Labile Glycosylated Hemoglobin Contributes to Hemoglobin A₁ as Measured by Liquid Chromatography or Electrophoresis

David M. Nathan

The utility of glycosylated hemoglobin (HbA₁) measurement as an index of chronic control in diabetes can be adversely affected by interference from a labile glycosylated fraction that changes rapidly with acute changes in blood glucose concentration. I used a "high-pressure" liquid-chromatographic assay and a newly developed electrophoretic assay to quantitate the contribution of this labile fraction. If erythrocytes are incubated at 22 °C in isotonic saline for 12 h before hemolysis, the labile fraction is eliminated. Its contribution is similar as measured by both assays: 2–3.5% of total HbA₁ in normal cells and 7–9.5% in diabetic cells. A 3-h incubation of erythrocytes with glucose produces acute changes in apparent HbA₁ concentrations by both assays, but such changes can be eliminated by the incubation in saline. Although current methods are time consuming, the labile glycosylated hemoglobin must be removed when the sample is prepared for HbA₁ measurement by liquid chromatography and electrophoresis if results are not to be factiously high.

Measurement of the minor glycosylated fractions of hemoglobin—designated hemoglobins A₁₅, A₁₆, and A₁₇, and collectively known as "fast hemoglobin"—provides, in effect, an integrated index of mean blood glucose concentrations during the six to eight weeks before the blood sample is collected (1). Positive interference from a labile glycosylated hemoglobin fraction that changes rapidly with acute changes in blood glucose concentration has been described for some assay methods but not for others (2–4). A new electrophoretic method (Corning Medical and Scientific, Medfield, MA 02052) for measuring the total concentration of fast hemoglobin, derived from the work of Allen et al. (5), has been characterized as reliable, accurate, and efficient, and not affected by minor changes in temperature, buffer pH, or ionic strength (6, 7). While comparing the electrophoretic method with an accepted "high-pressure" liquid-chromatographic (HPLC) method, discrepancies in the results led me to investigate the contribution of the labile glycosylated hemoglobin fraction in each assay.

Materials and Methods

The HPLC method was adapted from that previously described by Cole et al. (8), hemoglobins A₁₅, A₁₆, A₁₇, and A₀ (adult hemoglobin) being separately eluted on the basis of charge differences. I used a Model 110A Liquid Chromatography System (Beckman Instruments, Inc., Fullerton, CA 92634). All assays were performed at 25 °C. The buffers were the same as previously published (8).

The electrophoretic method relies on the electro-endosmotic separation of "fast" from normal adult hemoglobin on agar gels as previously described (6). Supplies for the electrophoretic method are all commercially available (Corning Medical and Scientific).

Procedures

Patients. The specimens of whole blood were obtained from 15 personnel and 40 patients with no history of diabetes and normal blood glucose concentrations during fasting. Diabetic patients (n = 150) were patients of our Diabetes Clinic, being treated by diet, oral agents, or insulin. Samples were obtained at random times during the day.

Hemolyseate preparation for HPLC method. Whole blood was collected in EDTA-containing tubes and stored at 4 °C until the sample was prepared, which was done within 24 h. The samples were centrifuged (1320 X g, 5 min) and all of the supernatant fluid was aspirated and discarded, leaving 0.5 mL of packed erythrocytes. The cells were either washed once with 5 mL of isotonic saline (non-incubated) or incubated for 14 h at 25 °C with 5 mL of isotonic saline (incubated samples). Such incubation of samples eliminates the labile fraction of glycosylated hemoglobin (2). After washing or incubation, the samples were centrifuged and the saline was aspirated. Samples were hemolyzed in 1.5 mL of distilled, de-ionized water, and glycolipids then were extracted into 1.5 mL of toluene by vigorous shaking. To separate the tolule, glycolipid, hemolysate, and cell ghosts the mixture was centrifuged (1320 X g, 4 °C for 30 min). The hemolysate was diluted with phosphate...
buffer (80 mmol/L, pH 6.8) and 20 μL (10 μg of hemoglobin) was applied to the HPLC column.

