Improved Method for Determining Erythrocyte Creatine by the Diacetyl-
\( \alpha \)-naphthol Reaction: Elimination of Endogenous Glutathione Interference

Philip K. Li, Josephine T. Lee, Cal-Shuang Li, and Ganesh Deshpande

We describe a simple, accurate, and reproducible method for determining erythrocyte creatine. The method is free from glutathione inhibition and is adaptable to use with standard spectrophotometers as well as centrifugal analyzers. A clear filtrate, essentially free of protein, hemoglobin, and glutathione, is prepared from 0.1 mL of packed erythrocytes by treatment with \( \text{Ba(OH)}_2 \) and \( \text{ZnSO}_4 \), then reacted with diacetyl-\( \alpha \)-naphthol. The standard curve for this method is linear from 10 to 500 mg/L. We show that endogenous sulphydryl species such as erythrocyte glutathione will interfere with the creatine-diacetyl-\( \alpha \)-naphthol reaction. This observation confirmed a suspicion of underestimation of erythrocyte creatine by the method of Griffiths (1964). Added \( \text{p-chloromercuribenzoic acid} \) did not completely eliminate this inhibition. In the present method these interfering sulphydryl species are eliminated from the reaction mixture, thus obviating the need for \( \text{p-chloromercuribenzoic acid} \) and dialysis. The reference interval for this method is 42–80 mg/L.

Additional Keyphrases: reference interval - anemia - index to erythrocyte age - centrifugal analyzer

In hemolytic anemia, the reticulocyte count is the most frequently used indirect measure of the compensatory increase in erythrocyte production (1); it commonly is regarded as the deciding factor in establishing the need for a bone-marrow examination in the work-ups of normochromic and normocytic anemias (2). In practice, reticulocyte counting (3) has been associated with a degree of statistical error, possibly because of the subjective criteria used in identifying the young cells histologically. This problem has apparently hampered its diagnostic precision. Other tests aimed at detecting, directly or indirectly, the products of erythrocyte catabolism such as increased plasma hemoglobin, increased "indirect" bilirubin in serum, and the presence of hemosiderinuria all lack diagnostic specificity. Erythrocyte survival time requires the in vivo use of a radioactive material and a prolonged testing period that includes multiple blood drawings. Therefore, a simple, sensitive, and specific laboratory test is needed, one that could be substituted to measure the erythrocyte life span.

Griffiths and Fitzpatrick (4) reported that creatine content of erythrocytes is a sensitive index of the mean age of an erythrocyte population. Their observations were later confirmed by Fehr and Knob (5), who reported distinctly greater creatine content of young erythrocytes and showed a close correlation with reticulocyte counts and erythrocyte survival time. Later, Opalinski and Beutler (6) reported the correlation between creatine content and erythrocyte hexokinase (EC 2.7.1.1), an enzyme for which activity is also correlated with erythrocyte life span. These observations have aroused renewed interest in the measurement of creatine in erythrocytes (5, 7, 8).

Fehr and Knob (5) and others (7) measured erythrocyte creatine content by applying a continuous-flow method (9) involving the diacetyl reaction (10) in the presence of \( \alpha \)-naphthol in alkali. The saponin-treated hemolysates were allowed to flow through the donor side of the dialyzers. Because the hemolysates contain relatively large amounts of glutathione, which is dialyzable under the same conditions as creatine and could cause sulphydryl inhibition of diacetyl reaction (11, 12), we suspected that these erythrocyte creatine values were underestimated. This suspicion was later confirmed in a private communication with Smith et al. (7), who found erythrocyte creatine values to be higher after they incorporated \( \text{p-chloromercuribenzoic acid} \) (p-CMBA) into their pre-mixing saline line. Furthermore, as shown in tissues, the use of p-CMBA and sodium \( \text{p-hydroxymercuribenzoate} \) is also necessary to counteract the inhibitory effects of endogenous sulphydryl compounds (4, 9, 11–13). Therefore, for more accurate creatine values, the interference by endogenous glutathione should be eliminated.

Here we report a simple, accurate, and practical method for determining erythrocyte creatine, which is free from glutathione interference and compatible with routine spectrophotometers and centrifugal analyzers. Initially, a clear filtrate is prepared from a mixture of packed erythrocytes, barium hydroxide, and zinc sulfate. The filtrate, essentially free of protein and glutathione, is reacted with the diacetyl-\( \alpha \)-naphthol reagent, and the endpoint absorbance is measured at 520 nm.

