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## Editorial



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### National Diabetes Data Group: Report of the Expert Committee on Glucosylated Hemoglobin

**T**he slow chemical reaction between glucose and proteins leads to continuous production of non-enzymatically glucosylated proteins in blood and tissues. The steady-state level of glycosylation of specific proteins is proportional to both the average glucose concentration and the lifespan of the protein in the circulation or tissues. Because the level of glucosylated hemoglobin has been shown to provide an index of blood glucose concentration during the previous 1–2-mo period, it is being used increasingly in the clinical management of diabetes. There has been considerable confusion in this field regarding terminology, methodology, and interpretation of assays of glucosylated hemoglobin. In this report we review the relative merits and limitations of various procedures that have been developed for the measurement of glucosylated hemoglobins. We have attempted to identify preferred methods and reliable standards for the accurate and reproducible measurement of glucosylated hemoglobins. In addition, we present an assessment of the current value of glucosylated hemoglobin measurements in the detection and management of diabetes and guidelines for the use and interpretation of these measurements.

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#### THE MODIFIED HEMOGLOBINS

In normal human red blood cells, HbA ( $\alpha_2 \beta_2$ ) comprises over 90% of total hemoglobin. About 8% of hemoglobin is altered by various post-translational modifications. Negatively charged minor components have been identified by both electrophoresis and ion exchange chromatography and have been designated hemoglobins A<sub>1c</sub>, A<sub>1b</sub>, A<sub>1a2</sub> and A<sub>1a1</sub> in order of increasing negative charge. Collectively these hemoglobins have been designated HbA<sub>1</sub> (see APPENDIX). The chromatographic peak commonly designated HbA<sub>1c</sub> accounts

for about 4–6% of total hemoglobin and consists primarily (60–80%) of a stable covalent derivative containing glucose attached nonenzymatically to both  $\beta$ -chain N-terminal valine residues by a ketoamine linkage. This peak also contains varying amounts of chemically modified but nonglucosylated hemoglobin. Thus, about 3% of total hemoglobin in normal red blood cells contains glucose attached at the  $\beta$ -chain amino terminus. Normal red blood cells also contain about 0.3% of a labile precursor, commonly called pre-HbA<sub>1c</sub>, in which glucose is attached to the N-termini of the  $\beta$ -chain by a Schiff base linkage. In addition, glucose forms ketoamine adducts at other sites on hemoglobin, including certain lysine residues and the N-termini of the  $\alpha$ -chains. These modified hemoglobins cannot be separated from HbA<sub>0</sub> by ion exchange chromatography or by electrophoresis but are separable by affinity chromatography on phenylboronate resins. They comprise another 3–5% of hemoglobin in normal red blood cells and their concentrations are elevated in parallel with HbA<sub>1c</sub> in diabetic red blood cells. Together, glucose adducts at the  $\alpha$ - and  $\beta$ -N-termini and at lysine residues comprise the glucosylated hemoglobins. Hemoglobins A<sub>1a1</sub>, A<sub>1a2</sub>, and A<sub>1b</sub> contain modifications at the N-termini of the  $\beta$ -chains; their structures are not completely characterized. In addition to the glucosylated hemoglobins, other negatively charged components are found in normal as well as pathologic red blood cells including HbF ( $\alpha_2 \gamma_2$ ) and hemoglobins covalently modified by compounds such as cyanate, acetaldehyde, and certain drugs such as acetylsalicylic acid.

