buffers recommended for determining LD activity, we believe that caution should be exercised in their use.

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Glycosylation of Hemoglobin S and Hemoglobin C

To the Editor:

In a recent study (1) of diabetic patients with hemoglobins S and C (Hb S and Hb C), we showed a significant decrease in the proportion of glycosylated Hb, as measured by cation-exchange chromatography. We suggested (1) that these low values for glycosylated Hb might be caused by the inability of either Hb S or Hb C to undergo glycosylation or by their failure to be eluted from the column with the usual buffer solution.

To explore these two possibilities, we used an alternative, colorimetric method, in which glycosylated Hb is measured without first separating total hemoglobin into its various components. The method, first described by Fluckiger and Winterhalter (2), and recently modified by Gabbay et al. (3) to increase its sensitivity and reproducibility, is based on the fact that ketoamine-linked hexoses are liberated on heating under acidic conditions as furfural compounds, which are then quantitated colorimetrically as an adduct of 2-thiobarbituric acid.

Using a combination of colorimetric and microcolumn chromatographic methods, we have found evidence that both Hb S and Hb C undergo glycosylation, and that the rates of their glycosylation are similar to that for Hb A.

The patients we studied were from the Diabetic Center of The Montreal General Hospital. Blood specimens were drawn into an evacuated collection tube, with ethylenediaminetetraacetate as an anticoagulant, the percentage of abnormal hemoglobin was estimated by electrophoresis on acrylamide gel. The proportions of glycosylated Hb in the erythrocytes of each patient were determined by the modified colorimetric method (2, 3) and by a commercial cation-exchange microcolumn chromatographic procedure (Isolab, Akron, OH 44321) carried out at 23 ± 0.3 °C.

Table 1 shows the colorimetric and chromatographic values for glycosylated Hb in four patients with abnormal hemoglobin. For comparison, the results of a group of patients with normal Hb (Hb A), who had similar colorimetric values, are shown. Note that although the colorimetric values in each pair of patients (normal vs abnormal Hb) were similar, the chromatographic values were consistently lower in patients with abnormal hemoglobins.

Table 1. Glycosylated Hb in Diabetic Patients with Normal (Hb A) and Abnormal (Hb S and Hb C) Hemoglobins, as Measured by Column Chromatography and Colorimetry

<table>
<thead>
<tr>
<th>Abnormal Hb, % of total Hb</th>
<th>Colorimetry</th>
<th>Chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>26.0</td>
<td>26.0</td>
</tr>
<tr>
<td>Hb S, 40%</td>
<td>9.9</td>
<td>5.5</td>
</tr>
<tr>
<td>Hb S, 38%</td>
<td>11.4</td>
<td>7.3</td>
</tr>
<tr>
<td>Hb S, 41%</td>
<td>21.1</td>
<td>12.3</td>
</tr>
<tr>
<td>Hb C, 43%</td>
<td>18.3</td>
<td>10.8</td>
</tr>
<tr>
<td>Hb C, 42%</td>
<td>13.8</td>
<td>8.4</td>
</tr>
<tr>
<td>Hb S, 28%</td>
<td>8.2</td>
<td>5.8</td>
</tr>
<tr>
<td>Hb S, 38%</td>
<td>9.1</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* Colorimetry results expressed as absorbance at 443 nm per 10 mg of hemolysate hemoglobin.

Table 2. Percentage of Glycosylated Hb in Seven Patients with Abnormal Hb, as Measured by Column Chromatography and Colorimetry

<table>
<thead>
<tr>
<th>Abnormal Hb, % of total Hb</th>
<th>Absorbance</th>
<th>Col. chromat.</th>
<th>Corrected col. chromat.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb S, 40%</td>
<td>9.9</td>
<td>5.5</td>
<td>9.1</td>
</tr>
<tr>
<td>Hb S, 38%</td>
<td>11.4</td>
<td>7.3</td>
<td>11.7</td>
</tr>
<tr>
<td>Hb S, 41%</td>
<td>21.1</td>
<td>12.3</td>
<td>20.0</td>
</tr>
<tr>
<td>Hb C, 43%</td>
<td>18.3</td>
<td>10.8</td>
<td>18.9</td>
</tr>
<tr>
<td>Hb C, 42%</td>
<td>13.8</td>
<td>8.4</td>
<td>14.4</td>
</tr>
<tr>
<td>Hb S, 28%</td>
<td>8.2</td>
<td>5.8</td>
<td>8.0</td>
</tr>
<tr>
<td>Hb S, 38%</td>
<td>9.1</td>
<td>6.0</td>
<td>9.6</td>
</tr>
</tbody>
</table>

* Absorbance readings by the colorimetric method were converted to percentage glycosylated Hb by reference to a standard curve that correlated chromatographic and colorimetric values in subjects with normal hemoglobin.

To determine whether this decrease was proportional to the percentage of the abnormal hemoglobins, we corrected the chromatographic values by using the following formula (1):

\[
\text{(obtained chromatographic value x 100)} - \text{percentage of abnormal Hb)}
\]

\[
= \text{corrected chromatographic value.}
\]

As may be seen from Table 2, the corrected chromatographic values agreed well with the colorimetric values. With the current microcolumn chromatographic procedures, one can measure only those glycosylated Hb components that are eluted from the column with the usual buffer solution (4), whereas with the colorimetric (thiobarbituric acid) method all glycosylated hemoglobins, regardless of their nature, are determined. Thus, using a combination of these two methods, we found that in patients with Hb S or Hb C the column-chromatographic procedure underestimates the percentage of glycosylated Hb. Further, the decrease in glycosylated Hb as measured by the chromatographic method was propor-
tional to the percentage of the abnormal Hb in each patient. We interpret this to mean that both Hb S and Hb C are indeed glycosylated, at rates that are similar to that for Hb A.