Barium hydroxide was added to provide a pH above the isoelectric point (pl) of glutathione, thus promoting the formation of insoluble precipitates when zinc sulfate was added. The formation of barium sulfate also promoted complete removal of glutathione, as a result of adsorption. Although glutathione is a tripeptide, in solution it behaves somewhat like a larger charged protein molecule. This salting-out phenomenon was first reported by Somogyi (14).

We show that the glutathione has been effectively removed from the hemolysates by the deproteinization treatment. We have studied the various apparent effects of glutathione, nonprotein, and guanido nitrogenous compounds on the diacetyl-\( \alpha \)-naphthol reaction. We also assessed the use of p-CMBA in this method.

Materials and Methods

Apparatus

For the routine assay method, we used a computer-backed Gilford 102 System (Gilford Instrument Laboratories, Oberlin, OH 44074) and a Gilford automatic pipetter/diluter for fluid handling. For the centrifugal analysis, we used the Rotochem IIA/36 (Instrument Division, Travon Laboratories,

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4 Approximately 900 mg/L of packed erythrocytes, 90% in reduced form (GSH), the rest in oxidized form (GSSG).

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Inc., Savage, MD 20863) and the Rotofill II for fluid handling.

Specimens
All whole-blood specimens were collected in heparin- or EDTA-containing tubes. Clinical specimens, when stored at 4°C undisturbed, could be validly assayed after as long as 10 days. Because erythrocytes of various ages differ in fragility, we recommend that hemolyzed specimens not be used.

Reagents
For deproteinization: Ba(OH)₂ solution (Reagent 1), 47.3 g/L, prepared from Ba(OH)₂ · 8H₂O in cool, boiled water. It is standardized against Reagent 2. Restandardize if a visible precipitate appears during storage.
ZnSO₄ solution (Reagent 2), 50 g/L, prepared from ZnSO₄·7H₂O. Although the exact concentrations of Reagents 1 and 2 are not critical, the alkali must neutralize the zinc sulfate precisely, volume for volume, when titrated; phenolphthalein is the indicator (15). On the basis of titration, the solution that is more concentrated is diluted to match the other.
For standard assay: Alkali solution (Reagent 3). Dissolve 160 g of anhydrous Na₂CO₃ in 1 L of 1.5 mol/L NaOH. Store in an amber-colored polyethylene bottle at room temperature.
α-Naphthol (Reagent 4), 8 g/L, prepared freshly by dissolving a preweighed vial of 128 g of α-naphthol (Sigma Chemical Co., St. Louis, MO 63178; cat. no. N-1000) in 16 mL of Reagent 3. It should give no more than a faint straw color. Preweighed α-naphthol vials should be stored in a desiccator.
Diacetyl (Reagent 5), 1 mL/L, prepared by diluting 1 mL of liquid stock reagent (Sigma; cat. no. D-3634) in 1 L of water. Working solution is stable. Handle stock in the hood only.
Diacetyl-α-naphthol working solution (Reagent 6). Prepare freshly by mixing 16 mL of Reagent 4, 2 mL of Reagent 5, and 7 mL of H₂O. It is stable for up to 4 h at room temperature. Discard if solution appears brownish.
Working standard (Reagent 7), 80 mg/L, prepared from 800 mg/L stock standard. The working standard is stable for at least five days. Prepare stock standard from the anhydrous creatine obtained by drying creatine monohydrate (Sigma; cat. no. C-3630) at 100°C for 3 h. The stock standard is stable for several months at 4°C.
Controls. We prepared aqueous normal (Reagent 8) and above-normal (Reagent 9) controls from an independently prepared stock creatine solution to approximate concentrations of 50 and 200 mg/L. Aliquots of 0.5 mL, stored frozen, are stable for three months. We could not find satisfactory commercial material to serve as controls. Because protein matrix did not affect analytical recovery, we did not include it in the preparation of controls.

