# Glycosylated Hemoglobin in Normal Subjects and Subjects with Maturity-onset Diabetes

## Evidence for a Saturable System in Man

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SUMMARY

**Concentrations of glycosylated hemoglobin (GHb) are elevated in diabetes mellitus and are believed to reflect previous metabolic control. To better define possible determinants of GHb in man, we investigated the relationship between GHb and both fasting plasma glucose (FPG) and basal insulin (IRI) in 42 normal subjects and 29 patients with maturity-onset diabetes. Concentrations of GHb in diabetic subjects (12.7 ± 3.4, x ± S.D., per cent total hemoglobin) were significantly higher than in normal subjects (8.2 ± 1.2, $p < 0.001$). In normal subjects, FPG ($r = 0.52$) and GHb ($r = 0.58$) (both $p < 0.001$) correlated with age. GHb did not correlate with IRI in either group. However, GHb was closely associated with FPG in both normal ($r = 0.60$, $p < 0.001$) and diabetic ($r = 0.85$, $p < 0.001$) subjects. Linear regression analysis of the data for the two groups combined was highly significant ($r = 0.91$, $p < 0.001$). However, the slope of the regression line for GHb versus FPG seen in normal subjects was significantly steeper than that of diabetic patients ($p < 0.005$). A curve describing a nonenzymatic saturable model was also found to fit the data of the two groups combined ($r = 0.85$, $p < 0.001$), suggesting the possible existence of a saturable system for glycosylation in man. DIABETES 27:834-39, August, 1978.**

The $A_1$ group of hemoglobins ($HbA_{1a-c}$) consists of rapidly migrating minor components representing less than 10 per cent of normal adult human hemoglobin.[1,2] Various moieties of hemoglobin $A_1$ are characterized by the presence of glucose ($HbA_{1c}$) or glucose metabolites ($HbA_{1a}$) linked to the amino terminal of the hemoglobin $\beta$-chain.[3,4] Formation of these glycosylated hemoglobins (GHb) is a postsynthetic modification of HbA, occurring slowly, nonenzymatically, and relatively irreversibly throughout the life cycle of the erythrocyte.[5] Levels of GHb are elevated as much as twofold in diabetic subjects[6,7] and have been said to reflect the degree of metabolic control of the individual, as measured by quantitative glycosuria or fasting and postprandial blood glucose values.[8,9]

Most prior clinical studies have examined GHb in juvenile or adult insulin-treated diabetics,[8-12] a group of patients in whom metabolic stability is often less than optimal.[13] To better define the possible determinants of GHb in man, we examined the relationships between GHb and both fasting plasma glucose (FPG) and basal plasma insulin (IRI) in two groups of subjects considered to be metabolically more stable throughout the life cycle of the erythrocyte: normal individuals and untreated or noninsulin-dependent maturity-onset diabetics. The results of this study demonstrate a strong correlation between GHb and FPG, independent of basal IRI, throughout the range of glycemia observed. Moreover, in normal subjects, an age-dependent increase of both FPG and GHb was observed.

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## MATERIALS AND METHODS

Forty-two normal subjects and 29 diabetic patients were studied. Normals ranged in age from 25 to 91 years and had levels of FPG between 80 and 110 mg. per deciliter; none had a family history of diabetes. The diabetic subjects, aged 30 to 84 years, were male patients treated in either the diabetes or medical clinics of the Seattle Veterans Administration Hospital. All had manifested fasting hyperglycemia on two or more occasions at least three weeks apart. Levels of FPG at the time of the study ranged from 120 to 399 mg. per deciliter. Most had been followed for months to years, and all were considered to be clinically stable. The majority were obese and had been prescribed weight-reduction diets as their sole means of treatment. Three of the diabetics were taking oral hypoglycemic agents at the time of the study and none had been treated with insulin.

In 12 normal and 14 diabetic subjects, all male, specimens were obtained after an overnight fast by means of an intravenous line kept patent with a saline solution. Four samples drawn 15 minutes apart while the subject was supine were analyzed for glucose and insulin as described below, and the results were averaged to determine basal values. In the other subjects, single fasting blood specimens were obtained after the individual had been sitting quietly for 10 minutes. All blood specimens were collected in 5-ml. EDTA tubes, iced immediately, and centrifuged at 4° C. to separate plasma and cells. Plasma glucose was determined by the ferricyanide method on an Auto-Analyzer. Plasma insulin (IRI) was determined by a double antibody modification of the method of Morgan and Lazarow.[14] Hemolysates were prepared from the red blood cells the same day by the method of Carrell and Kay[15] and incubated overnight at 4° C. in a 9:1 ratio with 10X developer no. 6.[16] The hemolysates were chromatographed on Bio-Rex 70 in duplicate by the method of Trivelli[7] as modified by Gabbay.[9] Glycosylated hemoglobin fractions were expressed as per cent of total hemoglobin eluted. The interassay coefficient of variation of this method in our laboratory is 2.7 per cent. By this technique we have found total hemoglobin $A_1$ ($HBA_{1a+b+c}$) to correlate closely with its principal glycosylated $HbA_{1c}$ fraction ($r = 0.98$, $p < 0.001$). Therefore, while we recognize that not all components of hemoglobin $A_1$ are glycosylated, in the analyses to follow all data are expressed as total GHb. Statistical correlations were by standard methods, and comparisons of groups were made with the Student's *t*-test.

