Automated Procedure for Assay of 2,3-Diphosphoglycerate in Red Cells by Measuring Enzymatically Released Inorganic Phosphorus

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An automated technique is reported for measuring erythrocyte 2,3-diphosphoglycerate (DPG). We have automated a specific method, in which the DPG phosphatase activity of phosphoglycerate mutase is used and potentiated by phosphoglycolate. Precision, accuracy, recovery, and comparability with a reference method are discussed.

Additional Keyphrases  phosphoglycerate mutase • P₁ measurement after release • normal values • hypoxia • colorimetry • blood banking • AutoAnalyzer • phosphoglycolate

The recent awareness of the importance of DPG¹ in the metabolism of red blood cells has heightened interest in methods of analysis for that compound. Concentrations of DPG in red cells are increased in conditions of hypoxia produced at high altitudes, or by chronic lung disease, congenital heart diseases, or anemia (1). Also, the oxygen-delivering capability of stored banked blood seems to be improved when the DPG concentrations are maintained (2) or restored (3).

Rose and Liebowitz (4) observed that DPG phosphatase activity of muscle PGM is stimulated greatly by phosphoglycolic acid.

\[
\text{DPG} \xrightarrow{\text{PGM}} \text{phosphoglycolate} + P₁
\]

These observations formed the basis for the determination of DPG by measuring inorganic phosphorus released. One mole of P₁ is released per mole of DPG hydrolyzed, and this phosphorus is measured colorimetrically.

The present study is concerned with automation of the Rose and Liebowitz assay with the AutoAnalyzer [Technicon Instruments Corporation, Tarrytown, N.Y. 10591]. We have incorporated into this procedure an important modification by Maeda (5), in which the conversion of adenosine triphosphate to inorganic phosphate is suppressed by EDTA. This method is preferable to similar manual methods (4, 5), owing to the improved precision of automation. It is a more direct measurement of DPG than other methods. Many of the enzyme systems necessary in other procedures are eliminated.

We compared this method with the Grisolia method (6), which is also an AutoAnalyzer method.

Materials and Methods²

Apparatus

A manifold and flow system were assembled for use with components of the AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N.Y. 10591) (Figure 1). A Sampler II, Pump I, colorimeter, and recorder in the linear mode were used. A transparent Plexiglas dialysis tank of standard size was used. All the mixing coils preceding dialysis were placed inside the dialysis tank to provide a constant temperature of 37°C (Figure 1). Pulse suppressors were placed on all lines being pumped into the dialysis tank. A special flowcell, 1.5 mm × 15 mm, was used (available from Acculab, Palisades, N.Y. 10964).

Reagents

Glycylglycine-K⁺ buffer, mol wt 132.1, 2 mmol/liter. Dissolve 0.264 g of glycylglycine (No. G-1002; Sigma Chemical Co., St. Louis, Mo. 63116) in 950 ml of water. Adjust the pH to 7.5 with molar KOH and dilute to 1 liter with water.

Phosphoglycolic acid, tetracyclohexy lammonium salt, mol wt 452, 10 mmol/liter. Dissolve 226 mg of

¹ Nonstandard abbreviations used: DPG, 2,3-diphosphoglycerate; PGM, phosphoglycerate mutase; EC 2.7.5.3; Pᵢ, inorganic phosphorus; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; and PGA, 3-phosphoglycerate.

² The manufacturers' name and products are given as scientific information only and do not constitute an endorsement by the United States Government.
phosphoglycolic acid, tetracyclohexylammonium salt (No. 1295D; General Biochemicals, Chagrin Falls, Ohio 44022) in water and dilute to 50 ml. Dispense into several small tubes and freeze at 

−20°C.

Phosphoglycerate mutase. From rabbit muscle. Obtained as a crystalline suspension (No. P-8252, Sigma; or No. 15432PAQ, Boehringer-Mannheim, New York, N.Y. 10021) in 2.4 or 2.6 mol/liter ammonium sulfate, depending on the supplier. Use the mutase directly from the bottle. Attention should be paid to the concentration in mg of protein per milliliter. Ten milligrams of mutase protein should be used for 50 ml of the enzyme mixture described below. The mutase will cause erratic results if too highly concentrated.

