Blood Bank (Seattle, Washington). The blood (clotting was prevented by acid-citrate-dextrose, ACD Formula A) had been stored at 4°C for 2 to 4 weeks. Several milliliters of each blood specimen were hemolyzed separately and the enzymic activity and electrophoretic mobility in starch gel electrophoresis (14) were meas-
Ion Exchange—DEAE-cellulose (0.78 and 0.9 meq per g) and CM-cellulose (0.6 meq per g) were purchased from Bio-Rad. DEAE-Sephadex (A-50, 3.5 meq per g, 40- to 120-μ particle size) and CM-Sephadex (C-50, 4.5 meq per g, 40- to 120-μ particle size) were from Pharmacia, Uppsala.

Calcium Phosphate Gel—Hydroxyapatite was prepared by the method of Tiselius, Hjertén, and Levin (15).

Buffer—Phosphate buffer was Na₂HPO₄-KH₂PO₄; Tris buffer was Tris-HCl; acetate buffer was acetic acid-sodium acetate; glycine buffer was glycine-NaOH.

Chemicals—NAD, NADP, D-glucose 6-phosphate, D-galactose 6-phosphate, D-gluconate, D-glucose 1-phosphate, D-fructose 1-phosphate, D-fructose 1,6-diphosphate, and glucosamine 6-phosphate were obtained from Sigma, Calbiochem, Nutritional Biochemicals, and Mann. Guanidine hydrochloride and p-dimethylaminobenzaldehyde were recrystallized twice.

Glucose 6-Phosphate Dehydrogenase Assay—The dehydrogenase activity was measured by the initial rate of reduction of NADP at 25°. The increase of absorbance at 340 μM was recorded on a Gilford automatic recording spectrophotometer. The reaction mixture used for routine assay contained: 7 × 10⁻⁴ M NADP, 7 × 10⁻⁴ M D-glucose 6-phosphate, 7 × 10⁻³ M MgCl₂, 0.05 M Tris buffer (pH 8.0), and enzyme. In crude hemolysate, which contains both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP oxidoreductase decarboxylating, EC 1.1.1.44), the difference of the reduction rate of NADP between the reaction mixture containing 7 × 10⁻⁴ M D-glucose 6-phosphate and 7 × 10⁻⁴ M 6-phospho-D-gluconate and the reaction mixture containing only 6-phospho-D-gluconate was used to calculate glucose 6-phosphate dehydrogenase activity (16). The compositions of the reactions mixtures used for determination of pH dependence, Michaelis constant, and substrate specificity are specified below.

Since the specific activity of the enzyme rapidly declined to a lower value when the enzyme was diluted below 1 mg of purified enzyme per ml (see "Inactivation of Glucose 6-Phosphate Dehydrogenase by Dilution"), it was practically impossible to examine all of the enzymic properties with fully active enzyme.

All the assays were made with the enzyme that had been diluted to 10 to 20 μg per ml in 0.01 M phosphate buffer (pH 7.2) or 0.05 M acetate buffer (pH 6.0) containing 5 × 10⁻⁴ M NADP, 10⁻⁴ M EDTA, and 10⁻⁵ M mercaptoethanol, and was kept at 0-°4 for several hours. After this incubation, the activity of the purified enzyme remained unchanged at least for 2 weeks at 0-°4 and was about 25% as active as fully active enzyme (see below). In studies on dilution effect and turnover rate, the assays were made immediately (within 5 sec) after dilution of a concentrated fully active enzyme solution (>5 mg per ml).

Enzyme activity is defined as micromoles of NADP reduced per min at 25°. Specific activity is the number of enzyme units per mg of protein. Protein was assayed by Lowry’s method (17), with crystalline bovine serum albumin as a standard.

Experiments and results

Isolation of Glucose 6-Phosphate Dehydrogenase

All the procedures were carried out in the cold or in an ice bath. All the buffer solutions used for the preparation contained 10⁻⁴ M mercaptoethanol, 10⁻³ M EDTA, and 2 × 10⁻⁵ M NADP, unless otherwise stated.

Hemolysis of Red Cells

About 2 liters of blood were centrifuged at 15,000 × g for 20 min, and supernatant plasma was removed by a pipette attached to an aspirator bottle. The red cells were washed twice with centrifugation with 2 volumes of 0.15 M NaCl containing 10⁻³ M EDTA, and were lysed by mixing with an equal volume of water containing 10⁻³ M EDTA, 10⁻³ M mercaptoethanol, and 50 ml of toluene. After vigorous shaking for a few minutes, they were centrifuged (15,000 × g for 30 min), and the hemolysate was collected by a pipette attached to an aspirator bottle. The precipitated debris was suspended in about 2 volumes of water and centrifuged. The extract was combined with the first hemolysates. Starting from 2 liters of whole blood, 2 to 2.5 liters of hemolysates were obtained.