The findings of low values for glycosylated Hb in patients with Hb S and Hb C by column-chromatographic methods is of particular practical significance when one considers that about 8% of black Americans are heterozygous for Hb S and 2 to 3% for Hb C.

In the present studies we have shown that the decrease in the concentrations of glycosylated Hb by the column-chromatographic method is proportional to the percentage of the abnormal hemoglobin. Consequently, the current chromatographic procedure can be used for the accurate determination of glycosylated Hb in patients with Hb S and Hb C, if the above-shown corrections are made to compensate for the percentage of the abnormal Hb.

I thank Mrs. Pauling Read for her excellent technical assistance.

References

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Simplified Colorimetry of Urinary Homovanillic Acid

To the Editor:
A colorimetric method for urinary homovanillic acid (HVA) was reported (1). We have simplified the method and calculations by use of a constant instead of a variable urine volume, eliminating the use of an internal standard, and the inclusion of a recovery control. Our experience with this simplified method is reported.

Specimen collection: A 24-h urine specimen was collected in 10 mL of concentrated HCl, stored at 4 °C, and assayed within a week.

Extraction: Into duplicate 50-mL extraction tubes, pipette 3 mL of urine and 1.5 mL of water for each test and test blank; for each standard and standard blank, pipette 0.01 mL of a solution containing 10 nmol of HVA per liter in 10 ml of HCl, and 4.5 mL of water. To all tubes add 0.5 mL of 6 mol/L HCl and 1 g of NaCl. Mix well. Add 30 mL of chloroform to each tube and shake them vigorously for 10 min. Centrifuge lightly to separate the phases and aspirate the aqueous (upper) layer. Transfer 25 mL of the chloroform solution to an extraction tube containing 3 mL of 41 mmol/L tris(hydroxymethyl)methylamine (Tris) buffer, pH 8.5–9.0, and shake vigorously for 10 min. Centrifuge lightly and transfer 2.5 mL of the aqueous (upper) layer to test tubes. Add 0.5 mL of the color reagent, which is 12 mmol/L 2-nitroso-1-naphthol-4-sulfonic acid in ethanol/water (40/60 by vol), to each tube. Then add 0.5 mL of 2 mol/L HCl to the test blanks and the standard blank and 0.5 mL of nitrous acid (mix 0.4 mL of freshly prepared 360 mmol/L sodium nitrite with 10 mL of 2 mol/L HCl) to each test and standard, shake, and allow to stand at room temperature for exactly 20 min. A recovery and recovery blank containing 2.4 mL of the Tris buffer, 0.01 mL of the HVA standard, 0.5 mL of the color reagent, and either 0.5 mL of 2 mol/L HCl or 0.5 mL of nitrous acid were assayed in duplicate with each batch. The absorbance was read at 500 nm vs the recovery blank. HVA excretion (mmol HVA/mmol creatinine) was calculated:

\[
\frac{AT - ATB}{100} \times \frac{1}{A_S - A_{SB}} \times 144
\]

where \(AT = \text{absorbance of the test, } ATB = \text{absorbance of the test blank, } AS = \text{absorbance of the standard, } A_{SB} = \text{absorbance of the standard blank, and } AR = \text{absorbance of the recovery.}\)

In this assay, increasing quantities of HVA standards gave an increased color, which was linear over the range 2 to 300 nmol of HVA per tube after extraction (\(r = 0.99 \) for eight data points, and absorbance = 0.0025 [nmol] – 0.025).

Color formation for samples with low HVA reached a maximum more rapidly than samples with high HVA and then decreased slowly. Therefore the absorbance should be measured within 20 min. The intra- and inter-assay coefficients of variation were 6.4% (n = 9) and 9.4% (n = 11), respectively. The accuracy of the batch was established as follows (a) on addition of HVA to urine, the curve was found to be linear to 250 nmol of HVA per tube (r = 0.94, six data points, absorbance = 0.0014 [nmol] + 0.006); and (b) the curve for dilutions of a urine with above-normal HVA was linear (\(r = 0.91, \) six data points, absorbance = 0.0049 [dilution] + 0.02). The mean recovery of the standard was 87% (n = 17), with a range of 72 to 113%.

The time required to assay one specimen is 3 h and four specimens with two controls can be assayed in one working day. In nine months, seven new cases of neuroblastoma have been diagnosed in our laboratory, of which five had an increased HVA and normal values for vanillylmandelic acid (VMA) and two had above-normal values for both HVA and VMA.

Reference

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Elimination of Pyruvate Interference in the Enzymic Determination of Amylase

To the Editor:
Hanson and Yasmineh (1) recently evaluated the Enzymatic Amylase—DS Reagent (cat. no 682427; Palmar Chemicals, an operation of Beckman Instruments, Inc., Carlsbad, CA 92008) and reported that amylase (EC 3.2.1.1) activity in serum or urine is spuriously decreased by increased pyruvate concentration, leading to grossly erroneous results. They also noted that pyruvate interference varies with reagent lot and with the time elapsed since reconstitution of the reagent. To overcome these problems, they recommend that laboratories establish the concentration of pyruvate that interferes with each different reagent lot under their particular operating conditions—i.e., pyruvate must be measured in all specimens in which amylase is to be measured. Furthermore, if the pyruvate concentration in the sample is found to exceed the tolerance of the Enzymatic Amylase—DS Reagent, it follows that amylase activity would have to be re-assayed by some alternative method that is not affected by pyruvate. All this would increase the laboratories' workload and result in reporting results for amylase.

Their data (1) indicate that the pyruvate interference is caused by the oxidation of NADH during the catalyzed