Disodium EDTA, 10.0 mmol/liter. Dissolve 3.722 g in 975 ml water, adjust the pH to 7.5 with molar KOH, and dilute to 1 liter with water.

Enzyme mixture. This should be prepared daily, filtered through Whatman No. 2 filter paper and kept chilled during use. Fifty milliliters, for 150 min running time, is prepared as follows:

<table>
<thead>
<tr>
<th>Amount</th>
<th>Working enzyme mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 ml</td>
<td>PGM (5 mg protein/ml)</td>
</tr>
<tr>
<td>4.0 ml</td>
<td>phosphoglycolic acid, 10 mmol/liter</td>
</tr>
<tr>
<td>2.0 ml</td>
<td>EDTA, 10.0 mmol/liter, pH 7.5</td>
</tr>
<tr>
<td>42.0 ml</td>
<td>glycyglycine-K⁺ buffer, 2 mmol/liter, pH 7.5</td>
</tr>
</tbody>
</table>

50.0 ml

Blank mixture. Add 4 ml of 10.0 mmol/liter EDTA to 96 ml of glycyglycine-K⁺ buffer, 2 mmol/liter.

Aminonaphthol reagent. This is a modification of Technicon Formula AR-24-58. To prepare the stock solution, dissolve 120 g of sodium bisulfite (or 109.6 g of sodium metabisulfite) and 4 g of anhydrous sodium sulfite in water by heating to 50°C. Add 2 g of 1-amino-2-naphthol-4-sulfonic acid; stir until dissolved. Dilute to one liter with water, filter, and dispense into 100-ml portions. The working solution is prepared by diluting the stock solution 10-fold with water and adding 0.5 ml of “Levor IV” per liter. Mix thoroughly. Stable at room temperature for two months.

Molybdate–ascorbic acid. This is a modification of the Maeda et al. method (5). Dilute 7 ml of 0.57 mol/liter ascorbic acid (prepared daily) to 50 ml with 3.40 millimolar ammonium molybdate in 0.5 molar H₂SO₄. Chill constantly in an ice bath while using. Prepare only that amount that will be used immediately.

Ammonia water, 0.9 mmol/liter. Dilute 6.0 ml of 15 molar ammonium hydroxide to 100 ml with water to prepare the stock solution. Dilute 1 ml of stock solution to a liter with water for the working solution.

Sampler water. Add 0.5 ml of “Brij 35” to 1 liter of water. Mix thoroughly.

Standards

(a) DPG, 0.5 mmol/liter. Dissolve 41.7 mg of DPG, mol wt 833.9 (No. 321025, Calbiochem, San Diego, Calif. 92112) in ammonia water and dilute to 100 ml with the same.

(b) Inorganic phosphate, 0.5 mmol/liter. Dissolve 68 mg of KH₂PO₄ in ammonia water and dilute to 100 ml with the same.

From the above stocks the following serial dilutions are prepared with ammonia water: 0.05, 0.10, 0.20, 0.30, 0.40, and 0.50 mmol/liter.

All the chemicals were obtained commercially and were of reagent grade. De-ionized, glass-distilled water was used. Reagents should be refrigerated unless specified otherwise.

Procedure

1. Collect venous blood with EDTA anticoagulant.
2. Determine its hematocrit.
3. Prepare a hemolysate by diluting whole blood 20-fold with ammonia water.
4. Store hemolysate at −20°C before use.
5. Start pump and establish a baseline with the enzyme reagent line in the blank mixture and
aminonaphthol and molybdate–ascorbate lines in their respective containers. The phosphate standards and the whole blood hemolysates are run at 30 samples per hour to provide the blank values.

6. Replace the blank mixture with the enzyme mixture. Establish a baseline, then run the standards and samples also at 30/h.

7. Subtract the blank value from the sample value, then divide by the hematocrit value to obtain results as mmol/liter of packed cells.