DEAE-cellulose Column

Two to 2.5 liters of the hemolysates were placed on a DEAE-cellulose column (in a funnel with porous glass filter 10 cm in diameter) prepared from 100 g of DEAE-cellulose powder buffered with 0.005 M phosphate buffer (pH 6.4 to 6.5). The column was washed with 2 liters of 0.006 M phosphate buffer (pH 6.4 to 6.5). This procedure removed most of the hemoglobin and 6-phosphogluconate dehydrogenase, while glucose 6-phosphate dehydrogenase remained on the column. The enzyme was eluted with 2 liters of 0.1 M phosphate buffer (pH 5.8) containing 0.5 M NaCl. In order to prevent hydrolysis of the enzyme by contaminating proteolytic enzymes, diisopropylfluorophosphosphate (final concentration, 4 × 10⁻⁴ M) or e-amino-n-caproic acid (final concentration, 10⁻⁴ M) was added to the eluents. After adjusting the pH to 6.2 with 0.5 M NaHPO₄, solid ammonium sulfate (350 g per liter) was added to the eluents. The process from the beginning of washing of the red cells to the precipitation of the enzyme by ammonium sulfate was completed within 12 hours. After being kept in the cold overnight, the precipitate was collected by centrifugation (20,000 × g for 30 min).

Carboxymethyl Cellulose Column Chromatography

The precipitate obtained in the previous step (from a total about 11 liters of blood) was suspended in about 250 ml of 0.005 M phosphate buffer (pH 5.8), dialyzed against the same buffer, and centrifuged (37,000 × g for 15 min). The supernatant solution (total, about 350 ml) was placed on a CM-cellulose column (5 × 35 cm) buffered with the same buffer as above. The enzyme was eluted with increasing concentrations of NaCl from 0 to 0.3 M and increasing pH. The gradient was produced by adding 0.05 M phosphate buffer (pH 6.4) containing 0.3 M NaCl into a mixing chamber which contained 1 liter of 0.005 M phosphate buffer (pH 5.8) (fixed initial buffer volume). The flow rate was 1 ml per min. An elution pattern is shown in Fig. 1. The major enzyme fraction was treated with ammonium sulfate (380 g per liter).

First Calcium Phosphate Gel Column Chromatography

The precipitate obtained in the previous step was suspended in 0.01 M phosphate buffer (pH 6.8) and dialyzed against the same buffer. The dialyzed solution (75 ml) was placed on a calcium
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**Fig. 1.** Elution pattern from a CM-cellulose column. Partially purified glucose 6-phosphate dehydrogenase (obtained through a DEAE-cellulose column) was placed on a CM-cellulose column (5 x 35 cm) and eluted with increasing pH and concentration of NaCl. •—•, absorbance; ▲—▲, dehydrogenase activity.

**Fig. 2.** Elution pattern from a calcium phosphate gel column. Partially purified glucose 6-phosphate dehydrogenase (peak from a CM-cellulose column) was placed on a calcium phosphate gel column (2.5 x 30 cm) and eluted with phosphate buffer (pH 6.8) of increasing concentration. •—•, absorbance; ▲—▲, dehydrogenase activity.

**Fig. 3.** Elution pattern from a DEAE-Sephadex column. Partially purified glucose 6-phosphate dehydrogenase (peak from first calcium phosphate gel column) was placed on a DEAE-Sephadex column (1.5 x 35 cm) and eluted with increasing concentration of NaCl. •—•, absorbance; ▲—▲, dehydrogenase activity.

**Fig. 4.** Elution pattern from a calcium phosphate gel column. Partially purified glucose 6-phosphate dehydrogenase (peak from first DEAE-Sephadex column) was placed on a calcium phosphate gel column (1.5 x 30 cm) and eluted with phosphate buffer (pH 6.8) of increasing concentration. •—•, absorbance; ▲—▲, dehydrogenase activity.

Phosphate gel column (2.5 x 30 cm) buffered with 0.01 M phosphate buffer (pH 6.8), and was eluted with phosphate buffer (pH 6.8), the concentration of which was gradually increased from 0.01 M to 0.2 M. The gradient was produced by adding 0.2 M buffer to a mixing chamber which contained 500 ml of 0.01 M buffer (fixed initial buffer volume). The flow rate was 30 ml per hour. An elution pattern is shown in Fig. 2. Glucose 6-phosphate dehydrogenase was eluted at phosphate buffer concentration ranging from 0.065 M to 0.12 M. The bulk of the enzyme fraction was precipitated with ammonium sulfate (350 g per liter).

**First DEAE-Sephadex Column**

The precipitate obtained in the previous step was suspended in 0.02 M phosphate buffer (pH 6.4) and dialyzed against the same buffer. The dialyzed solution (35 ml) was placed on a DEAE-Sephadex column (1.5 x 35 cm) buffered with 0.02 M phosphate buffer (pH 6.4) and was eluted with increasing concentrations of NaCl. The gradient was produced by adding 0.02 M phosphate buffer (pH 6.4) containing 0.25 M NaCl to a mixing chamber which contained 400 ml of 0.02 M phosphate buffer (pH 6.4) containing 0.05 M NaCl (fixed initial buffer volume).

An elution pattern is shown in Fig. 3. This procedure removed the bulk of yellow material and the enzyme fraction became almost colorless. The dehydrogenase fraction was precipitated with ammonium sulfate (350 g per liter).

**CM-Sephadex Column Chromatography**

The enzyme precipitate was dissolved in 0.005 M phosphate buffer (pH 5.8) and dialyzed against the same buffer. The dialyzed solution (12 ml) was placed on a CM-Sephadex column...
(1 × 30 cm) buffered with 0.005 M phosphate buffer (pH 5.8), and was eluted with increasing concentrations of NaCl and rising pH. The gradient was produced by adding 0.02 M phosphate buffer (pH 6.4) containing 0.15 M NaCl to a mixing chamber which contained 400 ml of 0.005 M phosphate buffer (pH 5.8) (fixed initial buffer volume). Flow rate was 15 ml per hour. The enzyme was eluted between 100 and 400 ml of effluent. This fraction was collected and treated with ammonium sulfate (350 g per liter).

