Maleimide as an Inhibitor in Measurement of Erythrocyte Glucose-6-phosphate Dehydrogenase Activity

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Erythrocyte glucose-6-phosphate dehydrogenase activity is measured with a centrifugal analyzer by use of a commercial reagent kit and of the reaction glucose-6-phosphate + NADP⁺ → 6-phosphogluconolactone + NADPH. Rate of production of NADPH is measured and related to hemoglobin concentration. Maleimide is added to inhibit further production of NADPH in a secondary reaction by endogenous 6-phosphogluconate dehydrogenase. The method is compared with others that are designed to circumvent the secondary reaction by either (a) addition of excess phosphogluconate dehydrogenase to drive the secondary reaction to completion or (b) inhibition of endogenous phosphogluconate dehydrogenase by 2,3-diphosphoglycerate. The present method has the advantages that reaction rate more quickly becomes linear and reagent cost is less as compared with other methods. The within-run coefficient of variation was 3%. The various methods investigated showed good statistical correlation.

Additional keyphrases: centrifugal analyzer • "kit" methods • screening • normal values • inherited disorders

Clinical implications of deficiency of erythrocyte glucose-6-phosphate dehydrogenase (G6PD) have been extensively studied and described in detail (1–4). Quantitative assay of this activity has proved to be difficult. In the reaction sequence, glucose-6-phosphate is oxidized as follows:

Glucose-6-phosphate + NADP⁺ → 6-phosphogluconolactone + NADPH

The 6-phosphogluconolactone is subsequently converted to 6-phosphogluconate by lactonase (EC 3.1.1.17), and then to ribulose-5-phosphate + CO₂ by 6-phosphogluconate dehydrogenase (PGD). This results in production of more than 1 mol of NADPH per mole of glucose-6-phosphate consumed, and therefore in overestimation of G6PD activity (5–7).

Several strategies have been proposed to obviate the secondary reaction of PGD. Glock and McLean (5) suggest that both PGD activity and PGD plus G6PD activity be measured, and G6PD activity calculated as the difference between the two measurements. The major disadvantage of this technique is that in specimens deficient in G6PD, activity is determined from small differences between two large measurements, and so there can well be large errors and poor precision (8).

In a second approach, proposed by Dror et al. (6), excess PGD is added to the reaction mixture, resulting in quantitative conversion of glucose-6-phosphate to final products, and production of 2 mol of NADPH per mole of substrate. This method was adapted to the centrifugal analyzer (9, 10). It suffers, however, from a relatively long lag period before the rate of production of NADPH becomes constant.

Finally, the secondary reaction may be circumvented by inhibiting endogenous PGD, with resulting production of 1 mol of NADPH per mole of substrate. Catalano et al. (10) used 2,3-diphosphoglycerate to inhibit PGD and adapted the method to the centrifugal analyzer. Inhibition of PGD by maleimide has also been reported (11).

The present studies were undertaken to provide a rapid, precise, accurate, and relatively inexpensive assay for screening large populations for G6PD deficiency. The studies of Catalano et al. (10) are extended to include use of a commercial reagent kit with the centrifugal analyzer. Maleimide is added to inhibit endogenous PGD. This modification is compared with methods in which excess PGD or 2,3-diphosphoglycerate is used to circumvent the secondary reaction of endogenous PGD, and advantages of its use in screening programs for detection of G6PD deficiency are evaluated.

Materials and Methods

Materials

The G6PDH Stat-Pak Kit was obtained from Calbiochem (San Diego, Calif. 92112) and reconstituted according to the manufacturer's instructions. Concentrations of reconstituted reagents are as follows: tris(hydroxymethyl)aminomethane buffer, pH 7.5, 50 mmol/liter; magnesium salt, 7.4 mmol/liter; glucose-6-phosphate, 3.8 mmol/liter; NADP⁺, 4.5 mmol/liter.

Maleimide was obtained from Aldrich Chemical Co., Milwaukee, Wis. 53233.

6-Phosphogluconate dehydrogenase, 2,3-diphosphoglycerate, di-tris(hydroxymethyl)aminomethane salt; and
NADP+ were obtained from Sigma Chemical Co., St. Louis, Mo. 63178.

DeltaTest Control, a comprehensive chemistry control serum containing two concentrations of constituents, including G6PD, was purchased from Princeton Biomedix, Princeton, N.J. 08540.

All other chemicals were reagent grade.

Instrument

A CentrifiiChem Model 400 centrifugal analyzer with Pipetor (Union Carbide Corp., Tarrytown, N.Y. 10591) was used.

Samples

Blood specimens in ethylenediaminetetraacetic acid-containing tubes, collected for routine hematological analysis, were obtained from the Hematology Laboratory of the Institute of Pathology, Kings County Hospital Center, Brooklyn, N.Y. Specimens were screened for anemia before analysis; those with abnormally low hemoglobin or hematocrit were not used in routine studies.

Reagents

Assay with added PGD: 6-Phosphogluconate dehydrogenase was added to reconstituted G6PDH Stat-Pak to a final concentration of 100 U/liter. One unit is defined by the manufacturer as that activity capable of oxidizing 1.0 μmol of 6-phosphogluconate to ribulose-5-phosphate + CO2 at pH 7.4 at 37 °C.