Sample preparation for the electrophoretic method. The samples for the electrophoretic method were either prepared directly from the anticoagulated whole blood by adding 50 to 150 μL of a hemolyzing reagent (saponin, 1 g/L, in distilled, de-ionized water) according to the manufacturer's directions (non-incubated), or were incubated for 14 h at 25 °C in isotonic saline and then prepared (incubated samples).

In vitro experiments. Erythrocytes were incubated with phosphate-buffered (10 mmol/L) isotonic saline, pH 7.4, and various concentrations of D-glucose at 37 °C for 3 h. The samples were prepared with and without saline incubation and assayed by both methods.

Plasma glucose assays were performed with a glucose oxidase (EC 1.1.3.4) method.

Results

HPLC method. In the absence of incubation in saline, the mean proportions of hemoglobin A\textsubscript{1c} (HgbA\textsubscript{1c}) and total fast hemoglobin (HgbA\textsubscript{1}) concentrations in normal erythrocytes, were 5.07% (SD 0.64%) and 6.78% (SD 1.1%), respectively (Table 1). In the diabetic population, the corresponding values were 9.72% (SD 2.04%) and 11.5% (SD 2.48%). These results agree closely with published results obtained by several different methods (8–11). The between-assay coefficients of variation for a normal and a diabetic sample, based on seven to 10 repeated assays for each, were <3%.

Overnight incubation of samples in isotonic saline decreased the mean HgbA\textsubscript{1c} and A\textsubscript{1} concentrations in the normal and diabetic populations, a greater decrement being observed in the case of the diabetics. In normal erythrocytes, the mean HgbA\textsubscript{1c} and A\textsubscript{1} concentrations of incubated samples were 4.91% (SD 0.78%) and 6.47% (SD 1.0%), respectively. In diabetics, the mean HgbA\textsubscript{1c} and A\textsubscript{1} concentrations decreased to 9.05% (SD 1.86%) and 10.4% (SD 2.17%), respectively. The decrease in HgbA\textsubscript{1} after incubation in saline was 2.3% for the normal hemolysates, 9.6% in the hemolysates from diabetics. There was no statistically significant correlation between plasma glucose concentration and absolute change in HgbA\textsubscript{1} concentration on incubation in saline (r = 0.03).

Electrophoretic method. The electrophoretic method is easily performed and considerably faster than the HPLC method. Eight samples could be assayed as quickly as two by the liquid-chromatographic method. On the basis of eight repeated assays, the inter-assay coefficients of variation for a normal and diabetic sample were 9 and 5.5%, respectively, similar to that reported by Menard et al. (6).

The mean HgbA\textsubscript{1} concentrations of non-incubated samples for normals and diabetics were 6.99% (SD 0.69%) and 12.6% (SD 3.42%). Incubation in saline decreased the mean HgbA\textsubscript{1} concentrations to 6.74% (SD 0.80%) for normals and 11.7% (SD 2.71%) for diabetics, decreases of 3.6% and 7.1%, respectively.

As with the HPLC method, there was no significant correlation of plasma glucose concentrations with HgbA\textsubscript{1} decrease after incubation in saline (r = 0.09).

Correlation of HPLC and electrophoretic methods. HgbA\textsubscript{1} concentrations as determined with the electrophoretic method compared favorably with those by the HPLC method only if similarly prepared samples were compared. HgbA\textsubscript{1} in 40 non-incubated samples (range of percentages: 7–17) analyzed by both methods correlated well (r = 0.944, p < 0.001 by Student's t-test). An almost identical result was obtained (r = 0.940, p < 0.001) when HgbA\textsubscript{1} results for 55 incubated samples assayed with both methods were compared. However, if the samples for HPLC were incubated in saline and the electrophoretic samples were prepared according to the manufacturer's specifications, which do not include a saline incubation, the correlation decreased to r = 0.415 for 43 normal samples and r = 0.83 for 55 samples from diabetics; the differences were great enough to spuriously increase HgbA\textsubscript{1} concentrations that were normal by the HPLC method into

Table 1. Comparison of Non-Incubated and Saline-Incubated Samples Assayed by Two Methods