Second Calcium Phosphate Gel Column Chromatography

The precipitate was dissolved in 0.01 M phosphate buffer (pH 6.8) and dialyzed against the same buffer. The dialyzed solution (about 15 ml) was placed on a calcium phosphate gel column (1.5 × 30 cm) buffered with 0.01 M phosphate buffer (pH 6.8), and was eluted by increasing concentrations of buffer. The gradient was produced by adding 0.2 M phosphate buffer (pH 6.8) to a mixing chamber which contained 500 ml of 0.01 M phosphate buffer (pH 6.8) (fixed initial buffer volume). Flow rate was 20 ml per hour. An elution pattern is shown in Fig. 4. The enzyme fraction was treated with ammonium sulfate (350 g per liter). After this stage of purification, the protein was easily denatured on the surface of glassware and in foam. All glassware, including the chromatographic column, was siliconized by treatment with dimethyldichlorosilane (Applied Science Laboratories). The enzyme solution was treated extremely gently, avoiding foam as much as possible.

Fractionation with Ammonium Sulfate

The precipitate obtained in the previous stage was dissolved in the smallest feasible amount of 0.05 M phosphate buffer (pH 6.4), and a small amount of insoluble material was removed by centrifugation. Saturated ammonium sulfate solution was added drop by drop to the supernatant solution until the solution became slightly turbid. After keeping overnight, the suspension was centrifuged. Most of the enzyme activity remained in the supernatant, and a small amount of inactive precipitate was discarded again by centrifugation.

Saturated ammonium sulfate was added to the supernatant solution until it became slightly turbid, and it was kept at room temperature for a few hours. The precipitate, which contained about 70% of the activity, was collected by centrifugation at room temperature (first precipitate). Saturated ammonium sulfate solution was added to the supernatant solution until it became slightly turbid at 0°C. The enzyme was collected by centrifugation after keeping the solution at room temperature for a few hours (second precipitation). Both precipitates were combined and used for further purification.

The specific activity of the enzyme at this stage of purification (specific activity of fully active enzyme was more than 570, and specific activity after partial inactivation by dilution was 127 to 140) was much higher than that of the most highly purified preparation so far reported (113 units), which was assumed to be about 80% pure (9). The enzyme preparation showed a single protein band on disc electrophoresis (Canalco model 12) in polyacrylamide gel. Homogeneity of the enzyme preparation was examined by analytical centrifugation in a Spinco model E centrifuge. The sedimentation pattern indicated that the preparation contained a major high molecular weight fraction together with a minor lower molecular size fraction.

FIG. 5. Elution pattern from a DEAE-Sephadex column. Partially purified glucose 6-phosphate dehydrogenase (peak from second calcium phosphate gel column) was placed on a DEAE-Sephadex column (1 × 30 cm) and eluted with increasing concentration of NaCl. ——, absorbance; ▲—▲, dehydrogenase activity.

Second DEAE-Sephadex Column Chromatography

The precipitate obtained in the previous step was dissolved in 0.02 M Tris buffer (pH 8.0), and the solution was dialyzed against the same buffer and placed on a DEAE-Sephadex column (1 × 30 cm) buffered with 0.02 M Tris buffer (pH 8.0). The enzyme was eluted by a gradient obtained by adding the buffer containing 0.5 M NaCl to a mixing chamber which contained 400 ml of the buffer (fixed initial buffer volume). An elution pattern is shown in Fig. 5. The major dehydrogenase fraction (260 to 325 ml of the effluent) was precipitated by ammonium sulfate (350 g per liter).

Crystallization and Precipitation by Ammonium Sulfate

The precipitate was dissolved in about 1 ml of 0.05 M acetate buffer (pH 6.0) and a small amount of insoluble material was discarded after centrifugation. Saturated ammonium sulfate solution was added to the supernatant until the solution became turbid. The suspension was kept at room temperature for a few hours, and centrifuged at room temperature. The supernatant, which contained less than 2% of the total activity, was discarded. The precipitate was dissolved in the smallest feasible amount of 0.05 M acetate buffer (pH 6.0) and ice-cold saturated ammonium sulfate solution was added drop by drop until the solution became slightly turbid. The enzyme suspension was kept in an ice bath for a few days. A silky sheen appeared and fairly homogeneous tiny needle crystals were observed (Fig. 6).

However, more than 70% of the enzyme activity still remained in the supernatant. If more ammonium sulfate solution was added or if the supernatant was kept at room temperature for several hours in order to precipitate all the enzyme, the precipitate must be essentially the identical protein.

The specific activity of the enzyme at this stage of purification (specific activity of fully active enzyme was more than 570, and specific activity after partial inactivation by dilution was 127 to 140) was much higher than that of the most highly purified preparation so far reported (113 units), which was assumed to be about 80% pure (9). The enzyme preparation showed a single protein band on disc electrophoresis (Canalco model 12) in polyacrylamide gel. Homogeneity of the enzyme preparation was examined by analytical centrifugation in a Spinco model E centrifuge. The sedimentation pattern indicated that the preparation contained a major high molecular weight fraction together with a minor lower molecular size fraction.
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Figure 6. Micrograph of glucose 6-phosphate dehydrogenase crystals (see text). $\times \sim 650$. 