Assay with added 2,3-Diphosphoglycerate: 2,3-Diphosphoglycerate was added to G6PDH Stat-Pak before reconstitution, the final concentration used in routine studies being 4.5 mmol/liter.

Assay with added maleimide: Maleimide was added to G6PDH Stat-Pak before reconstitution, the final concentration used in routine studies being 4 mmol/liter.

Reagents for "standard" and "enzyme-linked" assays of Catalan et al. were prepared as described (10).

Procedures

Lysis of cells: Blood was allowed to settle overnight, or in some cases centrifuged gently (1000 X g, 5 min), to pack the cells. A 25-μl volume of packed cells was lysed with 0.5 ml of
the digitonin/NADP solution described by Catalano et al. (10).

*Measurement of enzyme activity:* Lysate, 10 µl, and 50 µl of water were pipetted by the CentriChem pipettor into sample cavities of the transfer disk, and 350 µl of reagent was pipetted into reagent cavities. The zero cuvette contained water; the first cuvette, reagent alone. Water and reagent blanks are automatically subtracted from specimens by the CentriChem.

All reactions were run at 37 °C in the Rate mode, using the 340-nm filter. Other settings for each method are given in the text. Enzyme activity (micromoles of NADH produced per minute per liter of lysate) was calculated as described by Catalano et al. (10). Methods in which excess PGD was added assumed production of 2 mol of NADPH per mole of glucose-6-phosphate, and results obtained were divided by 2. Methods in which maleimide or 2,3-diphosphoglycerate were used assumed production of 1 mol of NADPH. Activity of patient's specimens is expressed as international (IUB) units (U) per gram of hemoglobin.

*Measurement of hemoglobin concentration.* After the final printout of enzyme activity, the filter was changed to 550 nm; the absorbance was stored and referenced to a water blank. Concentration was calculated as described (10).

**Results**

*Effects of addition of excess PGD:* Measurement of G6PD activity with the PGD assay is shown in Figure 1. A lag period of 16–18 min is observed before a constant rate of reaction is obtained. The period of constant activity extends for 8–10 min, during which time activity may be measured. Activity is enhanced by increasing exogenous PGD to 200 U/liter, but the lag period is not shortened (Figure 2). Similar results are obtained for increasing amounts of specimen assayed.

A similar pattern of activity is obtained with the "enzyme-linked" assay of Catalano et al. (10), but there are some differences. The lag phase is reduced to 6–8 min of reaction time, and a shorter period of peak activity—3 to 5 min—is seen. Enzyme activity is nearly linear during this period, but begins to decline rapidly after about 12 min of reaction time. Enzyme activity during the period of linearity is consistently lower than that obtained with PGD assay.

**Table 1. Within-Run Precision of G6PD Methods**

<table>
<thead>
<tr>
<th>Method with added</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
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<tr>
<td>PGD</td>
<td>8.76</td>
<td>0.09</td>
<td>0.98</td>
</tr>
<tr>
<td>2,3-Diphosphoglycerate</td>
<td>9.83</td>
<td>0.20</td>
<td>2.02</td>
</tr>
<tr>
<td>Maleimide</td>
<td>9.41</td>
<td>0.29</td>
<td>3.03</td>
</tr>
</tbody>
</table>

* Assay conditions as shown in Figure 6.

**Fig. 5. Assay with added maleimide**

Patients' specimens with various activities. Maleimide concentration, 4.0 mmol/liter. Time delay, 3 s. Results recorded in "Absorbance" mode at 1 min intervals for 9 min. After the final printout, instrument logic was reset, and further results recorded for 5 min at 1 min intervals.

Assays using inhibitors of endogenous PGD: In contrast to the findings described above, inhibition of PGD by 2,3-diphosphoglycerate or maleimide permits rapid measurement of G6PD activity. As shown in Figure 3, addition of 4.5 mmol of 2,3-diphosphoglycerate per liter results in a short lag period, followed by nearly linear rate of reaction maintained for nearly 20 min. PGD in specimens with normal or above-normal activities is not completely inhibited, but the small increases with time in rate of reaction does not affect interpretation of the clinical status of the patient. The effect of varying 2,3-diphosphoglycerate concentration is shown in Figure 4. Results indicate that a concentration of 4.5–5.0 mmol of 2,3-diphosphoglycerate per liter is optimal for assay; other concentrations result in differences in inhibition of PGD and poor precision of assay. At concentrations exceeding 6.0 mmol/liter the reaction rate declines steadily after the initial measurement at 1 min of reaction time.

The assay with maleimide is represented in Figure 5. Specimens with activity as low as 10 U/liter up to approximately 120 U/liter have constant reaction rates for more than 15 min of reaction time. Specimens containing greater activities show a small decrease in activity after 6–9 min, followed by a constant rate to 15 min. The range of linearity of activity with respect to enzyme concentration was studied with serial dilutions of DeltaTest Control Serum. Results showed that the assay is linear to at least 300 U/liter, under conditions described in Figure 5. Best results were obtained when measurements were taken in the first 6 min of reaction time, following a 1-min lag period.