At the end of a run, the system should be cleaned by placing all lines into NaOH (0.15 mol/liter) and pumping for 10 min. A 60-min water rinse should follow. Failure to perform this wash will result in clogging of the dialyzer and poor bubble patterns. A rising baseline usually indicates coating of the flowcell and a dirty system in general. This is corrected by the NaOH washout.

**Automated Grisolia Method**

In the method of Grisolia et al. (6) whole blood samples are diluted 1000-fold with ammonia water. We prepared the two reagent mixtures, A and B, slightly differently from Grisolia et al.

Mixture A is prepared by combining: 50 ml of 125 millimolar EDTA-K⁺, pH 7.0; 100 ml of 1 molar Tris-PO₄ buffer, pH 7.3: 7.5 ml of 1 molar MgCl₂; 25 ml of 0.1 molar ADP (1171, Calbiochem) and 125 ml of 0.2 molar 3-phosphoglycerate (P₇G). The barium salt of PGA (No. P-8627, Sigma) is purified as described by Grisolia et al. (6). This 0.2 molar PGA is dispensed into 25- and 50-ml portions, which are frozen.

Mixture B contains the enzymes and is prepared by mixing: 0.5 ml of pyruvic kinase from rabbit skeletal muscle (No. P-1381, Sigma); 0.25 ml of PGM (No. 15432EPAQ, Boehringer-Mannheim); 1.25 ml of enolase (No. 32472, Calbiochem); and 31.25 mg of bovine serum albumin. This mixture is diluted to 50 ml with water and divided into 2.5-ml portions, which are then frozen.

Before use, 50 ml of mixture A is combined with 5 ml of mixture B, and the resulting mixture is diluted to 100 ml with water.

2,4-Dinitrophenylhydrazine (No. 1266, Eastman Chemical Co., Rochester, N.Y. 42650), 2.52 mmol/liter, is prepared in HCl, 1 mol/liter.

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**Results and Discussion**

**Precision.** The day-to-day precision of the automated method was determined from the results of 63 measurements of a single whole blood sample (Table 1). The samples were stored at 4°C during this time. These measurements were performed on four different days over a seven-day period.

The study was limited to seven days to minimize problems that might result from instability of sample. The within-run precision of the automated method was determined by analyzing whole blood, DPG standards, and phosphate standards as randomly ordered replicates (Table 1). Each of the three categories of samples were run at three different concentrations; the statistics are compared in Table 1.

**Recovery.** DPG recovery was measured by adding five different concentrations of DPG aqueous standard to a whole blood hemolyase (Table 2). These analyses were all performed in triplicate. The mean recovery was 97.4%, and recovery was good in all ranges.

**Comparison with other methods.** Originally we evaluated this method by comparing it with the manual method of Rose and Liebowitz (4). Similar values were obtained for each method by using aqueous standards; however, these comparisons were not extensive.

Our precision with the Grisolia method has been good. A within-run analysis of 25 blood specimens yielded a standard deviation of .037 in our laboratory. Grisolia et al. (6) found a standard deviation of .098 over a period of days. These data compare favorably with those of the Rose and Liebowitz method (Table 1).

The DPG concentrations of whole blood from 21 people were determined by both the automated method under study and by the automated method of Grisolia et al. (6), the latter being the reference method. Eight of these samples were from normal people in our laboratory, and 13 were from hospitalized patients. Correlation between the two methods is shown in Figure 2. The correlation coefficient (r) for these data is .924. When only the normal people from our laboratory were used for comparisons, correlation was slightly

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**Table 1. Statistics for the Analytical System**

<table>
<thead>
<tr>
<th></th>
<th>Between-run</th>
<th>Within-run</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
<td>Blood</td>
<td>DPG std</td>
<td>PO₄ std</td>
</tr>
<tr>
<td>No. of specimens</td>
<td>63</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mean concn</td>
<td>4.19</td>
<td>2.95</td>
<td>4.24</td>
<td>4.94</td>
</tr>
<tr>
<td>Variance</td>
<td>.0450</td>
<td>.0030</td>
<td>.0110</td>
<td>.0060</td>
</tr>
<tr>
<td>SD</td>
<td>.212</td>
<td>.054</td>
<td>.104</td>
<td>.077</td>
</tr>
<tr>
<td>Coeff. variation</td>
<td>5.05</td>
<td>1.37</td>
<td>2.45</td>
<td>1.56</td>
</tr>
</tbody>
</table>