Measurement of Sedimentation Constant

Ultracentrifugation experiments were carried out in a Spinco model E centrifuge. The protein was dissolved in 0.05 M acetate buffer (pH 6.0) containing $10^{-3}$ M EDTA, $10^{-3}$ M mercaptoethanol, and $2 \times 10^{-4}$ M NADP at a concentration of about 0.3%. Schlieren patterns of the enzyme showed single and symmetrical sedimentation boundaries (Fig. 7).

The sedimentation constant ($s_{20, w}$) was calculated as 10.0 S.

Measurement of Molecular Weight

The molecular weight of the enzyme and subunit molecular size were determined by the sedimentation equilibrium method (18) with the use of Rayleigh interference optics in a Spinco model E centrifuge.

The protein solutions used for the determination were as follows.

- **Solution I**—The enzyme was dissolved in 0.05 M acetate buffer (pH 6.0) containing $10^{-3}$ M EDTA, $10^{-3}$ M mercaptoethanol, and $2 \times 10^{-4}$ M NADP, and dialyzed against the same buffer. The concentration of the protein was 0.015% and 0.008%, and its specific activity was 170 to 180.

- **Solution II**—The enzyme solution (about 8 mg per ml) was mixed with 10 volumes of ice-cold 60% saturated ammonium sulfate containing 0.05 M H$_2$SO$_4$. After keeping for 10 min at 0°, the mixture was centrifuged. The precipitate was suspended in the same ammonium sulfate solution as above, kept for 10 min at

**Table I**

<table>
<thead>
<tr>
<th>Steps of purification</th>
<th>Total volume</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity of glucose-6-P dehydrogenase</th>
<th>Accumulative purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysate</td>
<td>~15 x 10$^8$</td>
<td>2.06 x 10$^8$</td>
<td>5.75 x 10$^8$</td>
<td>2.1 x 10$^2$</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-cellulose column effluent</td>
<td>~1.3 x 10$^8$</td>
<td>24.6 x 10$^8$</td>
<td>5.5 x 10$^8$</td>
<td>&lt;100</td>
<td>82</td>
</tr>
<tr>
<td>Ppt with (NH$_4$)$_2$SO$_4$ and resuspended in buffer</td>
<td>300</td>
<td>13.7 x 10$^8$</td>
<td>5.5 x 10$^8$</td>
<td>7.5 x 10$^2$</td>
<td>142</td>
</tr>
<tr>
<td>CM-cellulose column effluent</td>
<td>65</td>
<td>5.35 x 10$^8$</td>
<td>3.9 x 10$^8$</td>
<td>14 x 10$^2$</td>
<td>495</td>
</tr>
<tr>
<td>First calcium phosphate gel column effluent</td>
<td>5.5 x 10$^8$</td>
<td>3.7 x 10$^8$</td>
<td>3.7 x 10$^8$</td>
<td>4.02 x 10$^2$</td>
<td>6.72</td>
</tr>
<tr>
<td>First DEAE-Sephadex column effluent</td>
<td>1.85 x 10$^8$</td>
<td>3.5 x 10$^8$</td>
<td>3.5 x 10$^8$</td>
<td>6.22</td>
<td>2,350</td>
</tr>
<tr>
<td>CM-Sephadex column effluent</td>
<td>13</td>
<td>55</td>
<td>3.2 x 10$^8$</td>
<td>58</td>
<td>20,600</td>
</tr>
<tr>
<td>Ppt with (NH$_4$)$_2$SO$_4$ and dissolved in buffer</td>
<td>280</td>
<td>37</td>
<td>7.5 x 10$^8$</td>
<td>150</td>
<td>55,000</td>
</tr>
<tr>
<td>Second calcium phosphate gel column effluent</td>
<td>3.5</td>
<td>28</td>
<td>(3.7 x 10$^9$)$^a$</td>
<td>(74)$^a$</td>
<td>(26,100)$^a$</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ fractionation, dissolved in buffer</td>
<td>3 x 10$^8$</td>
<td>16 x 10$^8$</td>
<td>(3.5-4.1 x 10$^9$)$^a$</td>
<td>(127-146)$^a$</td>
<td>(45,000-51,500)$^a$</td>
</tr>
<tr>
<td>Second DEAE-Sephadex column effluent</td>
<td>65</td>
<td>18</td>
<td>3 x 10$^8$</td>
<td>570</td>
<td>32,300</td>
</tr>
<tr>
<td>Crystallization and precipitation</td>
<td>1.7</td>
<td>15.6</td>
<td>11.5 x 10$^8$</td>
<td>201,000</td>
<td>54,500</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2.8 x 10$^9$)$^a$</td>
<td>258,000</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Ratio of specific activity of the preparations taking that of hemolysate as 1.00.

