The Reduced Pyridine Nucleotide Dehydrogenases of Human Erythrocytes

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Kiese (1) reported the presence of an enzyme in erythrocytes that would catalyze the reduction of methemoglobin by reduced triphosphopyridine nucleotide in the presence of methylene blue. This enzyme was purified further by Huennekens et al. (2) and reported to be active both with TPNH and, to a lesser extent, with DPNH. It was postulated that this enzyme was responsible for the physiological maintenance of hemoglobin in the red cell in the reduced state, on the assumption that some unknown electron carrier was present in red cells that could act in the same manner as methylene blue does in the assay system.

Sharbo and Falcione (3) purified this TPNH dehydrogenase further and reported it to be essentially free of heme protein.

A DPNH dehydrogenase with methemoglobin reductase activity has been isolated from normal human red cells (4). This enzyme is lacking in red cells of persons with hereditary methemoglobinemia (5). A second DPNH dehydrogenase, prepared from methemoglobinemic red cells, has quite different properties from the DPNH dehydrogenase of normal cells, as well as being present in lesser amount (6).

Evidence will be presented here that there are four reduced pyridine nucleotide dehydrogenases in human red cells: TPNH dehydrogenase A, TPNH dehydrogenase B, DPNH dehydrogenase I, and DPNH dehydrogenase II. The two TPNH dehydrogenases resemble each other closely, but are separable by chromatography. DPNH dehydrogenase I is the major "dihydrogenase" of human red cells previously described (4), while DPNH dehydrogenase II is a minor component previously purified from methemoglobinemic cells (6).

Described here are the preparation of two TPNH dehydrogenases from normal human red cells. Certain samples of these preparations contained considerable DPNH dehydrogenase activity. The properties of these four dehydrogenases were compared and their possible role in methemoglobin reduction was estimated.

**Experimental Procedure**

**Enzyme Assay**—TPNH dehydrogenase was determined at 30° in 3 ml of solution containing 0.06 μmole of 2,6-dichlorophenolinodophenol, 50 μmoles of Tris-HCl, pH 7.5, 1 μmole of EDTA, 500 μmoles of KCl, 0.2 μmole of TPNH, and enzyme. Absorbance at 600 μm was measured for 10 minutes. Under these conditions the rate of reaction was nearly zero order with respect to dye concentration. Rate of reaction was defined as the initial reaction rate in absorbance change per minute minus the reaction rate found for the same system without enzyme. A unit of activity was that amount of enzyme calculated to give a change in absorbance of 1 unit per minute; specific activity was units of enzyme per mg of protein.

DPNH dehydrogenase activity was determined in the same way except that KCl was not added to the system. Methemoglobin reduction was determined in 0.15 ml of solution at 38° containing 36 μg of methemoglobin, 20 μmoles of potassium phosphate, pH 7.35, 2 μmoles of TPNH or DPNH, and enzyme. Aliquots of 0.02 ml were withdrawn at 20-minute intervals, and methemoglobin was determined by the method of Evelyn and Maltoly (7). Cytochrome c reduction was determined by measuring absorbance at 550 μm in 3 ml of solution containing 0.12 μmole of cytochrome c, 50 μmoles of Tris-HCl (pH 7.55), 500 μmoles of KCl, 1 μmole of EDTA, 0.2 μmole of TPNH, and enzyme at 30°.

Purified DPNH dehydrogenase I was prepared as previously described (4). Protein was determined spectrophotometrically (8), with a correction applied for heme proteins (4). Flavin was determined by fluorophotometry (9). Methemoglobin was crystallized from human red cells after treatment with nitrite.

**Purification of Enzyme**—Cells from outdated blood from a blood bank were washed three times with an equal volume of buffered sodium chloride solution (1 volume of 0.1 m potassium phosphate, pH 7.4, plus 9 volumes of 0.9% NaCl) and were frozen until used. All subsequent operations except chromatography were carried out at 5°. All Tris-HCl buffers used were at pH 8.65, and all contained 1 mM EDTA.

