Review
Evaluation of Glycosylated Hemoglobin in Diabetic Patients

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Measurement of glycosylated hemoglobin has gained increasing use in the management of patients with diabetes. A number of clinical studies have demonstrated that HbA_{1c} is a reliable index of diabetic control.[1-5] Because of recent advances, such as the development of programmable pumps for the continuous administration of insulin, improved control is now feasible and can be documented by a reduction in HbA_{1c}.[6-8] With increasing experience in measurement of HbA_{1c}, a number of clinical investigators have expressed reservations about its utility in the assessment of diabetic control. Specifically, the finding of rapid changes in the level of HbA_{1c}[6-9] has raised questions concerning the kinetics of its formation.

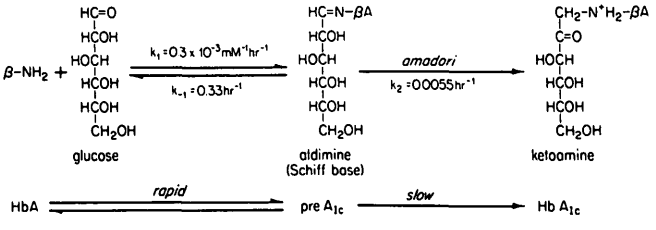
In this brief review, I will discuss the structure of glycosylated hemoglobins and the mechanisms of synthesis. An understanding of the various factors responsible for the formation of glycosylated hemoglobin is useful in comparing the various methods utilized for its measurement as well as in interpretation of results.

STRUCTURE AND SYNTHESIS

HbA_{1c} is the most abundant minor component of hemoglobin in normal red cells and is elevated as much as threefold in diabetic red cells. The term A_{1c} should be reserved for HbA, which has glucose attached to the N-terminus of the β-chain by a ketoamine linkage.[10-12] As shown in Figure 1, glucose is also linked in the same way to other sites on the hemoglobin molecule, such as the N-terminus of the α-chain and certain lysine residues,[13,14] but these modified components cannot be separated by conventional chromatographic or electrophoretic techniques. In diabetic patients, such glucose adducts are increased in parallel with HbA_{1c}.[15] In addition, small amounts of negatively charged hemoglobins (HbA_{1a1} and HbA_{1a2}) are formed by the attachment of sugar phosphates to the N-terminus of the β-chain.[16] HbA_{1b} may be a further modification of HbA_{1c}. Unlike the glucose adducts, these other minor components are not reliably elevated in hyperglycemic patients.

Biosynthetic studies in vivo indicate that HbA_{1c} (as well as the other glycosylated minor components) is formed slowly and continuously throughout the life span of the red cell.[18] Thus, levels of HbA_{1c} should provide an integrated measure of blood glucose levels over the preceding 2–3 mo. This is the rationale for its use in the assessment of diabetic control.

Recent experiments have delineated the chemical steps involved in the synthesis of HbA_{1c}.[19] As shown in the following scheme, the aldehyde group of glucose forms a reversible Schiff base linkage with the N-terminus of the β-chain. This labile adduct, pre-A_{1c}, can then undergo an Amadori rearrangement to the stable ketoamine (A_{1c}). The presence of the aldimine and the kinetics of its formation and dissociation have been documented by incubations of HbA with purified [^{14}C]-D-glucose in the absence and presence of cyanoborohydride, a reducing agent that selectively traps the adduct in the Schiff base form. Rate constants shown in the above diagram were derived from these experiments. The aldimine (pre-A_{1c}) is sixty times more likely to dissociate to HbA and free glucose than to undergo the Amadori rearrangement. From a knowledge of the life span of the red cell, the mean blood glucose level, and the three rate constants (shown above), the levels of pre-A_{1c} and A_{1c} in normal



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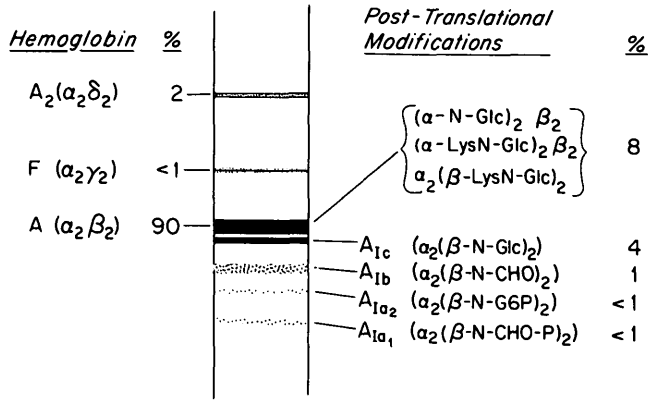


FIGURE 1. Hemoglobin components in normal human red cells, separated by isoelectric focusing. Glycosylated hemoglobins are shown on the right.

red cells are estimated to be 0.4% and 4%, respectively, values that are in good agreement with direct measurements of the reversible and stable components of HbA_{1c} (see below).

