Glycated Protein Update: Implications of Recent Studies, Including the Diabetes Control and Complications Trial

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On the basis of the results of the Diabetes Control and Complications Trial (DCCT), the American Diabetes Association (ADA) now recommends tight control of blood glucose to near-normal concentrations as the primary goal for most eligible insulin-dependent diabetic patients. In the DCCT, intensive therapeutic intervention was based on frequent self-monitoring of blood glucose and monthly measurements of glycohemoglobin. The importance of glycohemoglobin assessments serves to highlight the present inadequacies in laboratory measurements of this analyte, which hinders wide implementation of the ADA recommendations. Clinical interventions aimed at achieving the DCCT's published therapeutic goals may place patients at a significantly increased risk for life-threatening hypoglycemia, if the therapy is based on nonstandardized laboratory results. Clinical laboratories will now be under increasing pressure to provide reproducible, standardized measurements of glycohemoglobin, a goal that recent research has shown to be realistic, if widespread interlaboratory calibration is adopted. Finally, recent advances in measuring glycated serum proteins appear to warrant reevaluation of such assays during future intensive therapy trials, as potentially important tools for fine-tuning tight blood glucose control.

Indexing Terms: glycohemoglobin/blood glucose/fructosamine

Diabetes mellitus is estimated to affect >14 million people in the US (1), with a total economic burden exceeding $40 billion per year. Despite the discovery of insulin >70 years ago, there remains a high incidence of severe debilitating complications, including retinopathy, neuropathy, renal failure, and atherosclerosis. It has long been believed that tight control of blood glucose would decrease diabetes-induced sequelae, but until recently this had been demonstrated only in gestational diabetes mellitus (GDM) (2). The Diabetes Control and Complications Trial (DCCT) (3, 4) now confirms a direct relationship between the degree of blood glucose control and the risk of late renal, retinal, and neurological complications in patients with insulin-dependent diabetes (IDDM).

The DCCT study of 1441 patients evaluated the effects of conventional vs intensive blood glucose control regimes over an average of >6 years. The goal in the intensive therapy group was to maintain near-normal concentrations of blood glucose. This was achieved by self-monitoring of blood glucose and injections of insulin at least four times daily, and required highly motivated patients who interacted frequently with a team of health care workers. Patients were seen by a physician once a month, at which time blood glucose control was confirmed by measurement of glycohemoglobin (gHb). At the end of the study the intensive therapy group revealed 34–76% lower incidence of clinically meaningful retinopathy, 60% less neuropathy, and 56% less albuminuria than controls treated conventionally, albeit at the cost of a threefold increase in severe hypoglycemic episodes (4).

On the basis of these data, the American Diabetes Association (ADA) now recommends as optimum therapy for eligible insulin-dependent diabetics, blood glucose control at least equal to that achieved in the intensive therapy cohort, and furthermore suggests that the same may be true for patients with non-insulin dependent diabetes (NIDDM) (5). Similar intervention has been previously recommended for GDM (6).

Physicians will now be under increasing pressure to treat all IDDM patients, unless specifically contraindicated, with intensive glucose-control programs. This will have a major impact on clinical laboratories, given that gHb measurements played a central role in the DCCT. The selection of gHb measurements as a means of monitoring diabetic control was based on an extensive literature documenting its tight correlation with concentrations of blood glucose averaged over time (7). Thus the participating physicians set their patients' therapeutic goals principally in terms of a target gHb value, and assessed long-term diabetic control by this index. The DCCT results vindicated this approach by showing that in patients receiving intensive intervention therapy, the risk of progressive retinopathy, a major complication of diabetes, was directly proportional to the average gHb concentration. Moreover, the risk of life-threatening hypoglycemia was inversely related to the gHb value (4).
If the ADA recommendations are widely adopted, there will be a greatly increased need for accurate and precise assays that reflect blood glucose concentrations integrated over time. Although gHb is an excellent measure of average glycemia, clinical laboratories employ many diverse methods to quantify it, some of which are labor-intensive, slow, relatively expensive, and imprecise. Furthermore, most assays are not well standardized (8–11), such that results from one laboratory are not necessarily interchangeable with those from another. Therapeutic alterations based on such technical discrepancies may place patients in intensive treatment regimes at significant risk of life-threatening hypoglycemia. The DCCT, to avoid this potential danger, employed a single core laboratory for all assays of gHb. This arrangement is clearly not suited to general use.