Procedure
Pre-assay treatment of specimens: Centrifuge 1.0 mL of anticoagulated whole blood at 2000 × g for 10 min. Aspirate the plasma and buffy coat and gently mix the packed erythrocytes. Carefully pipet 0.1 mL of packed erythrocytes and wash out into 0.7 mL of premeasured H₂O. Mix contents and allow to stand for 2 min before adding 0.1 mL of Reagent 1. Subsequently, add 0.1 mL of Reagent 2 slowly to the mixture with a constant swirling action while the precipitate is forming. Vortex-mix the entire contents and allow to stand for an additional 2 min. Separate the supernate from the pink-colored precipitate by a second centrifugation at 2000 × g for 10 min and then assay with the routine assay method.
Routine assay method: Before each run, we checked the Gilford 102 System according to the manufacturer’s specifications and programmed it according to Table 1. For each test sample the fluids were delivered according to Table 2. After allowing all tubes to stand for 15–20 min at room temperature, we read the endpoint absorbances at 520 nm. The data were computed automatically by the manufacturer’s "endpoint program":

\[
F = \text{calculated factor} = \frac{C_u}{(A_u - A_{rb})}
\]

where

\[
C_u = \text{conc. of standard } \times 10 \text{(dilution factor for Ba(OH)₂-ZnSO₄ treatment)}
\]
\[
A_u = \text{absorbance of standard}
\]
\[
A_{rb} = \text{absorbance of reagent blank at position 0}
\]

Subsequent concentrations for unknowns and controls were computed from their net absorbances, after corrections for the reagent blank, to yield creatine results in mg/L of packed erythrocytes:

\[
C_u = F \times (A_u - A_{rb})
\]

where

\[
C_u = \text{conc. of unknown (uncorrected)}
\]
\[
A_u = \text{absorbance of unknown}
\]

To convert the measured creatine into mg/L of packed erythrocytes, it was necessary to introduce a correction term of 1.087, based on the observation (n = 200) that the crudely prepared packed erythrocytes gave a mean microhematocrit value of 92 (5). Thus, the true erythrocyte creatine content was obtained by multiplying the uncorrected value by this correction term.

Centrifugal analysis: Prepare supernate the same as in the routine assay method. Reactions are completed in the transfer disc by mixing 100 μL of standards, supernates, or controls with 500 μL of diacetyl-α-naphthol working reagent (Reagent 6). After letting it sit for 15–20 min at room temperature, load the transfer disc into the cuvette assembly and complete the run according to the manufacturer’s "endpoint II" program. The correction term of 1.087 is also used in these final calculations, as discussed above.

Other Studies
Thin-layer chromatographic identification of glutathione.

<table>
<thead>
<tr>
<th>Sample no/content</th>
<th>Reagent pump (reagent 8)</th>
<th>Sample pump (as specified)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0, reagent blk.</td>
<td>500 μL</td>
<td>100 μL H₂O</td>
</tr>
<tr>
<td>1, standard*</td>
<td>500 μL</td>
<td>100 μL reagent 7</td>
</tr>
<tr>
<td>2, etc., controls</td>
<td>500 μL</td>
<td>100 μL specimen</td>
</tr>
</tbody>
</table>

* Factor calculated and entered in microprocessor subsequent to reading.
We confirmed the presence or absence of glutathione in a supernate or filtrate by thin-layer chromatography. A total of 50 μL of material was spotted at several points before the chromatograms were developed. Spotted silica gel plates were developed in a solvent system of n-butanol/water/acetac acid (60/25/15, by vol) for 2 h. Ninhydrin spray (2 g/L in ethanol) was the visualizing agent.

**Specificity evaluations.** We evaluated the specificity of our assay system with respect to glutathione, nonprotein, and guanido nitrogenous compounds, as well as certain other metabolites. Two creatine solutions, 50 and 150 mg/L, were supplemented with the material of interest to a concentration exceeding the physiological range in erythrocytes. The supplemented and unsupplemented creatine solutions were analyzed according to the routine assay method as described.

Glutathione inhibition of diacetyl reaction. We studied the interference of glutathione with the diacetyl-α-naphthol reaction by the routine assay method. Solutions of known creatine concentration were supplemented with glutathione to give concentrations of 900–4000 mg/L of packed erythrocytes. The inhibitory effects of glutathione were evaluated from the observed analytical recoveries.

Protective effect of p-CMBA. We also studied the protective effect of p-CMBA with regard to the glutathione inhibition. Solutions of known creatine concentrations were evaluated by the routine assay method in the presence or absence of glutathione and the simultaneous presence or absence of p-CMBA.