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#### NOMENCLATURE

Because of rapid developments in methodologies for measuring glucosylated hemoglobins and differences in what is actually measured in various assay procedures, there has been confusion and inconsistency in nomenclature in this field. Difficulties with nomenclature have arisen largely from the interplay between operational and structural characterizations of modified hemoglobins. Thus, HbA<sub>1c</sub> was originally described operationally as a unique chromatographic or fast electrophoretic species of hemoglobin. However, after the structural identification of glucose at the  $\beta$ -chain N-terminal

valine residue, the terms HbA<sub>1c</sub> and glycosylated hemoglobin were often used interchangeably. It is now recognized that other species of hemoglobin, including HbA<sub>0</sub>, contain glucose bound in ketoamine linkage to lysine residues of both the  $\alpha$ - and  $\beta$ -chains, and also that chromatographically isolated HbA<sub>1c</sub> contains hemoglobin species other than the adduct of glucose at the  $\beta$ -N-terminus. A similar confusion was introduced by the use of HbA<sub>1</sub> or "fast hemoglobin" as a synonym for glycohemoglobins. The terms are not equivalent since modifications other than glycosylation may generate fast hemoglobins, and glycosylation of lysine residues in hemoglobin does not yield fast hemoglobins.

To clarify the confusion and imprecision in the use of nomenclature referring to modified hemoglobins in diabetes, we present a glossary of definitions (see APPENDIX) based on current understanding of the structure and properties of these species. Use of a uniform and consistent nomenclature is essential for effective communication and comparison of results.

#### METHODS FOR MEASUREMENT OF GLUCOSYLATED HEMOGLOBINS

**Assay precision.** A number of methods are available to measure glycosylated hemoglobin. Most of the available assays have been shown to provide acceptable precision when performed correctly. The results of the various procedures appear to correlate strongly with one another and thus should be equally useful for monitoring blood glucose control. Each method has specific advantages and disadvantages for the clinical laboratory. For the individual laboratory, the choice of a method will depend on a number of factors including available equipment and expertise, cost, number of samples to be analyzed, and transport requirements. Problems with poor assay precision over time have created skepticism about the clinical utility of glycosylated hemoglobin. These problems can be best addressed by careful attention to quality control. Regardless of the specific assay method employed, optimal clinical utility of glycosylated hemoglobin necessitates a high degree of precision. This is dictated by the fact that glycosylated hemoglobin is an indirect measure of mean blood glucose concentration; each 1% change in glycosylated hemoglobin reflects a large change in mean plasma glucose (25–35 mg/dl). Thus, it is important to have a measurement technique that can resolve small differences in glycosylated hemoglobin levels. It is highly desirable to have an assay with intra- and interassay coefficients of variation <5%. Each assay method should include a set of carefully maintained internal controls appropriate to the specific assay. The controls should include a minimum of two samples, one with glycosylated hemoglobin in the normal range and the other in the diabetic range. Each laboratory should establish its own nondiabetic (normal range) reference interval. Carefully performed studies indicate that this interval is relatively narrow ( $\leq 2\%$  glycosylated hemoglobin, e.g., HbA<sub>1c</sub> 4–6%).

Assays can be divided into several categories based on the method of differentiating glycosylated and nonglycosylated

hemoglobin species. The principles involved include: (1) separations based on charge differences, (2) determinations based on chemical analysis, and (3) separations based on structural differences.

**Methods based on charge differences.** Ion exchange chromatographic methods include procedures ranging from those that employ disposable microcolumns and measure HbA<sub>1c</sub> to expensive, high-performance automated systems that quantify several minor hemoglobin species. Electrophoretic methods may employ various stationary phases, including citrate agar or isoelectric focusing. All of these methods require strict attention to assay-specific details. For example, ion exchange chromatographic methods are very sensitive to small variations in temperature, pH, and ionic strength. Agar gel electrophoretic and isoelectric focusing methods require special attention to consistency of the solid-phase preparation, sample loading, and the quantification by densitometric scanning.

Each of the methods that separate hemoglobins based on charge differences are subject to interference by a wide variety of modifications of hemoglobin that result in similar changes in charge. For example, glucose forms a reversible adduct with hemoglobin (labile adduct, or "pre-HbA<sub>1c</sub>"). This adduct is always present in blood and its level depends on ambient blood glucose concentration. Failure to remove this fraction before assay will result in variable and falsely elevated test results. All of these methods may also be greatly affected by inappropriate sample handling and storage. Since both the accuracy and reproducibility of ion exchange and electrophoretic methods are affected by the pre-HbA<sub>1c</sub> fraction, these assays should be performed only on samples suitably treated to remove pre-HbA<sub>1c</sub>. Clinicians using commercial laboratories should be familiar with the assay procedures employed and should establish that interference by pre-HbA<sub>1c</sub> is excluded. In turn, commercial laboratories should be directed to employ procedures that exclude interference by the pre-HbA<sub>1c</sub> fraction.