To 2 liters of washed cells were added 38 liters of water. The solution was adjusted to pH 7.4 with 2 m Tris, and 11.2 kg of (NH₄)₂SO₄ were added. The solution was filtered through a Sharples centrifuge and the residue in the bowl was discarded. Then 4.2 kg of (NH₄)₂SO₄ were added to the solution, and it was again filtered through the Sharples centrifuge. The residue in the bowl was dissolved in 350 ml of 0.1 m Tris-HCl. This solution was centrifuged at 30,000 × g, and the precipitate was discarded; the supernatant liquid was designated Fraction 2.

Fraction 2 was dialyzed for 5 hours in 2 liters of 0.01 m Tris-HCl; this dialysis was repeated for a second 5-hour period in fresh 0.01 m Tris-HCl. Potassium sulfate (200 ml of a 1.0% solution) was added, and the solution was diluted to 1500 ml. The precipitate was removed by centrifugation and was discarded. To the solution were added 420 g of (NH₄)₂SO₄; after centrifugation, the precipitate was discarded. Then 157 g of (NH₄)₂SO₄ were added, and the solution was centrifuged. The precipitate was dissolved in 20 ml of 0.1 m Tris-HCl to give Fraction 3.

Fraction 2 was dialyzed for 5 hours in 1 liter of 0.01 m Tris-HCl.
HCl, and the chromatography was repeated on a Ca₃(PO₄)₂ column, 0.5 cm in diameter and 5 cm deep. To the column were added successively 1 ml of 0.01 M Fraction 4B. The second TPNH dehydrogenase was treated similarly to give a precipitate between 50 and 70% saturation with (NH₄)₂SO₄. Fractions of 2 ml each were collected. Those fractions with specific activity greater than 5 were combined, and the enzyme was precipitated between 55 and 70% saturation with (NH₄)₂SO₄. The precipitate was dissolved in 1 ml of 0.1 M Tris-HCl to give Fraction 5B. The purification procedure is summarized in Table I.

### RESULTS

#### Differentiation of Dehydrogenases

The two TPNH dehydrogenases were closely similar in properties except for the difference in chromatographic behavior. To test the possibility that the separation might be the result of overloading the column, two columns were prepared and half as much Fraction 3 was added to one as to the other. The recovery of enzyme from 1 volume of Fraction 3 was 11.6 units of dehydrogenase A and 23.0 units of dehydrogenase B, and from 0.5 volume of Fraction 3, 5.7 units of dehydrogenase A and 16.0 units of dehydrogenase B.

The two DPNH dehydrogenases differed not only in chromatographic behavior but also in kinetic properties. The effects of pH, temperature, and substrate concentration on the two enzymes were quite different.

The ratios of activity with deaminodihydrolipoamide DPNH to that with DPNH were: DPNH dehydrogenase I, 0.97, and DPNH dehydrogenase II, 0.15. The activity of purified DPNH dehydrogenase I with TPNH was only 1% of that with DPNH (4). We were not able to prepare DPNH dehydrogenase II that was similarly free of TPNH activity, although TPNH dehydrogenase B fractions were obtained which had a ratio of activity with DPNH to that with TPNH of 0.01. This ratio in various TPNH dehydrogenase B preparations varied from 0.01 to 0.60.

The data on DPNH dehydrogenase II described above were obtained on an enzyme preparation (Fraction 5B) with a DPNH:TPNH ratio of 0.48. Both TPNH dehydrogenase fractions were red and both absorbed strongly at 410 nm. From the ratio of absorbance at 410 and 280 nm, 12% of the protein of dehydrogenase A and 47% of the protein in dehydrogenase B were calculated to be heme protein. TPNH dehydrogenase A contained no more than 1 mole of flavin per 6.8 x 10⁶ g of protein. TPNH dehydrogenase B contained 15 times this amount of flavin, but this could be adsorbed to a small amount of glutathione reductase which was present (11). Enzyme prepared by the method of Huennekens et al. (2) was similar in properties to dehydrogenase B.