<table>
<thead>
<tr>
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<th>HPLC</th>
<th>Electrophoresis</th>
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<tbody>
<tr>
<td></td>
<td>Non-Incubated</td>
<td>Incubated (#)</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Normal (n = 40)</td>
<td></td>
<td></td>
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<tr>
<td>A\textsubscript{1c}</td>
<td>5.07 (0.64)(^b)</td>
<td>4.91 (0.78)</td>
</tr>
<tr>
<td>A\textsubscript{1}</td>
<td>6.76 (1.1)</td>
<td>6.47 (1.0)</td>
</tr>
<tr>
<td>Diabetics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A\textsubscript{1c}</td>
<td>9.72 (2.04)</td>
<td>9.05 (1.86)</td>
</tr>
<tr>
<td>A\textsubscript{1}</td>
<td>11.5 (2.48)</td>
<td>10.4 (2.17)</td>
</tr>
<tr>
<td>(n = 115)</td>
<td>(n = 55)</td>
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\(^a\) With isotonic saline, 25 °C, 14 h. \(^b\) % of total hemoglobin (and SD).

*Fig. 1. Measurement of hemoglobin A\textsubscript{1} concentration by liquid chromatography and by electrophoresis after incubation (3 h, 37 °C) with various concentrations of glucose.*

Erythrocytes were then either immediately hemolyzed or incubated with isotonic saline (14 h, 25 °C) before assay. The r value is for the correlation between glucose and hemoglobin A\textsubscript{1} concentrations in the absence of saline incubation.
the diabetic range with the electrophoretic method. This phenomenon occurred most frequently in patients with no known previous history of diabetes who were experiencing stress-related hyperglycemia at the time the blood was sampled. The absolute change in HgbA1 concentration after saline incubation as measured by HPLC correlated well with the absolute change by electrophoresis (r = 0.820, p < 0.05).

In vitro studies. The 3-h incubation of erythrocytes from a normal volunteer with glucose in various concentrations resulted in an increase in HgbA1 concentrations as measured by either HPLC or the electrophoretic method, an increase that correlated significantly with the glucose concentration measured in the medium (Figure 1). Saline incubation of the glucose-exposed cells eliminated the contribution of the labile fraction to the HgbA1 concentration as measured by either assay method.

Discussion
The enthusiasm for measurements of glycosylated protein as an index to chronic blood glucose control has led to the development of numerous clinical assays. As experience with them has accrued, problems have been encountered with each (12), the most common being the recognition of a labile fraction of glycosylated hemoglobin, the proportion of which can change rapidly with acute changes in glucose concentration. The labile fraction, presumably the unstable aldimine formed before the Amadori rearrangement that yields the stable ketoamine structure, has been observed both in animal models of diabetes and in man (2, 4, 13). The presence of such a rapidly changing fraction measured in the HgbA1 fraction detracts from the clinical usefulness of glycosylated hemoglobin assay. Nondiabetic patients with transient hyperglycemia associated with stress and otherwise well-controlled diabetics with a few hours of hyperglycemia, will have spuriously increased hemoglobin A1c concentrations because of the presence of the labile fraction. This paper demonstrates the need to eliminate the labile fraction before assay by either of two techniques.

Goldstein et al. (2) and others (3) have demonstrated that incubation of the erythrocytes in saline or dialysis of the hemolysate against saline can effectively eliminate the contribution of this labile fraction in the liquid chromatographic method. Using similar methods to eliminate the labile fraction, I have shown its contribution to be quantitatively similar as measured by either of the two different methods, but I found no correlation between the decrement in HgbA1c on incubation in saline and the in vivo glucose concentration as Goldstein et al. did. This discrepancy may be due to the kinetic pattern of labile HgbA1 synthesis and disposal, to the poor predictive value of a single glucose determination, or to some other factor.

Our experience with the electrophoretic method was similar to that of Hayes et al. (7). The assay compares well with the HPLC method if the sample is prepared in the same way for both assays. Advantages of the electrophoretic method include speed, reproducibility, and simplicity. Unfortunately, the need to incubate samples with isotonic saline to remove the labile fraction complicates the procedure, but is necessary to preserve the clinical utility of HgbA1c measurement. More rapid methods of eliminating the labile fraction are under active investigation.

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References