A second issue raised by the DCCT is the problem that gHb reflects blood glucose concentrations averaged over the previous 6–8 weeks (7). If patients are to be assessed more frequently than this, there is the possibility that assays more sensitive to rapid blood glucose fluctuations may provide significant additional information. Over the last decade several assays of glycated serum proteins have been proposed and evaluated for this purpose, but their clinical utility remains controversial.

The central role of tight control of blood glucose in intensive intervention programs now necessitates a major reassessment of the manner in which laboratories assay and report results for glycated proteins (including gHb). We propose that there is now an urgency for laboratories to provide more accurate and precise measures of gHb, and to reevaluate the potential role of other glycated serum proteins in monitoring therapy of diabetic patients.

**Chemistry of Protein Glycation**

Protein glycation starts with the nonenzymatic, reversible condensation of a sugar aldehyde or ketone group with a free, protein-derived amino group to form a labile Schiff base. Equilibrium is reached within a few hours and is proportional to the ambient glucose concentration. Schiff-base adducts rearrange slowly to form more-stable ketoamine “Amadori” products, taking 4–6 weeks to reach equilibrium (7). Amadori products may undergo further irreversible rearrangements over a period of months, reacting with free amino groups to form fluorescent cross-linked advanced glycation end (AGE) products. These structures are highly heterogeneous and are readily detected on proteins such as Hb and collagen. They accumulate with age, reflect increased blood glucose, and are increased in patients with poorly controlled diabetes mellitus. AGE products on basement membrane proteins are believed by some investigators to disrupt normal vascular function and to be a major contributing factor in the genesis of late diabetic complications (12). If it is true that protein glycation plays an etiological role in diabetic vascular disease, then this may provide a pathophysiological basis for the clinical observation that the average gHb concentration is directly related to the risk of late diabetic retinopathy (4).

The proportion of a given protein that carries an Amadori product is related to the half-life of the protein and the blood glucose concentration integrated over time. On extracellular molecules the glycated products are derived almost exclusively from glucose, whereas intracellular proteins may react with many different native and phosphorylated sugars. However, in cells that are freely permeable to glucose, such as erythrocytes (RBCs), the major source of glycation products is glucose (7).

**Glycohemoglobin**

Glycation may occur at several amino acid residues on various types of Hb (e.g., HbA, A, and F), and the products are termed gHb or glycated Hb. In particular, chromatographic analysis of HbA reveals a number of minor species, HbA1a, HbA1b, and HbA1c, that are collectively known as HbA1, or fast hemoglobins. HbA1c is the specific Amadori product of glucose with the N-terminal valine of each β chain of HbA, comprising ~80% of HbA1 and about half of the total gHb. It is this fraction that is best correlated with average blood glucose concentrations. Given the long half-life of RBCs, the proportion of HbA1c is an index of blood glucose concentration over the preceding 6–8 weeks, with reference intervals being ~4–6% of total Hb, depending on the assay used (7).

In 1986 the ADA recognized the clinical importance of gHb and recommended at least biannual measurements for the follow-up of all types of diabetes (6). This encouraged the development and marketing of a plethora of assays by various companies. Recent College of American Pathologists (CAP) surveys report that >15 assays are now commonly used in American clinical laboratories (13, 14), all based on one of four basic principles: ion-exchange chromatography, electrophoretic separation, affinity chromatography with phenylboronate matrices, or newly developed immunoassays based on monoclonal antibody technology. These assays differ widely in accuracy and precision and lack standardization, so that results are both method- and laboratory-specific (for recent comprehensive reviews see references 8–11). In the DCCT this problem was well recognized and avoided by the use of a single central laboratory for all HbA1c assessments. CAP surveys of both hospital and physician office laboratories reveal two major trends that may offer potential solutions: The first is a move towards automated technologies: In 1991 only ~19% (151 of 792) of CAP survey laboratories used automated methodologies (13), compared with at least 47% (639 of 1346) in 1993 (14). Most automated methods offer markedly better precision than manual methods. This holds true for newer automated HPLC affinity-based assays not reported in the CAP surveys (15). The second is a trend to manufacturer standardization. The Abbott Vision and IMx systems (Abbott Labs., N. Chicago, IL) measure gHb, but are calibrated with a calibrator assayed for HbA1c by the ion-exchange HPLC method (16, 17); results are reported as “standardized HbA1c”. Because the Vision, IMx, and ion-exchange...
HPLC (e.g., Bio-Rad (Richmond, CA) Diamat) methods are used by ~40% (535 of 1346 laboratories) of CAP survey laboratories (14), this may represent a move towards adoption of an industry standard. It is hoped that other manufacturers will follow suit, as recent research clearly shows that calibration and standardization result in better long-term precision, decreased lot-to-lot variability, and improved interassay precision—as well as allowing direct comparison of data produced by the various methods (8–11). However, the encouraging trends towards automation and standardization were not evident in a recent European survey, in which 102 laboratories used 16 different methodologies for determining gHb concentrations (11).