#### Specificity

None of the four enzymes had dehydrogenase activity with any of the following substrates: dihydrolipoamide, glucose 6-phosphate, 6-phosphogluconate, lactate, malate, or glyceraldehyde 3-phosphate.

#### Effect of pH

The effect of pH on the B enzyme is shown in Fig. 1. The A enzyme gave results indistinguishable from those shown. The ratio of activity with DPNH compared to that with TPNH was not constant. Below pH 6.5 the enzymes were unstable, and the A enzyme was less stable than the B enzyme. TPNH dehydrogenase activity was lower when Tris buffers were used instead of phosphate.

### Table I

**Purification of TPNH dehydrogenases**

<table>
<thead>
<tr>
<th>Fraction and treatment</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Specific activity (µmol/min/mg protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolyzed cells</td>
<td>2,000</td>
<td>650,000</td>
<td>0.0009</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>550</td>
<td>15,000</td>
<td>0.017</td>
<td>45</td>
</tr>
<tr>
<td>Protamine, (NH₄)₂SO₄</td>
<td>50</td>
<td>1,220</td>
<td>0.10</td>
<td>22</td>
</tr>
<tr>
<td>4A. Chromatography, (NH₄)₂SO₄</td>
<td>2.4</td>
<td>2.2</td>
<td>6.5</td>
<td>3</td>
</tr>
<tr>
<td>4B. Chromatography, (NH₄)₂SO₄</td>
<td>2.5</td>
<td>11.2</td>
<td>3.2</td>
<td>6</td>
</tr>
<tr>
<td>5B. Chromatography, (NH₄)₂SO₄</td>
<td>1.0</td>
<td>1.8</td>
<td>7.4</td>
<td>3</td>
</tr>
</tbody>
</table>

A column, 2.0 cm in diameter, was filled to a depth of 20 cm with Ca₃(PO₄)₂ gel (10). To this were added successively 10 ml of 0.01 M Tris-HCl, enzyme solution, 30 ml of 0.01 M Tris-HCl, and 150 ml of 0.01 M Tris-HCl containing 1% (NH₄)₂SO₄. The chromatography was performed at room temperature with a slight vacuum (450-mm pressure of Hg) applied to the lower end of the column; 10-ml fractions were collected. Two colored bands were evident on the column, a lower orange-red band and an upper red-brown band. TPNH dehydrogenase A activity was eluted in advance of the orange-red band. DPNH dehydrogenase I activity was eluted between the two colored bands, and TPNH dehydrogenase B activity after the red-brown band. The fractions containing DPNH dehydrogenase I were of high specific activity, suitable for further purification of the enzyme (4). Fractions containing TPNH dehydrogenase A with specific activity greater than 1.0 were combined. The enzyme in these fractions was precipitated between 50 and 70% saturation with (NH₄)₂SO₄, then dissolved in 1 ml of 0.1 M Tris-HCl to give Fraction 4B.

Fraction 4B was dialyzed for 5 hours in 1 liter of 0.01 M Tris-HCl, and the chromatography was repeated on a Ca₃(PO₄)₂ gel column, 0.5 cm in diameter and 5 cm deep. To the column were added successively 1 ml of 0.01 M Tris-HCl, enzyme, 10 ml of 0.01 M Tris-HCl, and 25 ml of 0.01 M Tris-HCl containing 0.5% (NH₄)₂SO₄. Fractions of 2 ml each were collected. Those fractions with specific activity greater than 5 were combined, and the enzyme was precipitated between 55 and 70% saturation with (NH₄)₂SO₄. The precipitate was dissolved in 1 ml of 0.1 M Tris-HCl to give Fraction 5B. The purification procedure is summarized in Table I.