**Results and Discussion**

**Pre-Assay Treatment of Specimens**

The clear filtrate obtained from the described protocol was essentially free of protein and interfering sulfhydryl species. With a Coomassie Brilliant Blue procedure, we confirmed that the protein content in the filtrate is less than that of the detection limit of this procedure (<20 mg/L). Direct and indirect evidence also confirmed the removal of glutathione from the filtrates.

**Analytical Variables**

**Linearity.** The present procedure gave a linear response to creatine concentration between 10 and 50 mg/L of packed erythrocytes. Our dynamic effective range is adequate for normal and above-normal samples, including the extremely high values in patients with autoimmune hemolytic anemia.

**Analytical recovery.** Recovery was assessed by supplementing 12 hemolysates with known amounts of creatine before the addition of Ba(OH)₂ and ZnSO₄. At the concentrations studied, 50, 100, and 200 mg/L, mean recovery was 101% (range 95–106%). Substituting water for the Ba(OH)₂ and ZnSO₄ reagents did not affect the recovery of the aqueous creatine standards.

**Precision.** Within-run precision was assessed by assaying 21 replicates of normal and above-normal specimen pools. Day-to-day precision was estimated over a period of 21 days from the same pools of packed erythrocytes that had been apportioned and stored frozen. Results obtained in precision studies are summarized on Table 3. Use of aqueous creatine solutions without pre-assay treatment improved precision in all cases.

**Comparison of methods.** Regression parameters for comparison of 25 blood samples (creatinine range: 30 to 640 mg/L) by the routine assay method (x) and the centrifugal analysis method (y) are: slope = 0.87, intercept = 10.9, standard error of estimate = 9.5, and correlation coefficient = 0.992. We conclude that our two methods correlate well.

**Specificity Evaluation**

Results of the specificity evaluation are summarized on Table 4. At the concentrations tested, only glutathione interfered with the diacetyl-α-naphthol reaction. A change in absorbance of less than 2% was considered noninterfering. Reduced glutathione, at the concentration found in normal erythrocytes, produces a significant negative interference (Figure 1).

**Thin-Layer Chromatographic Identification of Glutathione**

Results obtained from the thin-layer chromatograms are summarized in Table 5. The detection limit for glutathione was about 2 μg. On the basis of these observations and other indirect evidence, we conclude that there are, indeed, significant amounts of glutathione in erythrocytes that can be effectively removed by the Ba(OH)₂–ZnSO₄ deproteinization treatment. Our data also suggest that the endogenous glutathione, if not removed, will pass through the dialyzer membranes and inhibit the diacetyl-α-naphthol reaction in the Griffiths method (9) for the determination of erythrocyte creatine. We did not study the effect attributable to the oxidized form of glutathione, owing to its relatively insignificant presence in erythrocytes, but believe that it is removed concurrently with reduced glutathione.

<table>
<thead>
<tr>
<th>Table 3. Assay Precision</th>
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<tbody>
<tr>
<td>Sample</td>
</tr>
<tr>
<td><em>Within-run (n = 21)</em></td>
</tr>
<tr>
<td>Normal pool</td>
</tr>
<tr>
<td>Above-normal pool</td>
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<tr>
<td><em>Day-to-day (n = 21)</em></td>
</tr>
<tr>
<td>Normal pool</td>
</tr>
<tr>
<td>Above-normal pool</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 4. Specificity Evaluation</th>
</tr>
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<tbody>
<tr>
<td>Compound studied</td>
</tr>
<tr>
<td>Ammonia</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
<tr>
<td>Creatinine</td>
</tr>
<tr>
<td>2,3-Diphosphoglyceric acid</td>
</tr>
<tr>
<td>Glutathione, oxidized</td>
</tr>
<tr>
<td>Guanidine</td>
</tr>
<tr>
<td>Guanidoacetic acid</td>
</tr>
<tr>
<td>Lactate</td>
</tr>
<tr>
<td>Methionine</td>
</tr>
<tr>
<td>Pyruvate</td>
</tr>
<tr>
<td>Urea nitrogen</td>
</tr>
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</table>

* No interference observed.