Irrespective of the method used, the exact analyte measured, or the method of standardization, gHb results are affected by a number of factors: (a) patients with hemolytic disease exhibit a significant decrease of gHb by all methods; (b) high gHb values have been reported in iron-deficiency anemia, probably due to the increased proportion of old RBCs; (c) various hemoglobinopathies affect most of the assay methods—importantly, HbF may vary in diabetes and pregnancy (7, 18–20); and (d) another source of artifact, carbamylated Hb, is present in significant amounts in renal failure, which is common in diabetic patients (21). Overall, the least affected methods are those based on immunoassay or affinity chromatography (11). However, if an abnormal Hb is present that reduces RBC survival, gHb will be decreased regardless of the analytical method used. gHb values may still be useful in the presence of abnormal hemoglobins, but they must be compared with previous results obtained by the same method in a given patient. Clearly the gHb goals set in the DCCT would not be appropriate for such patients.

GHB assay technology is advancing rapidly, with the development of fully automated assays that can provide accurate and precise results in either the clinical laboratory or the physician's office. Despite these advances, however, the question remains whether gHb by itself provides the best index for monitoring diabetic control on a frequent basis.

Glycated Serum Proteins

If patients are to be monitored at intervals of <6–8 weeks, there may be a need for assays that are more sensitive than gHb to short-term fluctuations in blood glucose concentrations. This applies particularly to GDM, where short-term blood glucose control critically affects fetal well-being and the risk of congenital abnormalities (2). Furthermore, pregnancy may affect the rate of turnover of RBCs and the proportion of maternal HbF, rendering gHb measurements difficult to interpret in pregnant women (20, 22). These problems have led to the development of assays of glycated serum proteins. Many of these proteins have half-lives of 1–4 weeks, and the extent of their glycation reflects the mean plasma glucose concentrations over the preceding 2–3 weeks—with the caveat that gross changes in protein concentration and half-life may have large effects on the proportion of protein that is glycated. Thus, results obtained may be invalid in the presence of cirrhosis of the liver, nephrotic syndrome, dysproteinemias, or after rapid changes in acute-phase reactants (23).

Approaches to measuring glycated serum proteins have been reviewed (24) and include the use of various colorimetric procedures and chromatographic methods. Only two methods, the nitroblue tetrazolium colorimetric procedure (the "fructosamine" assay) and phenylboronate affinity chromatography, have been extensively evaluated, yet their use and clinical value remain controversial.

In 1982 Johnson et al. (25) introduced the fructosamine assay as a quick, inexpensive, reproducible assay that did not require preincubation of specimen to dispose of the labile Schiff base fraction, therefore making the procedure easily automated. The assay measures the ability of total glycated serum proteins to reduce the dye nitroblue tetrazolium under specific alkaline conditions that exclude other reducing substances in serum. Early work indicated that fructosamine results were significantly higher in diabetics than in normal individuals and were sensitive to changes in blood glucose concentrations over a period of 2–3 weeks (25). Over the succeeding decade, numerous clinical trials evaluated the assay as a screening or diagnostic test for diabetes and for monitoring glucose control in GDM, IDDM, and NIDDM (reviewed in 24, 26). During this period, the assay itself underwent extensive modification as various artifacts were identified. The data concerning the clinical value of this first-generation assay are thus extremely difficult to interpret. Nevertheless, Armbruster concluded in 1987 that "the fructosamine assay can provide clinically useful information for the detection and control of diabetes" (24). Fructosamine appeared at that time to be ideally suited for detecting short-term deterioration of diabetic control. In contrast, Windeler and Kobberling reviewed essentially the same data and determined that "the fructosamine test is not evaluated sufficiently to allow its clinical use . . . . the results presented so far do not suggest a reliable test" (26).