Fig. 1. Effect of pH on reaction rate of dehydrogenases of Fraction 5B. Conditions are those described in the text except for buffer. Reaction rate is given in micromoles per minute. Open symbols, Tris-HCl buffer; solid symbols, potassium phosphate buffer.

The ratios of activity with 2,6-dichlorophenolindophenol, cytochrome c, and methemoglobin were: A enzyme, 55,000:250: 1, and B enzyme, 55,000:160: 1. The dehydrogenases had no transglyceraldehyde 3-phosphate. All four dehydrogenases were active with glucose 6-phosphate, 6-phosphogluconate, lactate, malate, or glycerol-oxyde 3-phosphate.

The ratios of activity with deaminodihydrolipoamide DPNH to that with DPNH were: DPNH dehydrogenase I, 0.97, and DPNH dehydrogenase II, 0.15. The activity of purified DPNH dehydrogenase I with TPNH was only 1% of that with DPNH (4). We were not able to prepare DPNH dehydrogenase II that was similarly free of TPNH activity, although TPNH dehydrogenase B fractions were obtained which had a ratio of activity with DPNH to that with TPNH of 0.01. This ratio in various TPNH dehydrogenase B preparations varied from 0.01 to 0.60. The data on DPNH dehydrogenase II described above were obtained on an enzyme preparation (Fraction 5B) with a DPNH:TPNH ratio of 0.48.

Both TPNH dehydrogenase fractions were red and both absorbed strongly at 410 nm. From the ratio of absorbance at 410 and 280 nm, 12% of the protein of dehydrogenase A and 47% of the protein in dehydrogenase B were calculated to be heme protein. TPNH dehydrogenase A contained no more than 1 mole of flavin per 6.8 x 10⁶ g of protein. TPNH dehydrogenase B contained 15 times this amount of flavin, but this could be adsorbed to a small amount of glutathione reductase which was present (11). Enzyme prepared by the method of Huennekens et al. (2) was similar in properties to dehydrogenase B.
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Effect of Temperature—The effect of temperature on the T enzyme is shown in Fig. 2. The A enzyme gave similar results. DPNH dehydrogenase II activity showed little dependence on temperature with an apparent activation energy of 2700 cal per mole as compared with 5100 cal per mole for DPNH dehydrogenase I (4). The apparent activation energy of the TPNH reaction was 6900 cal per mole. Above 30°, the enzyme was inactivated. High ionic strength and the presence of the reduced nucleotides tended to minimize inactivation. DPNH dehydrogenase activity was more stable to heat than was TPNH dehydrogenase activity.

Effect of Substrate Concentration—Change in concentration of either TPNH or dye in the ranges studied had little effect on the reaction rate of dehydrogenase B (Fig. 3). The data in Fig. 3A were fitted to the equation (12)

\[
\frac{1}{V} = \frac{1}{V_{\text{max}}} + \frac{K_{\text{m}^\text{TPNH}}}{V_{\text{max}}} \left[\text{TPNH}\right] + \frac{K_{\text{m}^\text{DCI}}}{V_{\text{max}}} \left[\text{DCI}\right]
\]

where \( V \) is initial velocity, the brackets indicate molar concentrations of TPNH and DCI \(^3\) and \( V_{\text{max}}, K_{m^\text{TPNH}}, \) and \( K_{m^\text{DCI}} \) are constants. The lines were drawn in Fig. 3 with the constants taken as follows: \( V_{\text{max}} = 1.19 \mu \text{mole per minute}, K_{m^\text{TPNH}} = 5.2 \times 10^{-6} \text{ M}, \) and \( K_{m^\text{DCI}} = 5.8 \times 10^{-6} \text{ M}. \) TPNH dehydrogenase A gave similar results.