$^b$ Activity measured after dilution inactivation (see text). The concentrated enzyme solution was diluted (100- or 200-fold) in a buffer and kept at 0°-4° for several hours before the activity measurement.
0°, and centrifuged. This procedure eliminates tightly bound NADP from the enzyme and reduces enzyme activity (13). In this particular instance, the specific activity was 70 (60% inactivation) at a protein concentration of 0.03% and 35 (80% inactivation) at a protein concentration of 0.003% after keeping the enzyme solution for 1 hour at room temperature. The precipitate was dissolved in 0.05 M acetate buffer (pH 6.0) and dialyzed against the same buffer. The protein concentrations used for the molecular weight measurement were about 0.02% and 0.008%.

Solution III—The protein was dissolved in 4 M guanidine hydrochloride solution containing 0.05 M acetate buffer (pH 6.0), 10-3 M EDTA, and 2 M mercaptoethanol, and dialyzed against the same solvent. In this solvent, the enzyme had no enzymic activity. The protein concentration used for the measurement was about 0.02%.

Molecular weight was calculated by the equation (18)

\[ M = \frac{2.303 \times (d \log \Delta y/dr) mRT}{rAx \log (1 - \epsilon p)} \]

where \( R \) = gas constant, \( T \) = absolute temperature, \( m \) = magnification factor, \( rAx \) = radius to middle point of fringes, \( \epsilon \) = partial specific volume of protein, \( \rho \) = density of solvent, \( \omega \) = angular velocity, and \( d \log \Delta y/dr \) = slope of logarithm of fringe displacement with respect to radius.

If a given protein solution is homogeneous, plots of \( \log \Delta y \) against radius should be linear. If protein molecules reversibly dissociate into small subunits at a range of concentration used for measurement, the plots should be curvilinear. Higher \( d \log \Delta y/dr \) values should be obtained at larger rather than at smaller \( r \) values. Because the protein solution is more concentrated near the bottom of the cell, a greater fraction of the molecules should associate. By contrast, in the upper layer of the cell, the protein solution is more diluted and a greater part of the molecule should be in the dissociated form.

Some examples of the relationships between the logarithm of the fringe displacement and the radius obtained from the interference patterns are shown in Fig. 8, A to C. The interference patterns obtained in the solution of the native enzyme (Solution I described above) and the solution of the denatured enzyme (Solution III described above) showed no concentration dependence and indicated a high degree of homogeneity of protein size.

In protein Solution I, the molecular weight was estimated as 240,000 ± 8,000, based on a partial specific volume value of 0.731 ml per g, which was calculated for the amino acid composition of the enzyme according to the method of Cohn and Edsall (19).

In protein Solution III, the molecular weight was estimated as approximately 43,000 ± 3,000, correcting change of partial specific volume value due to denaturation and unfolding in guanidine-HCl (0.721 ml per g with correction) and amount of guanidine-HCl bound with protein (about 5%) (20). This result, together with the molecular weight value of undenatured active enzyme (240,000), indicates that the enzyme consists of six similar size subunits.

The interference patterns of the partially inactivated enzyme treated by acidic ammonium sulfate solution showed concentration dependence (Fig. 8B). The molecular weight was estimated as about 180,000 ± 10,000 near the bottom of the cell and as about 123,000 ± 10,000 in the upper layer of the cell. Thus the results indicate that removal of the bound NADP induces dissociation of the enzyme accompanied by inactivation.

Amino Acid Composition

The purified enzyme was dialyzed against water and lyophilized. The protein was hydrolyzed for 20 hours at 110° in hydrochloric acid of constant boiling point in an evacuated, sealed tube. The hydrolysates were evaporated to dryness under reduced pressure in the presence of NaOH.

In order to estimate the cystine (or cysteine) content, a sample of oxidized protein was subjected to amino acid analysis. The procedure used for the oxidation of the protein with performic acid was essentially that described by Schram, Moore, and Bigwood (21). After oxidation, the reaction mixture was diluted with 10 volumes of cold water, and the solution was immediately lyophilized. The samples were dissolved in a small amount of water, and the solution was again lyophilized and hydrolyzed as described above.

The amino acid analysis was performed with a Technicon amino acid analyzer.

Tryptophan was estimated by p-dimethylaminobenzaldehyde method (22, 23).

The amino acid composition of the enzyme is presented in Table II. The number of amino acid residues in 1 enzyme molecule (mol wt 240,000) and the number of amino acid residues per subunit, assuming tentatively that the enzyme consists of six identical subunits, are also given in the table.

Fig. 7. Schlieren pattern of purified glucose 6-phosphate dehydrogenase in the centrifuge at 50,740 rpm 32 min after reaching final speed at 20°. The concentration of the protein was about 0.3% in 0.05 M acetate buffer (pH 6.0) containing 10-3 M EDTA, 10-3 M mercaptoethanol, and 2 × 10-4 M NADP.
Some of the enzymic properties of glucose 6-phosphate dehydrogenase from human red cells have been examined previously by several investigators (4, 25, 26) with the use of partially purified enzyme preparations. Although most of the reported properties are probably genuine characteristics of the enzyme, some fundamental properties, i.e. optimal pH, Michaelis constant, and substrate specificity, as well as unique phenomena of dilution inactivation of the enzyme, were examined in more detail with the purified homogeneous enzyme preparation.

**Optimal pH**—The effect of pH was examined with acetate buffer (pH 5.0 to 6.0), Tris buffer (pH 7.0 to 9.0), and glycine buffer (pH 9 to 10). Phosphate buffer was not used since it inhibited the reaction. As Fig. 9 shows, the optimal pH for the enzymic reaction was pH 8.0.