Maleimide concentration is not critical. In most experi-

**Table 2. Statistical Correlation Among G6PD Methods**

<table>
<thead>
<tr>
<th>No. Detrs.</th>
<th>Method X</th>
<th>Method Y</th>
<th>Slope</th>
<th>y-intercept</th>
<th>r</th>
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</thead>
<tbody>
<tr>
<td>111</td>
<td>PGD assay</td>
<td>Maleimide assay</td>
<td>0.991</td>
<td>0.162</td>
<td>0.973</td>
</tr>
<tr>
<td>111</td>
<td>PGD assay</td>
<td>2,3-Diphosphoglycerate assay</td>
<td>0.953</td>
<td>-0.212</td>
<td>0.985</td>
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<tr>
<td>56</td>
<td>PGD assay</td>
<td>&quot;Enzyme-linked&quot; assay</td>
<td>0.802</td>
<td>0.113</td>
<td>0.997</td>
</tr>
<tr>
<td>56</td>
<td>2,3-Diphosphoglycerate assay</td>
<td>&quot;Enzyme-linked&quot; assay</td>
<td>0.801</td>
<td>0.818</td>
<td>0.991</td>
</tr>
<tr>
<td>56</td>
<td>assay</td>
<td>Maleimide assay</td>
<td>1.121</td>
<td>-0.207</td>
<td>0.994</td>
</tr>
</tbody>
</table>

* Conditions described in Figure 6A.  From Catalano et al. (10).  Conditions described in Figure 6B.
ments I used 4 mmol/liter final concentration (11), but results were essentially identical with concentrations of 3–5 mmol/liter.

Statistical comparison among methods: Within-run precision of three methods is shown in Table 1 (precision of the Catalano et al. “enzyme-linked” assay was not determined). Results show that good precision is obtained with all of the methods studied, the PGD assay yielding the lowest coefficient of variation. In more recent experiments a coefficient of variation of 1.3% for activities similar to those in Table 1 has been obtained for the maleimide assay if a time interval (ΔT) of 3 min rather than 1 min is used.

Statistical correlation studies among the four methods investigated, shown in Table 2, indicate that good agreement is obtained among the four assay methods.

Distribution of enzyme activity in normal populations: Results in Figure 6 show that distribution patterns for the different methods are generally similar. Of particular note is the occurrence of clusters of activity in each chart, corresponding to that anticipated for deficient, non-deficient, and mixed phenotypes. “Normal ranges” for the non-deficient populations were determined for each assay method by use of probability paper, and log-transformed data where applicable (12). Normal ranges for each method are included in Figure 6.

Discussion

In screening for G6PD deficiency, the clinician requires a rapid and precise assay, in order to establish a normal population range to assist in diagnosis of otherwise unexplained hemolytic anemia.

Methods used to date to assay G6PD have to some degree failed to fulfill the requirements of either rapidity of assay, precision, accuracy, or cost effectiveness. For example, the method recommended by the World Health Organization (13), which neglects the contribution of the secondary reaction of PGD, is not accurate. The Glock and McLean method (5) is not precise, nor when it was adapted to the centrifugal analyzer (CentrifChem) as described (10) was it able to achieve constant rate of either the PGD assay or combined PGD plus G6PD reactions. The “enzyme-linked” and “non-linked” assays described by Catalano et al. are more accurate and precise (9, 10), but require preparation of several reagents, and the “enzyme-linked” assay is complicated by a relatively long lag period. The PGD and 2,3-diphosphoglycerate assays as shown in this communication similarly suffer from several disadvantages; the former has a prohibitively long lag period, the latter requires scrupulous adherence to proper concentration for optimal inhibition of endogenous PGD. Reagents for both methods are relatively expensive.

The assay with added maleimide now described combines the speed and precision of the centrifugal analyzer with the convenience and economy of a commercial reagent kit. The secondary reaction of PGD is effectively inhibited, thus reducing the lag period observed with other methods, and permitting measurement to be made within several minutes of initiation of reaction. The assay is effective over a broad range of concentration of maleimide, thereby minimizing errors in preparation of reagent. Cost of the reagent is low as compared with other methods, making it suitable for application in screening programs.

Equally important is the finding that the maleimide assay is clinically equivalent to the other methods. The studies with randomly selected populations demonstrate that the maleimide assay is as capable of detecting moderate or severe G6PD deficiency as are the other methods investigated. Precision of the maleimide assay is acceptable, although somewhat poorer than the PGD or 2,3-diphosphoglycerate assays. Further modifications of procedure are expected to improve precision of the maleimide assay; these are currently under investigation.

In summary, erythrocyte G6PD can be effectively measured by inhibiting endogenous PGD by maleimide. Adaptation of the method to the centrifugal analyzer and use of a commercially available reagent kit facilitates the assay to be used in screening programs to detect individuals deficient in G6PD.

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References

11. Lohr, G. W., and Waller, H. D., Glucose-6-phosphate dehydroge-
13. Standardization of procedures for the study of glucose-6-phos-