The effects of substrate concentration on DPNH dehydrogenase II were those expected of a reaction in which a ternary complex is formed (Fig. 4). In this case, data were fitted to the equation (12)

\[
\frac{1}{V} = \frac{1}{k_3} + \frac{1}{k_3[D\text{PNH}]} + \frac{1}{k_3[\text{DCI}]} + \frac{k_{-3}}{k_3[D\text{PNH}][\text{DCI}]}
\]

The lines of Fig. 4 were drawn with the constants taken as follows: \( 1/k_3 = 1.2 \text{ minutes per } \mu \text{mole}, 1/k_3 = 0.2 \times 10^{-4} \text{ minute per } \mu \text{mole}, \) and \( k_{-3} = 2.6 \times 10^{-4} \text{ minute per } \mu \text{mole}. \) Similar results were obtained with a DPNH dehydrogenase purified from methemoglobinemic red cells (6).

Effect of Ionic Strength—Increasing ionic strength activated both TPNH dehydrogenases (Fig. 5), but inhibited both DPNH dehydrogenases. High ionic strength also inhibited the activity with TPNH of DPNH dehydrogenase I. When the activation by various salts was compared, (NH\(_4\))^+ and F\(^-\) were found to be most effective.

\(^3\) The abbreviation used is: DCI, 2,6-dichlorophenolindophenol.

High ionic strength had no effect on the shape of the pH curve of the TPNH dehydrogenases (Fig. 1), and had no appreciable effect on apparent activation energy (Fig. 2). In the absence of KCl, no rational equation relating rate to substrate concentration could be developed (Fig. 3A). Addition of KCl had no effect on dependence of rate on TPNH concentration, but did eliminate inhibition by TPNH. KCl also increased the maximum velocity of the reaction and the dependence of rate on dye concentration.

Methemoglobin Reduction Rates of reduction of methemoglobin by several reducing systems under conditions simulating those in the red cell are compared in Fig. 6. An excess of the reducing system was added to a concentrated methemoglobin solution prepared from nitrite-treated red cells. Each line represents the average of four experiments: one with recrystallized human methemoglobin and three with hemolysates from persons with methemoglobinemia due to DPNH dehydrogenase deficiency. DPNH dehydrogenase II was equally as effective as DPNH dehydrogenase I in reducing methemoglobin. From

Fig. 2. Effect of temperature on dehydrogenases of Fraction 5B. Conditions are described in the text. \( f_{\Lambda} \) is absolute temperature; reaction rate is given in micromoles per minute. ○, TPNH as substrate; □, TPNH with KCl omitted; △, DPNH as substrate.

Fig. 3. Effect of substrate concentration on TPNH dehydrogenase in the absence (A) and presence (B) of KCl; other conditions as in the text. \( V \) is initial reaction rate in micromoles per minute; brackets denote molar concentration. The lines from top to bottom represent dye concentrations of \( 4 \times 10^{-4}, 5.6 \times 10^{-4}, 10 \times 10^{-4}, \) and \( 40 \times 10^{-4} \text{ M}. \)

Fig. 4. Effect of substrate concentration on DPNH dehydrogenase II. \( V \) is initial reaction rate in micromoles per minute; brackets denote molar concentration. In A, lines from top to bottom represent dye concentrations of \( 4 \times 10^{-4}, 5.6 \times 10^{-4}, 10 \times 10^{-4}, \) and \( 40 \times 10^{-4} \text{ M}. \) In B, the lines from top to bottom represent DPNH concentrations of \( 1.8 \times 10^{-4}, 2.5 \times 10^{-4}, 4.9 \times 10^{-4}, \) and \( 35 \times 10^{-4} \text{ M}. \)
TPNH dehydrogenase, $0.5 \times 10^{-4}$ min$^{-1}$; and DPNH dehydrogenase II, $0.6 \times 10^{-4}$ min$^{-1}$.