*The units for DPG concentration are mmol/liter for standards and mmol/liter packed cells for blood.*
Table 2. Recovery of Added DPG

<table>
<thead>
<tr>
<th>Sample, mmol/liter</th>
<th>Actual value (DPG, mmol/liter)</th>
<th>Theoretical value</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.61</td>
<td>3.88</td>
<td>93%</td>
</tr>
<tr>
<td>2</td>
<td>4.95</td>
<td>5.05</td>
<td>98%</td>
</tr>
<tr>
<td>3</td>
<td>6.19</td>
<td>6.21</td>
<td>100%</td>
</tr>
<tr>
<td>4</td>
<td>7.23</td>
<td>7.37</td>
<td>98%</td>
</tr>
<tr>
<td>5</td>
<td>8.37</td>
<td>8.54</td>
<td>98%</td>
</tr>
</tbody>
</table>

Mean = 97.4%

* One ml blood + one ml standard, the concentration of the standard being the indicated number of mmol/liter.

better \((r = .975)\). Conversely, when four additional samples from sick patients who had abnormally high DPG values were included with the other samples (25 samples), the correlation was somewhat poorer \((y \text{ intercept } = 1.21; r = .904; \text{ slope } = 0.73)\). The \(t\) value \((1.22)\) indicated no significant difference when results for all 25 samples were compared for the two methods.

**Normal values.** We analyzed the blood of 25 healthy people. The mean value was 4.52 mmol/liter packed red cells, with a standard deviation of 0.4 and a range of 3.88–5.20. This compares well with the normal range found by Oski et al. (3) and by Grisolia et al. (6). Hellerstein et al. (7) have recently extensively studied normal values, as obtained by the Grisolia method. With special handling techniques they found somewhat lower values.

**Effects of dilution.** To prove that sample dilution does not alter the values by this method, we diluted single blood samples from 2 people 10-, 20-, 30-, and 40-fold with ammonia water. These samples were analyzed in duplicate. The results were multiplied by the dilution factor, and the means were calculated. The values for each sample were similar (Table 3) regardless of how high the dilution.

**Linearity.** The calibration curves of the DPG standard and the phosphate standards have excellent linearity. We find that 1 mmol of phosphate from the phosphate standard has the same absorbance as the phosphate from 1 mmol of hydrolyzed DPG.

**Chart recording.** Figure 3 is a typical chart recording. Carryover is negligible and baseline stability is excellent. No change was observed in peak heights of standards after a 1-h run. Carryover from the 5- to the following 2-mmol/liter standard (Figure 3) is 1.1% of the peak height of the latter.

We first ran our automated procedure without using EDTA and found that we obtained higher values than by other methods.

As noted by Maeda et al. (5), the EDTA isolates the reaction we are measuring from interference from ATP when it is converted to inorganic phosphate. After adding EDTA, we found the normal values to be the same as values obtained by other methods (3, 5, 6).

We found the original glycylglycine buffer concentration (10 mmol/liter) to be too high to work in the AutoAnalyzer system; it caused a gradual coating of the flowcell. The buffer was diluted to 2 mmol/liter and it became possible to run several hours before the manifold needed cleaning with 0.15 molar NaOH.
A special flowcell was used (1.5 mm × 15 mm). This flowcell was found to increase sensitivity and improve washout characteristics when compared with standard AutoAnalyzer flowcells. It also decreased reagent consumption.

In preparing the enzyme mixture, we discovered that the mutase could be used directly from the bottle and added to the mixture, thus eliminating the necessity of centrifuging it and replacing the supernatant fluid from the ammonium sulfate with glycglycine buffer (5). This is an important time saver.

The cost in reagents per sample with this procedure is approximately 40¢ per sample. The mutase is the most expensive reagent.

The primary advantages of this system are simplicity, low cost, and a more nearly direct measurement of DPG.

References