**Methods based on chemical analysis.** At the present time, the only available chemical method that is practical for the clinical laboratory is the hydroxymethylfurfural thiobarbituric acid (TBA) colorimetric test. Sugar released from hemoglobin during acid hydrolysis is quantitated by its color reaction with thiobarbituric acid. This assay is more specific for glycosylated hemoglobins than are methods based on charge differences and it provides a measure of glucose bound to both valine and lysine residues. The method is minimally affected by sample handling and storage conditions. Consistency in results requires elimination of free glucose and careful standardization of assay conditions, since the assay is nonstoichiometric. Fructose can be included as a standard for the assay.

**Methods based on structural differences.** Affinity chromatography using immobilized m-phenyl boronic acid separates glycosylated and nonglycosylated hemoglobins based on the formation of complexes between the immobilized phenylboronic acid and cis-diols in glucose-modified hemoglobin

species. The assay appears to be very specific for glycosylated hemoglobin and is minimally affected by sample handling and storage conditions, temperature, or pre-HbA<sub>1c</sub>. Results to date are promising, but clinical experience with the method is limited. The assay may be sensitive to minor changes in gel properties. Recent preparations of gels appear to show more consistency in performance than did earlier preparations. Gels from different manufacturers and different lots from the same manufacturer may be quite different.

*Assay standards.* The diversity of methods to determine glycosylated hemoglobin and procedural modifications and variations in these methods make the availability of a stable standard highly desirable. This standard should be stable to long-term freezing at  $-20^{\circ}\text{C}$  or storage in closed vials at  $4^{\circ}\text{C}$ , and should also be stable in open vials during routine laboratory use. In addition the optimal standard should give reproducible results, while the major methods should be used for determining post-translational modifications of hemoglobin (HPLC, disposable columns, TBA test, and affinity chromatography). Such a standard would permit comparison of results obtained by different methods in different laboratories and ensure long-term assay accuracy and precision. The optimum clinical utility of glycosylated hemoglobin measures cannot be appreciated until practical standards and reference systems are instituted.

#### CLINICAL APPLICATIONS

A major reason for scientific interest in the measurement of glycosylated hemoglobin is the potential application of this methodology to the assessment of the metabolic status of patients with diabetes mellitus. The major premise of this thesis is that the level of glycosylated hemoglobin is an accurate index of the integrated level of blood glucose in the recent weeks to months before testing; there is growing evidence in support of this premise. In the past several years, however, the clinical validity of glycosylated hemoglobin determination has come under criticism, in part because of a lack of reliability and reproducibility of the methods used and a less-than-full appreciation of the chemistry involved.

A reliable, reproducible standardized method of measuring glycosylated hemoglobin can be of considerable clinical utility in several situations: (1) The determination is invaluable in research projects where information on average blood glucose is an important contribution to a particular study; for example, when comparing groups of patients with diabetes or the levels in a given patient over time. The potential for using glycosylated hemoglobin measurement in the research area is enormous and includes such important topics as the natural history of diabetes mellitus, patient care assessment, and therapeutic interventions. (2) Measurement of glycosylated hemoglobin is useful in the ongoing care of patients with diabetes. It should be emphasized that measurement of glycosylated hemoglobin does not reflect the level of blood glucose in the patient's immediate past (hours to days) and should not be used to make clinical decisions related to the

immediate clinical situation (e.g., acute modification of insulin dose). The test does provide information about the average blood glucose of a given patient over the previous weeks to months and may be, in this context, a useful adjunct to the day-to-day assessment of blood glucose regulation afforded by blood and urine determinations. When the glycosylated hemoglobin value does not correspond to the physician's impression of the patient's metabolic status, further investigation is generally warranted.