It is not surprising that the first generation of fructosamine assays has not found general favor, given the number of major problems identified in the original assays. These include an apparent lack of specificity for glycated proteins (as much as 60% of any given result was found to be due to nonfructosamine reducing substances, now known to include urates); a lack of standardization among laboratories; difficulty in calibrating the assay; and susceptibility to interference by hyperlipidemia, common in patients with diabetes (27–30). Furthermore, as fructosamine values are affected by protein concentration, assay results were often reported relative to the serum protein concentration. Recent work reveals that absolute rather than relative fructosamine values may be a better index of average blood glucose, there being no direct relationship between serum protein and fructosamine concentrations (31).

Boehringer Mannheim (Indianapolis, IN) and Roche
Diagnostic Systems (Somerville, NJ) have recently modified their fructosamine assays to address these problems. Their second-generation assays now include increased detergent concentrations and uricase, and are calibrated with glycated lysine, which has properties closely resembling those of glycated proteins. The new assay yields reference intervals about one-eighth of those of the first-generation assay, reflecting enhanced specificity and closely agreeing with the HPLC/fructose method for assessing protein glycation. The new assay thus appears to be highly specific for fructosamine and free from interference by urates and triglycerides (32). Furthermore, standardization has been effectively achieved by the adoption of an industry standard. Fructosamine results are now reported without reference to the total serum protein, the normal variations in serum albumin concentration having been shown to be compensated by changes in this protein’s half-life (31). The only proviso is that the test is not recommended in the presence of dysproteinenia or when serum albumin is <30 g/L.

Preliminary clinical data tend to support the validity of the new fructosamine assay as a means of monitoring diabetic control, especially in patients involved in intensive intervention programs (33, 34), and a pilot study suggests it may have a role in screening for NIDDM in the elderly (35). Clearly, the second-generation fructosamine assay is a marked improvement over its predecessor, and its potential role in providing a rapid, cheap, reproducible assay for short-term control in intensive intervention programs warrants reassessment.

Several authors have explored alternative ways to measure serum protein glycation. The best documented of these is phenylboronate–affinity chromatography separation of glycated and native proteins, followed by assessment of total or specific proteins. Some debate has been generated whether total or individual serum proteins, each with its own half-life, should be measured: Immunoglobulins (36), calmodulin (37), and albumin (38) have been advocated for this. The findings that the half-life of albumin may vary, and that acute-phase reactants may rapidly alter the relative proportion of serum proteins, also serve to complicate the argument (31). Nevertheless, assays of either glycated serum albumin or total glycated serum protein discriminate reasonably well between diabetic and non-diabetic populations and provide an index of intermediate (2–3 weeks) glycemic control that is, in some cases, reportedly superior to the first-generation fructosamine assay (39, 40). The affinity chromatography method is not affected by lipemia, hemolysis, icterus, or type of anticoagulant used (41) and may be amenable to newer dedicated automated HPLC systems with improved assay performance.

An attractive alternative analytical approach is an enzyme-linked immunoassay based on monoclonal antibodies to glycated albumin (42). This methodology offers the potential for a rapid automated assay. Insufficient clinical data are at present available to assess its utility in practice.

Conclusions
With the release of data from the DCCT (4), and as confirmed by European studies and metaanalysis (43, 44), it is now apparent that tight blood glucose control should be the goal for all eligible IDDM, and possibly also NIDDM, patients. As previously observed with GDM, the evidence for the benefit of maintaining near-normal blood glucose concentrations in diabetic patients without specific contraindications seems incontrovertible. A major limiting factor in implementing these recommendations is likely to be the additional cost, and the onus is thus on the medical profession to develop inexpensive systems for monitoring and treating diabetic patients.

Clinical laboratories can respond by rapidly adopting fully automated, standardized measurements of gHb that will significantly assist in the interpretation of this assessment. The advantages of interlaboratory calibration and standardization are now well documented (8–11, 45). In addition, the potential utility of assays of glycated serum proteins should be reevaluated in future trials of intensive therapy regimes. While it is probable that gHb measurements will remain a central part of such programs, preliminary studies indicate that during rapid changes in blood glucose control, and especially in pregnancy (46, 47), assays of glycated serum proteins may provide important additional information. The DCCT data suggest that the gHb concentration is directly related to the risk of late complications such as retinopathy (4) and may be inversely related to the risk of severe hypoglycemia. Fine-tuning of long-term blood glucose control with the aid of both gHb and glycated serum protein assays may take on a major role in alleviating the morbidity of a disease that remains a major scourge of modern society.

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References