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7 Microprotein Rapid Stat Kit, Pierce Chemical Co., Rockford, IL 61105.
thione 900 mg/L, inhibition was 15–35%. This variability can be accounted for, in part, by the age of the working reagent. The extent of inhibition was found to be related to the molar concentration ratio of glutathione to creatine: the higher the ratio, the greater the effect. Specimens having a normal or subnormal creatine content were therefore affected more than those having above-normal content, and an upward shift in reference values can be expected. The inability of Ba(OH)$_2$-ZnSO$_4$-treated filtrates to inhibit the diacetyl reaction provided additional indirect evidence showing that the glutathione had been removed. Figure 1 shows the absorbance spectrum of a reaction mixture in the presence or absence of glutathione.

**Protective Effect of p-CMB**

In all cases, we observed that p-CMB protected against inhibition by glutathione but, as previously reported (11), could not reverse the inhibition completely. Our results indicate that the extent of protection is primarily dictated by the molar concentration ratio of glutathione to p-CMB, the optimal ratio being 2:1. Under these conditions, as much as 94% of the creatine can be recovered. In the absence of glutathione, addition of p-CMB had a small inhibitory effect, 3–6% of that of glutathione. The extent of inhibition seemed to be independent of the concentrations of either creatine or p-CMB. Perhaps this explains why the protection of p-CMB to glutathione inhibition is incomplete. These and other observations support the desirability of removing endogenous glutathione from the analytical mixture, thus obviating the use of p-CMB.

Additional indirect evidence for the removal of glutathione from the erythrocyte hemolysates by our deproteinization treatment is provided by the inability of p-CMB to increase the apparent creatine content of the supernate. On the contrary, apparent creatine content was increased by the use of p-CMB for analysis of a filtrate prepared by ultrafiltration or dialysis. Figure 1 shows the glutathione inhibition and p-CMB protection.

**Clinical Applications**

Reference intervals. We collected and analyzed venous blood samples from 59 subjects (well hospital employees and children and infants from our out-patient clinics). The concentration of erythrocyte creatine ranged from 42 to 80 mg/L of packed erythrocytes (mean 61, SD 19 mg/L). This range is somewhat higher than that previously reported (5, 7, 9). However, our preliminary data suggest that female subjects have a statistically significant higher range (20%) than that of the males (4, 5). We also noticed that values further increased in pregnancies. We will discuss the reference intervals with respect to age and sex in another communication when the evaluation of data is completed.

Other studies. Our preliminary studies with patients confirmed the close correlation of erythrocyte creatine concentration with the mean age of the erythrocytes (4, 5). When patients respond to therapy, the erythrocyte creatine concentrations frequently remain increased beyond the peak reticulocytosis. This observation is consistent with reports by Sasse et al. (16) and Opelinski and Beutler (6), indicating that the reticulocyte is too short-lived to be used as a guide to the mean age of a circulating erythrocyte population.

**Table 5. Thin-Layer Chromatographic Detection of Reduced Glutathione (GSH)**

<table>
<thead>
<tr>
<th>Sample description</th>
<th>Treatment</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH eq. soln, 2 µg/L</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>Hemolysate from packed erythrocytes</td>
<td>Ba(OH)$_2$-ZnSO$_4$</td>
<td>-</td>
</tr>
<tr>
<td>Hemolysate from packed erythrocytes</td>
<td>Ultrafiltration</td>
<td>+</td>
</tr>
<tr>
<td>Hemolysate from packed erythrocytes (supplemented with GSH 2000 mg/L)</td>
<td>Ba(OH)$_2$-ZnSO$_4$</td>
<td>-</td>
</tr>
<tr>
<td>GSH eq. soln, 2000 mg/L</td>
<td>Ba(OH)$_2$-ZnSO$_4$</td>
<td>-</td>
</tr>
<tr>
<td>Hemolysate from packed erythrocytes</td>
<td>Dialysis</td>
<td>+</td>
</tr>
</tbody>
</table>

* + = detectable, - = nondetectable.