METHODS FOR MEASURING GLYCOSYLATED HEMOGLOBIN

Since many clinicians and investigators are rightfully confused by the variety of techniques that are in use or that have been proposed, it is worthwhile to review these methods and compare their relative merits and drawbacks (Table 1).

Chromatographic techniques. Ever since glycosylated hemoglobins were first isolated by chromatography on amberlite (Biorex 70),[20] use of this cation exchange resin has been the method most often applied and, therefore, the standard to which other assays should be compared. Most chromatographic methods used in the clinic detect not only HbA_{1c} but also the other glycosylated hemoglobin components HbA_{1a1}, HbA_{1a2}, and HbA_{1b}.[16] In addition, the rapid assays also include pre-A_{1c}. The combination of these minor components are often called HbA_1 or "fast hemoglobin". Chromatographic methods are necessarily cumbersome and time consuming, but these concerns are minimized by the availability of simple and inexpensive disposable columns.[21,22] The reproducibility of these analyses depends upon maintenance of reasonably uniform temperatures (± 1°C).[23] Laboratories that have a large demand for HbA_1 measurements should consider a high performance liquid chromatography system that can be completely automated and provides highly reliable data with a turnaround time of approximately 20 min.[24,25] Interpretation of chromatographic data is thwarted by increased levels (>0.5%) of HbF, which co-chromatographs with HbA_{1c}, and by hemoglobin variants such as S,[26,27] C, D, G, etc. In addition, falsely high values for HbA_1 are obtained in patients with uremia,[28,29] and possibly in alcoholics[30] and patients with lead poisoning[31] or on high doses of aspirin.[32] Lactescent plasma will also cause falsely high values if whole blood is analyzed by a rapid chromatographic assay.[33]

Electrophoretic techniques. Two electrophoretic methods have been adapted for measuring HbA_{1c}. Isoelectric focusing on thin (1 mm) slabs (but not cylinders) of polyacrylamide gel provides a clean separation of HbA_{1c} from HbA_0[32,34] but the method requires a moderate level of technical skill and a high quality densitometer for quantitation. Electrophoresis on agar gel at pH 6.5[35,36] provides a much wider separation than that obtained by gel electrofocusing (see Figure 2), and quantitation is easier. Therefore agar gel electrophoresis is an attractive alternative to column chromatography for the routine measurement of HbA_{1c}.

Colorimetric method. Flückiger and Winterhalter[11] have devised a colorimetric test taking advantage of the fact that when A_{1c} is subjected to mild acid hydrolysis, 5-hydroxymethylfurfural (5-HMF) is released and can combine with thiobarbituric acid (TBA) to form a colored product. This method has been adapted for routine use[37–39] and generally gives reliable results. The TBA test has several advantages over chromatographic and electrophoretic methods. Because it is specific for ketoamine linked glucose, it is unaffected by the presence of HbF, hemoglobin variants, other post-translational modifications of hemoglobin, and, most importantly, pre-A_{1c}. Therefore, no preparation of the hemolysate is necessary to rid the specimen on pre-A_{1c}. The test requires only a simple colorimeter and inexpensive stable reagents. However, the TBA test is difficult to standardize because the yield of 5-HMF from HbA_{1c} is only about 30% and may be considerably less for glycosylated HbA.[13]

TABLE 1
Comparison of commonly used methods to measure glycosylated hemoglobin

Method	Precision	False increase due to	False decrease due to
Chromatographic		Pre-A_{1c}, A_{1a1}, A_{1a2}, A_{1b}. HbF, HbG, and other negatively charged hemoglobins. Uremia; ? alcoholism; ? high dose aspirin; ? lead poisoning. Lactescent plasma. Storage of hemolysate.	HbS, HbC, HbE, and other positively charged variants
Disposable columns	Good		
HPLC	Excellent		
Electrophoretic			
Gel electrofocusing	Good	Pre-A_{1c}	
Agar gel electrophoresis	Good		
Colorimetric (TBA test)	Good	Baseline color absorbance. De novo formation of 5-HMF in sample containing high glucose.	