A number of other areas in the evaluation and treatment of diabetes have been offered as potential areas of application of glycosylated hemoglobin measurements. One potential application is in the diagnosis of diabetes, but more data are needed to justify this application. Further study is also required to evaluate correlations between hemoglobin measurements and the complications of diabetes.

#### SUMMARY

*Nomenclature.* Standardized, well-defined nomenclature is essential for effective communication of the results of basic and clinical research on glycosylated hemoglobin in diabetes. Proper use of the terms defined in the APPENDIX should eliminate some problems and confusion in the literature.

*Test procedures.* A wide variety of assays and procedures is available for measuring glycosylated hemoglobins. When properly performed, suitable precision, long-term reproducibility, and correlation with glycemic control is achievable with each of the three basic types of assays, i.e., those based on charge or structural differences or on chemical analysis of hemoglobin. No single assay procedure appears to be clearly superior to others at this time, and excellent correlations are obtained among the results of each of the assay procedures. Thus, the selection of a method should be based on the expertise, equipment, and special needs of the individual laboratory.

*Assay precision and long-term reproducibility.* Problems with assay precision and reproducibility in the past have created undue skepticism about the clinical utility of glycosylated hemoglobin measurements. At present, intra- and interassay coefficients of variation of  $<5\%$  are attainable, and should be achieved in any laboratory performing these measurements. Interference by pre-A<sub>1c</sub> is a major source of variation in the determination of HbA<sub>1</sub> by ion exchange or by electrophoretic methods, and should be removed before these assays.

*Assay standards.* The development of a stable reference standard, useful in all types of glycosylated hemoglobin assays, is important to ensure long-term assay reproducibility and to facilitate the comparison of results between laboratories. The development of this standard should be identified as a major goal of future research.

#### APPENDIX: HEMOGLOBIN NOMENCLATURE

HbA. The major adult form of hemoglobin, a native unmodified tetramer consisting of two alpha and two beta chains ( $\alpha_2\beta_2$ ).

**HbA<sub>0</sub>.** The major component of HbA identified by its chromatographic and electrophoretic properties. Post-translational modifications, including glucosylation, do exist in this fraction but do not significantly affect the charge properties of the protein.

**HbA<sub>1</sub>.** Post-translationally modified, more negatively charged forms of HbA<sub>0</sub>, separable from HbA<sub>0</sub> by chromatographic and electrophoretic methods.

**HbA<sub>1a1</sub>, HbA<sub>1a2</sub>, HbA<sub>1b</sub>, HbA<sub>1c</sub>.** Chromatographically distinct components of HbA<sub>1</sub>.

**HbA<sub>1c</sub>.** Composed primarily of the adduct of glucose attached to the β-chain terminal valine residue by a ketoamine linkage.

**pre-HbA<sub>1c</sub>.** A labile form of glucosylated hemoglobin containing glucose attached by aldimine linkage at the β-chain terminal valine residue.

**"Fast" hemoglobin(s).** The total HbA<sub>1</sub> fraction, which, because of more negative charge, migrates more rapidly toward the anode on electrophoresis and elutes earlier on cation exchange chromatography than HbA<sub>0</sub>.

**Glucosylated hemoglobin.\*** Hemoglobin modified by glucose at α- and β-chain terminal valine residues and ε-amino groups of lysine residues.

**Glycosylated hemoglobin.\*** A generic term for hemoglobin containing glucose and/or other carbohydrate(s).

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\*Hemoglobin that chromatographs as HbA<sub>1</sub> or HbA<sub>1c</sub> is often referred to as glycohemoglobin or glycosylated hemoglobin. This is an inappropriate use of terminology since only a fraction of HbA<sub>1c</sub> is glucosylated hemoglobin. The glucosylated hemoglobin component of HbA<sub>1c</sub> actually varies from 60% to 90%, depending on the resolution of the chromatographic system employed. Because the nonenzymatic chemical linkage between sugar and proteins differs from that of enzymatic glycosylation of proteins, the term glycation has recently been recommended for the former species.

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