**Effect of Substrate Concentration**—The rate of the enzymic reaction was examined as a function of the concentration of NADP and glucose 6-phosphate. The usual Michaelis-Menten relationship was observed, since a Lineweaver-Burk plot (27) gave a straight line (Fig. 10). The apparent Michaelis constant for substrate was determined to be 0.1 M.

**Table II**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Percentage of amino acid residues</th>
<th>Assumed no. of residues per molecule</th>
<th>Assumed no. of residues per subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>10.85 ± 0.10</td>
<td>227</td>
<td>38</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.97 ± 0.17</td>
<td>49</td>
<td>16</td>
</tr>
<tr>
<td>Serine</td>
<td>4.07 ± 0.20</td>
<td>114</td>
<td>19</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>13.79 ± 0.16</td>
<td>286</td>
<td>43</td>
</tr>
<tr>
<td>Proline</td>
<td>5.82 ± 0.08</td>
<td>144</td>
<td>24</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.89 ± 0.05</td>
<td>164</td>
<td>27</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.88 ± 0.01</td>
<td>132</td>
<td>22</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>1.68</td>
<td>40</td>
<td>7</td>
</tr>
<tr>
<td>Valine</td>
<td>5.21 ± 0.01</td>
<td>126</td>
<td>21</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.86</td>
<td>52</td>
<td>9</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.37 ± 0.01</td>
<td>93</td>
<td>17</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.67 ± 0.23</td>
<td>193</td>
<td>32</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>8.48</td>
<td>180</td>
<td>30</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>6.46 ± 0.26</td>
<td>105</td>
<td>18</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.22 ± 0.09</td>
<td>117</td>
<td>19</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.06 ± 0.05</td>
<td>54</td>
<td>9</td>
</tr>
<tr>
<td>Arginine</td>
<td>7.02 ± 0.17</td>
<td>108</td>
<td>18</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>2.29</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Mean value and deviations of analysis of native and performic acid-oxidized protein. The contents of methionine sulfone and tyrosine of performic acid-oxidized protein have not been included in the mean value because of uncertainty of their recovery. Total of individual amino acid residues is taken as 100.

b The nearest integral number to the calculated number of amino acid residues per molecule based on molecular weight of 240,000.

c The nearest integrated number to the calculated number of amino acid residues per subunit, assuming tentatively that the enzyme consists of six identical subunits (see text).

d The recovery after hydrolysis is assumed to be 95% (24).

e The recovery after hydrolysis is assumed to be 90% (24).

f Calculated from the cysteic acid content in performic acid-oxidized protein. The recovery of cysteic acid is assumed to be 95% (21).

g Estimated by p-dimethylaminobenzaldehyde method.

h Not included in the total.
of enzyme. The molecular weight of the enzyme is about 240,000, the maximal turnover weight of the enzyme is about 2.2 X 10^5 moles of substrate per min per mole of contaminating in the samples. It was concluded that the apparent reduction of NADP should be attributed to D-glucose 6-phosphate itself. It was shown in Table III. Both D-galactose 6-phosphate and glucose 6-phosphate are fairly good substrates at the same concentration as 100%.

**Specific Activity and Rate of Turnover**—The specific activity of the fully active enzyme (see “Inactivation of Glucose 6-Phosphate Dehydrogenase Dilution”) measured in the reaction mixture containing 7 X 10^{-4} M D-glucose 6-phosphate, 7 X 10^{-4} M NADP, 7 X 10^{-3} M MgCl_2, and 0.1 M Tris buffer (pH 8.0) was 750. Based on this value, together with minor correction because of substrate concentration dependence described above, the approximate maximum specific activity that would obtain at optimal pH and under conditions of saturation of the enzyme with substrates was estimated as 900 at 25°. Since the molecular weight of the enzyme is about 240,000, the maximal turnover number is about 2.2 X 10^9 moles of substrate per min per mole of enzyme.

**Specificity of Substrate Analogues**—Among a number of carbohydrate phosphates examined, only D-galactose 6-phosphate and 2-deoxy-D-glucose 6-phosphate were oxidized by the enzyme. The Michaelis constants and relative maximum reaction rates estimated from Lineweaver-Burk plots for these compounds are shown in Table III. Both D-galactose 6-phosphate and 2-deoxy-D-glucose 6-phosphate were fairly good substrates at higher concentrations since their maximum reaction rates were 45% and 12% of D-glucose 6-phosphate. However, their Michaelis constants were much higher (8.0 X 10^{-3} M and 6.0 X 10^{-2} M) than that for D-glucose 6-phosphate (3.9 X 10^{-4} M). Thus, at lower concentrations, their reaction rates were very low. For example, relative rates were 2.4% for D-galactose 6-phosphate and 3.1% for 2-deoxy-D-glucose 6-phosphate at a concentration of 7 X 10^{-4} M, taking the rate of D-glucose 6-phosphate at the same concentration as 100%.

None of the following compounds were used as substrates: 6-phosphogluconate, D-glucose 1-phosphate, D-fructose 1-phosphate, D-fructose 1,6-diphosphate, D-glucoammine 6-phosphate.

NAD was a weak substrate for the dehydrogenase. In contrast to NADP, the reaction rate for NAD was reduced about 40% in the presence of 7 X 10^{-3} M MgCl_2. The Michaelis constant and relative maximum reaction rate for NAD are shown in Table III.