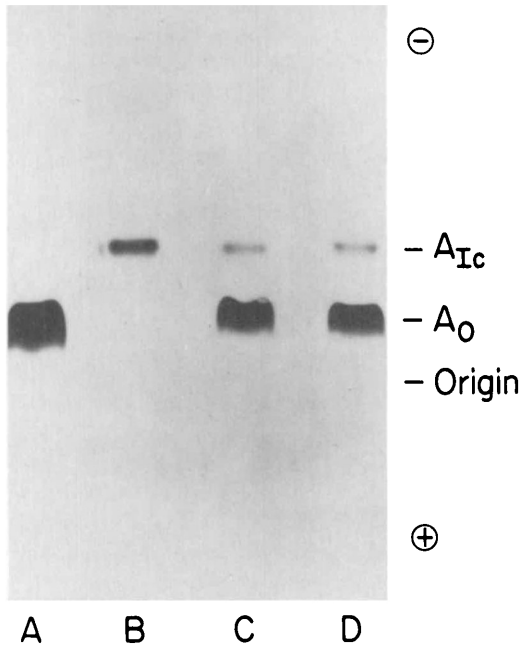


FIGURE 2. Separation of HbA_{1c} and HbA by agar gel electrophoresis in 0.1 M citrate, pH 6.3. (A) Purified HbA_0. (B) Purified HbA_{1c}. (C) Artificial mixture of HbA_{1c} and HbA_0 in a 1:20 ratio. (D) Normal hemolysate.

Falsely high values may result because of the de novo formation of 5-HMF as a result of condensation of free glucose (in high concentration) with hemoglobin.[40] Furthermore, the precision of the TBA test is limited by a variable degree of background color absorbance from nonglycosylated hemoglobin.

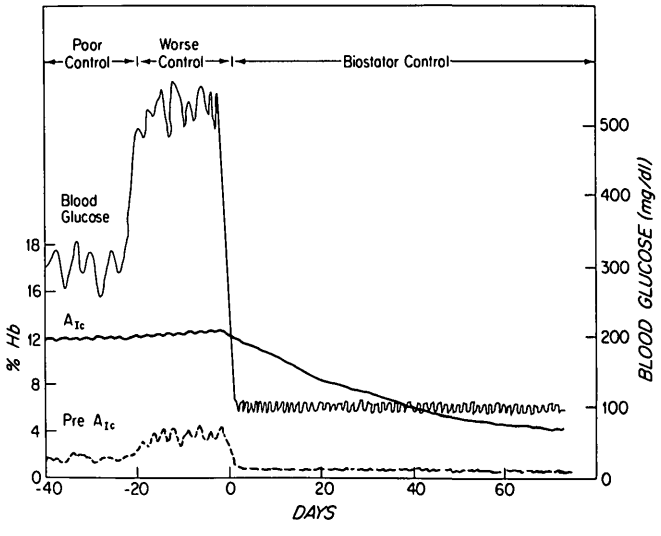
Other approaches that have been used to measure HbA_{1c} include a radioimmune assay[41] and a spectrophotometric test that takes advantage of the absorbance changes that occur when hemoglobin binds to organic polyphosphates.[42] Thus far, practical experience with these methods has been limited.

INTERPRETATION OF GLYCOSYLATED HEMOGLOBINS

Pre-A_{1c}. The kinetics shown above bear directly on interpreting clinical measurements of glycosylated hemoglobin. Most clinical assays involve the direct application of a blood sample or hemolysate to the column and the rapid elution of HbA_1 5–20 min later. According to the rate constant shown above for the dissociation of pre-A_{1c} to HbA_0 and glucose (k_{-1} = 0.33 h^{-1}), about 2 h would be required for half of the pre-A_{1c} to dissipate. Most column assays are run at room temperature and, therefore, the rate of dissociation of pre-A_{1c} should be even slower. Thus, nearly all of the pre-A_{1c} will remain intact during the rapid assay of glycosylated hemoglobins.