**Discussion**

TPNH dehydrogenase B and DPNH dehydrogenase II were difficult to separate, and were at first assumed to be one enzyme. It was found, however, that competition between the nucleotides did not occur. Under standard assay conditions for DPNH dehydrogenase, the following rates of reduction, in absorbance change per minute, were observed: $67 \ \mu$ TPNH, 0.114; $133 \ \mu$ TPNH, 0.112; $67 \ \mu$ DPNH, 0.058; and $67 \ \mu$ TPNH plus $67 \ \mu$ DPNH, 0.164.

The TPNH dehydrogenases of red cells are unusual among diaphorases in that they appear not to contain flavin. DPNH dehydrogenase II is unusual in its kinetic behavior, which is characteristic of the formation of a ternary complex of enzyme, DPNH, and dye. It may be assumed that the dye is a non-specific substrate, since methylene blue and ferricyanide are also substrates. The most likely explanation of the observed enzymatic activity is that the enzyme binds DPNH and the bound nucleotide reacts more rapidly with 2,6-dichlorophenolindophenol than does free nucleotide. The simplest formulation of the reaction is as follows.

$$E + DPNH \xrightarrow{k_1} E \cdot DPNH \xrightarrow{k_2} E \cdot DPN^+ + DCIHz \xrightarrow{k_3} E + DPN^+$$

When initial rates of reaction are considered, this formulation leads to Equation 2.

The measured rates of methemoglobin reduction by DPNH and TPNH dehydrogenases are comparable to those found in the intact red cell. In normal red cells in which hemoglobin has been oxidized to methemoglobin with nitrite, the rate of reduction of methemoglobin in the presence of glucose is from 10 to 15% per hour (13). In cells deficient in DPNH dehydrogenase, the corresponding rate of reduction is 2 to 5% per hour, indicating a 75% loss in reducing capacity. Under conditions simulating those in the red cell, the total of the first order rates of methemoglobin reduction measured for ascorbic acid, glutathione, and DPNH and TPNH dehydrogenases is $9.6 \times 10^{-4}$ min$^{-1}$, which is equivalent to 13.2% per hour. Of this total, DPNH dehydrogenase I accounts for 61%.

In methemoglobinemic individuals lacking DPNH dehydrogenase I, 15 to 45% of the total hemoglobin is present as methemoglobin (13). In persons with half the normal level of DPNH dehydrogenase (heterozygotes), no increase in methemoglobin is found (5). Thus a two-thirds loss in capacity to reduce methemoglobin in the red cell results in methemoglobinemia, while a one-third loss has no effect. Since each of the other reducing systems studied appears to comprise less than one-third of the total reducing capacity of the cell, absence of any of these systems would not be expected to cause methemoglobinemia. In fact, absence of ascorbic acid in scurvy and deficiency of TPNH in glucose 6-phosphate dehydrogenase deficiency do not cause methemoglobinemia. The only defects in reducing mechanisms of the red cell that could cause methemoglobinemia appear to be the absence of DPNH dehydrogenase I, deficiency of DPN itself, or the lack of some enzyme necessary to reduce DPN to DPNH.
SUMMARY

Four enzymes with reduced pyridine nucleotide dehydrogenase activity were demonstrated in human red cells. Two reduced triphosphopyridine nucleotide dehydrogenases with similar properties were separated by chromatography. These did not appear to contain flavin and were weak methemoglobin reductases. A reduced diphosphopyridine nucleotide dehydrogenase, previously demonstrated in methemoglobinemic red cells, was also purified from normal red cells.

The rates of reduction of methemoglobin by ascorbic acid, glutathione, and DPNH and TPNH dehydrogenases were determined. The DPNH dehydrogenase previously purified from normal red cells was calculated to account for two-thirds of the capacity of the human red cell to reduce methemoglobin. It was concluded that the only defects in reducing mechanism likely to produce methemoglobinemia are absence of DPNH dehydrogenase, of DPN, or of enzymes producing DPNH.

REFERENCES
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