**Inactivation of Glucose 6-Phosphate Dehydrogenase by Dilution**

As has been described in the section of purification procedures, the enzyme showed higher specific activity (and total activity) when it was more concentrated. This phenomenon was examined in more detail by the use of purified homogeneous enzyme preparation.

When the enzyme was diluted in a buffer (pH 6.0 to 8.0, acetate or Tris buffer) from a concentrated solution (more than 5 mg of enzyme per ml) to give a concentration less than 0.1 mg per ml, the specific activity decreased. The decrease was more rapid and slightly more severe at 0° than at 25° (Fig. 11). After a certain period of time in dilute solution, some of the lost activity.

**Table III**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Michaelis constant (K_m)</th>
<th>V_max (relative to glucose 6-phosphate)</th>
<th>V_max (relative to NADP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose 6-phosphate</td>
<td>3.9 X 10^{-4}</td>
<td>100</td>
<td>4.4 X 10^{-4}</td>
</tr>
<tr>
<td>D-Galactose 6-phosphate</td>
<td>8.0 X 10^{-4}</td>
<td>4b</td>
<td>4.4 X 10^{-4}</td>
</tr>
<tr>
<td>2-Deoxy-D-glucose 6-phosphate</td>
<td>6.0 X 10^{-3}</td>
<td>12</td>
<td>4.4 X 10^{-4}</td>
</tr>
<tr>
<td>NADP</td>
<td>4.0 X 10^{-3}</td>
<td>100</td>
<td>4.4 X 10^{-4}</td>
</tr>
<tr>
<td>NAD</td>
<td>4.0 X 10^{-3}</td>
<td>4.7</td>
<td>4.4 X 10^{-4}</td>
</tr>
</tbody>
</table>

*The reaction mixture contained 0.1 M Tris buffer (pH 8.0), 7 X 10^{-3} M MgCl_2, 7 X 10^{-2} M NADP, enzyme, and various concentrations of glucose 6-phosphate or its analogues.

The reaction mixture contained 0.1 M Tris buffer (pH 8.0), 7 X 10^{-3} M MgCl_2, 7 X 10^{-4} M d-glucose 6-phosphate, enzyme, and various concentrations of NADP.

The reaction mixture contained 0.1 M Tris buffer (pH 8.0), 7 X 10^{-4} M glucose 6-phosphate, enzyme, and various concentrations of NAD (without MgCl_2).
Glucose 6-Phosphate Dehydrogenase from Human Erythrocytes

DISCUSSION

Since the discovery of glucose 6-phosphate dehydrogenase (Zwischenferment) by Warburg and Christian in 1931 (28), the enzyme has been purified to varying degrees from a variety of sources. The enzyme has been crystallized from yeast (specific activity, 680) (29) and bovine udder (specific activity, 68) (30). However, the latter was not homogeneous judging from analytical ultracentrifugation. Homogeneity and molecular size (NADP-free enzyme, mol wt 102,400 ± 2,400 at infinite dilution) of the yeast crystalline enzyme were reported recently (31).

Kirkman (7) and Chung and Langdon (9) attempted to purify the enzyme from human red cells. The best preparation so far reported had a specific activity of 113, but the yield was only several per cent (9).

In this work, particular attention was paid to avoid hydrolysis and inactivation of the enzyme by proteolytic enzymes (the major proteolytic enzyme in hemolysates is plasmin) by diisopropylfluorophosphate and ε-aminocaproic acid in the process of purification and to improve the yield of the enzyme. Thus, after more than 20 steps of purification, a homogeneous enzyme preparation (specific activity, 750 in fully active state, 170 to 180 after dilution inactivation) was obtained in an over-all yield of about 50%.

Controversial results have been reported regarding the molecular size of glucose 6-phosphate dehydrogenase of human red cells. Kirkman and Hendrickson (12), using fairly crude enzyme preparations (0.3 to 0.6 unit per mg of protein), estimated the molecular size from the relative rate of sedimentation in sucrose gradient centrifugation and relative rate of diffusion through sintered glass, together with an arbitrary assumed value of partial specific volume of the enzyme: the molecular weight of the active NADP-bound enzyme was estimated as 105,000 and that for inactive (or less active) subunit as about one-half. Tsutsui and Marks (11) also carried out sucrose gradient centrifugation of the enzyme in the presence and absence of NADP. Their results were similar to those of Kirkman and Hendrickson (12).

On the other hand, Chung and Langdon (9, 13), using a more purified enzyme preparation (up to 113 units per mg of protein), estimated the molecular weight as 190,000 from a rough estimation of the sedimentation constant by ultracentrifugation and the diffusion constant by the porous diaphragm method. They observed that removal of NADP resulted in dissociation of the enzyme into inactive subunits with a molecular weight of about one-half of the native protein.

This discrepancy could be settled in the present work by more accurate measurement of molecular size of the enzyme under various conditions. Dilution of human red cell glucose 6-phas-
phate dehydrogenase below certain concentrations caused partial inactivation of the enzyme (75% inactivation of concentrations less than 0.3 mg per ml) (Fig. 12). This inactivation was entirely different from the inactivation of the enzyme due to removal of NADP (10-13), since NADP had no effect on the former inactivation, while the latter was prevented and reversed by NADP.