The interpretation of HbA_1 is not adversely affected by the inclusion of HbA_{1a1}, HbA_{1a2}, and HbA_{1b}. As shown in Figure 1, the sum of these components is much less than HbA_{1c} and does not change much in diabetes. In contrast, the inclusion of pre-A_{1c} in the measurement of A_1 or A_{1c} confuses the interpretation of the test. A number of puzzling clinical and experimental observations can be explained by taking pre-A_{1c} into account. First, the rather wide variation that has been reported for the normal level of glycosylated hemoglobin is largely due to the presence of varying amounts of pre-A_{1c}. The level tends to be high when a rapid column method is used, while the lowest normal values that have been reported have come from a prolonged column procedure that measures only HbA_{1c}, and not A_{1a1}, A_{1a2}, A_{1b}, or pre-A_{1c}.[16] Secondly, the level of total A_{1c} decreases about 10% after thorough dialysis of hemolysate[7,43] or after saline incubation of red cells.[44] Therefore, one of these two maneuvers should be used to rid the hemolysate of pre-A_{1c}. These experimental findings are in accord with the above prediction that in the absence of recent fluctuations in blood sugar, about 10% of the total A_{1c} should be in the labile precursor form pre-A_{1c}. Furthermore, the rate of decay in HbA_{1c} observed by Goldstein et al.[44] after incubation agrees well with the above value for k_{-1}(0.33 h^{-1}). Finally, the rapid fall in glycosylated hemoglobin within a day of instituting rigorous diabetic control can be explained by a prompt reduction in pre-A_{1c}. For example, when patients have been treated with an artificial beta-cell device (Biostator), prompt decreases in total A_{1c} of about 8%[6,8] have been observed. In poorly controlled diabetic patients who have wide fluctuations of blood glucose, the level of pre-A_{1c} can be highly variable. In contrast, the level of HbA_{1c} is very stable and changes very slowly. A hypothetical illustration is shown in Figure 3. When a brittle diabetic goes into a period of deteriorating control, the increased degree of hyperglycemia will be promptly reflected in higher levels of pre-A_{1c}. Upon institution of meticulous control with a Biostator device, the level of pre-A_{1c} should promptly fall to normal values. During these periods of wide fluctuations in blood sugar, the level of HbA_{1c} should remain nearly constant. The detailed clinical study of Compagnucci et al.[45] elsewhere in this issue verifies these predictions. In all patients the levels of A_{1c} remained constant during the period of observations. Fluctuations in "total A_{1c}" were due to variable amounts of a reversible component, which we know is the aldimine pre-A_{1c}. These investigators point out that in patients with relatively stable concentrations of blood sugar, the level of HbA_{1c} can be accurately predicted from random blood sugar measurements. In contrast, in patients with widely fluctuating blood

FIGURE 3. Hypothetical diagram showing the levels of blood glucose, HbA_{1c} and pre-HbA_{1c} in a patient with brittle diabetes who undergoes a period of increased hyperglycemia and then rapidly becomes normoglycemic when treated with a Biostator device.



glucose, the measurement of HbA_{1c} is much more valuable in providing an assessment of diabetic control not available from random blood sugar measurements.

Aging of red cells in vivo. The model for the formation of HbA_{1c} based on the above kinetic constants predicts that HbA_{1c} should accumulate in a nearly linear fashion as the red cell ages in vivo. When young and old red cells are separated by density centrifugation, the level of HbA_{1c} does increase directly with cell density,[46,47] but the change is not linear and does not extrapolate to zero in the least dense fraction. These findings can be explained by the fact that even the best techniques afford only a partial separation according to cell age[48] and that pre-A_{1c} makes up a relatively larger proportion of the total A_{1c} in the young cell population. Therefore, these studies do not imply either that glycosylation is favored in younger red cells or that the formation of HbA_{1c} is reversible.

The interpretation of glycosylated hemoglobin is predicated on the assumption of a normal red cell life span. Patients with hemolysis have a significant reduction in HbA_{1c}.[19,49] Diabetics may have a slight reduction of red cell life span,[50] particularly those with uremia or microangiopathic red cell morphology.[51] The observation that the slope of HbA_1 versus blood glucose is lower in diabetics than in normals may be due to slightly shortened life span in diabetic red cells rather than to a "saturable system."[5]

GLYCOSYLATED HEMOGLOBIN AND DIABETIC COMPLICATIONS

In view of the availability of open- and closed-loop systems for the institution of improved diabetic control, the measurement of glycosylated hemoglobins may prove useful in answering one of the fundamental questions in diabetes: Are the long-term complications of the disease related to the degree of control of hyperglycemia? Studies designed to answer this question should be concerned with the relative advantages and pitfalls of various methods used to measure glycosylated hemoglobin, as well as the interpretation of the results. On a more theoretical level, the same chemical reactions that are involved in glycosylation of hemoglobin may cause similar modifications in tissues responsible for diabetic complications. A number of proteins besides hemoglobin are modified by nonenzymatic glycosylation, particularly in diabetics. Examples include proteins of the red cell membrane[52,53] albumin and other serum proteins,[54–57] collagen,[58–61] and lens crystallins.[62–65] Recently, Cohen and colleagues[66] showed increased levels of ketoamine-linked glucose in the collagen of glomerular basement membrane from diabetic rats. The key question is whether or not these modifications have any effect on the functional behavior of the tissues so modified. The fact that these linkages are nearly irreversible may lead to a cumulative effect. Furthermore, ketoamine-linked glucose may be responsible for covalent cross-links in proteins,[67] thereby causing further alterations in function. Even though nonenzymatic glycosylation is a slow and seemingly innocuous modification, it has the potential for imposing significant functional alterations in proteins that turn over slowly and are exposed to high levels of glucose. A great deal of careful clinical and laboratory research is needed to unravel this puzzle.

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