**References**


CLINICAL CHEMISTRY, Vol. 28, No. 1, 1982
New Spectrophotometric Method for the Determination of Hemoglobin A₁ Compared with a Microcolumn Technique

Olov Wällinder, Gunnar Ronquist, and Pehr-Johan Fager

We compared a spectrophotometric kit method (Glycospec) for determination of glycosylated hemoglobin (HbA₁) with a microcolumn kit method (Bio-Rad). The Glycospec method is based on the change in absorbance when phytic acid binds to hemoglobin A. With glycosylated hemoglobin there is no such change because the binding is blocked by the sugar moiety. Inter-assay CVs were 2–6% for both methods. In healthy subjects the mean (±SD) value for HbA₁ was about 1% higher with the spectrophotometric than the microcolumn method. For samples from 122 diabetics the correlation between values for HbA₁ obtained with the two methods was acceptable (r = 0.89), although the spectrophotometric technique yielded 2–4% higher values, a difference at least partly due to the absence of 2,3-diphosphoglycerate from the spectrophotometric standards. Adding 1.8 mmol of D-per liter to these standards caused displacement of the standard curve; HbA₁ values then agreed well with those of the microcolumn method. The spectrophotometric procedure is easily automated, and therefore is well suited for large-scale analyses if problems with standards and calibration can be solved.

Additional Keyphrases: monitoring diabetes · chromatography, liquid · “kit” methods

Glycosylated hemoglobin (hemoglobin A₁) normally comprises about 6% of the total hemoglobin of the erythrocyte. The predominant subfraction of hemoglobin A₁ (HbA₁) is HbA₁c, formed by a slow non-enzymic glycosylation reaction during the life-time of the erythrocyte (1). Thus the percentage of HbA₁c reflects the average blood glucose concentration during the preceding four or five weeks (2, 3). Both HbA₁c and the total HbA₁ fraction are increased in diabetes mellitus, and determination of HbA₁c has become an important index of long-term diabetic control (4–6). The method most widely used for determining HbA₁c is based on chromatography through microcolumns (7, 8)—a method not ideally suited for routine clinical use because it is difficult to automate and is highly sensitive to minor changes in experimental conditions (9). Recently, a spectrophotometric assay based on a new principle was reported (10). This paper presents a comparison of the two methods with regard to reliability of results for normal subjects and diabetic patients. Various factors influencing the HbA₁c values were also studied.

Materials and Methods

The control group consisted of 18 ostensibly healthy blood donors, most of them men, with no clinical signs of diabetes mellitus. The 122 diabetic patients were regularly attending our outpatient service for diabetic care. They were being treated with insulin, sulfonylurea drugs, or diet alone. Blood samples for HbA₁c determinations, obtained after an overnight fast, were drawn in EDTA-containing tubes, unless otherwise stated. They were analyzed concomitantly by the microchromatographic and the spectrophotometric methods.

HbA₁c analysis by the use of microcolumns was performed with a commercial kit (Bio-Rad Laboratories, Richmond, CA 94804). Blood was drawn into EDTA-containing tubes, if not otherwise stated, and was mixed with a hemolyzing reagent. An aliquot of each hemolyzate was applied to the microcolumn. Adding the elution buffer rapidly eluted HbA₁c from the column, while the main part of the hemoglobin was retarded. The percentage of HbA₁c in the blood samples was determined by measuring the absorbance at 415 nm of the eluted HbA₁c fraction and the diluted hemolysate. The eluant buffer was kept at 22 °C, the temperature being continuously monitored with a microelectrode inserted into the resin. Values deviating from 22 °C were corrected according to a nomogram provided by the manufacturer.

The spectrophotometric assay is based on the fact that organophosphorus compounds such as 2,3-diphosphoglycerate (2,3-DPG) and inositol hexaphosphate (phytic acid) bind to the N-terminal amino acid of the two beta chains of hemoglobin A (10). This causes a conformational change of the hemoglobin molecule, a process that can be monitored spectrophotometrically. The prerequisite for such a change is available N-terminal residues. Hence, the spectrum of glycosylated hemoglobin is not changed by the organophosphorus compounds because of the blocking effect of the glucose moiety. The change in absorbance induced by phytic acid is thus inversely proportional to the percentage of glycosylated hemoglobin.

The spectrophotometric assay was done in accordance with the manufacturer’s instructions with a kit (Glycospec; Abbott Laboratories, North Chicago, IL 60064) and an Abbott automated bichromatic analyzer (the ABA-100). An aliquot of each blood sample (drawn in EDTA-containing tubes) or standard was pipetted into the ABA sample cups. Ten microliters of each sample was automatically dispensed with 500 µL of hemolyzing reagent into the appropriate cuvette and the dif-