The molecular weight of the enzyme measured by the sedimentation equilibrium method at concentrations of 0.2 mg and 0.08 mg per ml was about 240,000. The interference patterns showed no concentration dependence. This finding, and the sedimentation constant (10.0 S), indicated a value of 240,000 for the molecular weight of the fully active enzyme as well as of the partially inactive enzyme. It is unlikely that some metal contamination caused partial inactivation, since EDTA, well as of the partially inactive enzyme. It is unlikely that some metal contamination (820, 0.08 mg per ml was about 240,000. The interference patterns showed no concentration dependence. This finding, and the sedimentation constant (10.0 S), indicated a value of 240,000 for the molecular weight of the fully active enzyme as well as of the partially inactive enzyme. It is unlikely that some metal contamination caused partial inactivation, since EDTA, reducing agents, and various buffers showed no effect.

The partial inactivation by dilution which is NADP-independent may be attributed to structural change of the protein without dissociation into subunits. It should be mentioned that a similar phenomenon, i.e. partial inactivation by dilution without dissociation into subunits, has been observed in Bacillus subtilis l-alanine dehydrogenase (32) and l-lactate dehydrogenase (33).

If one assumes the shape of the protein to be a prolate ellipsoid, one can calculate its major dimensions from the molecular weight (240,000), the partial specific volume (0.731 ml per g), and the sedimentation constant (10.0 S). The major axes are then 30 and 4.3 nm, respectively.

The inactivation by removal of bound NADP can be attributed to reversible dissociation of the protein into subunits, since the molecular weight was estimated as 123,000 ± 10,000 in the upper layer of the cell and as 180,000 ± 10,000 near the bottom of the cell after removal of NADP from the enzyme.

The molecular weight value observed in guanidine-HCl (43,000 ± 3,000) indicates that the enzyme consists of six similar size subunits. It is not yet known whether or not these subunits are identical. This problem may be elucidated by peptide mapping of tryptic hydrolylates of the enzyme, which is under study in conjunction with investigations of genetically altered enzyme (A+). The number of tryptic peptides and the frequencies of lysine and arginine in the molecule (Table II) will give further information.

Considering the results of this work and the results reported by previous investigators, the following relationship between activity and configuration of the enzyme may be suggested.

The fully active enzyme (Hexamer I, mol wt 240,000), which has probably six tightly bound NADP per molecule, consists of six similar size subunits. By dilution, Hexamer I dissociates into two partially active (specific activity, less than 20%) subunits with bound NADP (Trimer I), which eventually reassociate to form a new partially active (specific activity, 25%) hexamer (Hexamer II). At higher concentrations (>3 mg per ml), Hexamer I is predominant, while at lower concentrations (0.007 to 0.3 mg per ml) most of the enzyme molecules are partially active Hexamer II. Hexamer I can be detected only in extremely diluted solution (<10-4 mg per ml), such as that used by Kirkman and Hendrickson (12) and Tsutsui and Marks (11) for sucrose gradient centrifugation.

Removal of bound NADP from the enzyme by acidic ammonium sulfate treatment and dilution to concentrations of <0.1 mg per ml induces dissociation of the enzyme into inactive (or very weakly active) trimers without bound NADP (Trimer II). At higher concentrations (>0.2 mg per ml), a partially active (<170 units per mg) hexamer without bound NADP (Hexamer III) is predominant (Fig. 8B). Higher temperature (25°C) rather than low temperature (0°C) favors this formation of partially active hexamers from trimers. When Trimer II is further diluted, it may dissociate into smaller subunits (12). These relations are summarized schematically in Fig. 13.

About 20 mmoles of NADP (13). From these values, one can estimate that 1 enzyme molecule (mol wt 240,000; stationary specific activity, 170 to 180) contains 4 to 5 NADP molecules. According to Kirkman (7), 0.13 to 0.15 × 10^9 units of enzyme contained 1 mole of NADP. This indicates that 1 enzyme molecule has about 6 NADP molecules.
Molecular size of glucose 6-phosphate dehydrogenase from other mammalian species seems to be similar to that of human enzyme, since Nevuldine and Levy (34), using gel filtration methods, estimated the molecular weight of rat mammary enzyme as about 130,000 in the dilute state and as about a quarter million at higher concentrations (>1.9 mg of protein per ml) in the presence of NADP.

It should be mentioned that human glucose 6-phosphate dehydrogenase of various tissues including erythrocytes is under control of a single structural gene linked to the sex chromosome (35); accordingly, glucose 6-phosphate dehydrogenase from various tissues should be structurally identical.

Michaelis constants of major substrates and relative rate of utilization of substrate analogues measured for the homogeneous enzyme as about 130,000 in the diluted state and as about a quarter million at higher concentrations (>1.9 mg of protein per ml) in the presence of NADP.

The specific activity of fully active human glucose 6-phosphate dehydrogenase (750) was similar to that of the crystalline enzyme from bovine mammary gland, which had a specific activity of 68 (30). Although the bovine enzyme has been obtained in crystalline form, it was not homogeneous in analytical centrifugation (30). At the same time, since the activity of the enzyme is so sensitive to variations in its environment, the activity of bovine enzyme might have been measured in a partially inactivated state. True specific activity of bovine enzyme may be of the same order as that of human enzyme.

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