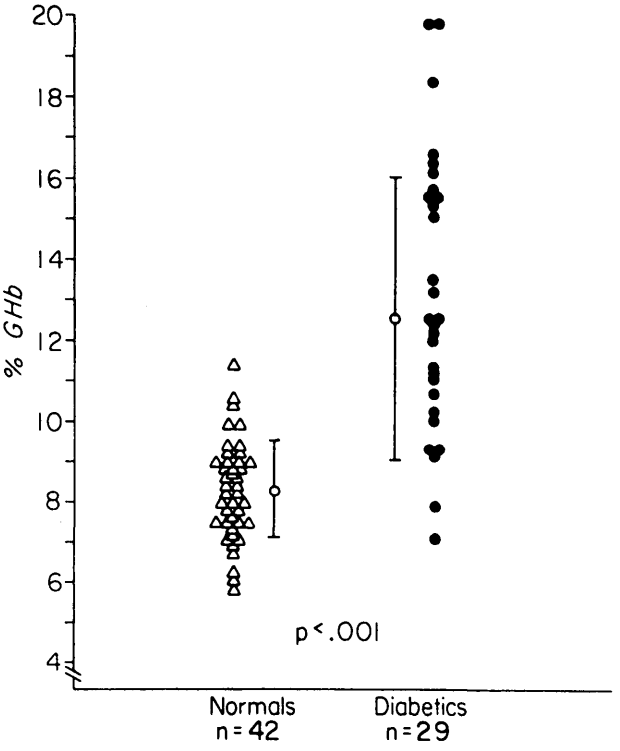


**FIG. 1. Shown is a scattergram of glycosylated hemoglobin levels in normal subjects and maturity-onset diabetics. The bars represent ± 1 S.D. from the mean.**

## RESULTS

A comparison of GHb levels in the normal and diabetic populations is shown in figure 1. The concentration of GHb in normals was 8.2 ± 1.2 per cent (x ± S.D., per cent total hemoglobin), and that in diabetics (12.7 ± 3.4) was significantly higher ($p < 0.001$). A positive correlation of FPG ($r = 0.52$) and GHb ($r = 0.58$) (both $p < 0.001$) with age was observed in the normal subjects (figures 2A and B). Despite this age-related increase, the percentage of GHb in 29 normals matched for age (51 ± 14 years) with diabetics (54 ± 14, p = N.S.) remained significantly lower (8.7 ± 1.0 per cent, $p < 0.001$). There was no correlation of FPG ($r = -0.05$) or of GHb ($r = 0.04$) (both p = N.S.) with age in diabetics.

Levels of GHb were found to be closely associated with those of FPG, as reflected by their linear relationship in the group of 29 stable maturity-onset diabetic patients ($r = 0.85$, $p < 0.001$, figure 3). Similarly, in the 42 normal subjects, a significant correlation was found between FPG and GHb ($r = 0.60$, $p < 0.001$, figure 3). For the normals and diabetics combined, a linear relationship exists between these two variables ($r = 0.91$, $p < 0.001$).

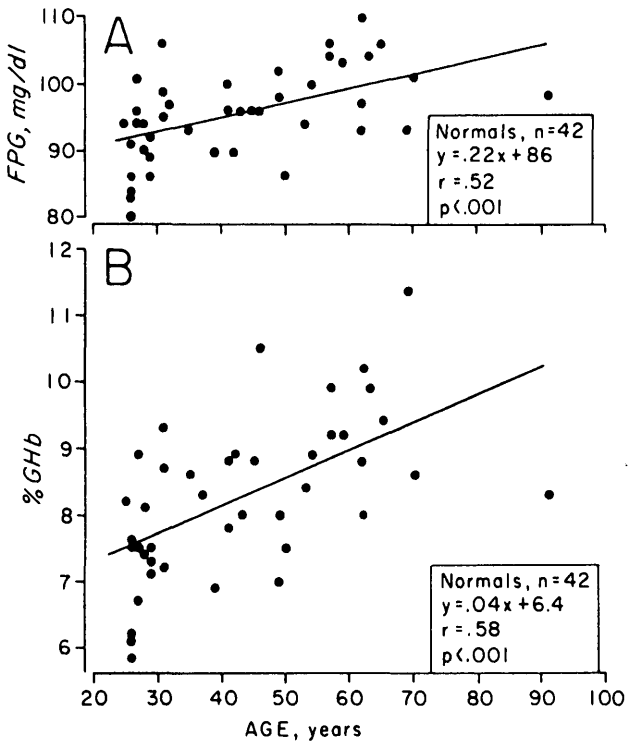


**FIG. 2. The relationships between age and both fasting plasma glucose (A) and glycosylated hemoglobin (B) in normal subjects.**

However, the slope of the regression line for GHb versus FPG in normals (0.105) is significantly steeper ($p < 0.005$) than that of diabetics (0.036, figure 3). This difference in regression slopes raises the possibility that the relationship between GHb and FPG is curvilinear rather than linear. We attempted, therefore, to fit a curve to our data by means of a standard equation, analogous to the Michaelis-Menten equation, describing a saturable system. From this equation, a least-squares curve was generated that also fit all the data significantly ($r = 0.85$, $p < 0.001$; see legend to figure 3). This curve predicts half-maximal saturation at an FPG of 180 mg. per deciliter and postulates a theoretic maximum of glycosylation of 23.1 per cent of total hemoglobin.

Basal IRI in the diabetics ($17 \pm 13$ $\mu$U. per milliliter, $n = 14$) did not differ significantly from that of normals ($10 \pm 5$, $n = 12$). Moreover, no correlation was found between GHb and IRI in the group of normals ($r = 0.27$, p = N.S.), diabetics ($r = -0.06$, p = N.S.), or both groups combined ($r = 0.22$, p = N.S.).

## DISCUSSION

This study confirms the findings of others[7,8,12] that levels of glycosylated hemoglobin are higher in maturity-onset diabetic subjects than in normals. In addition, GHb was found to be highly correlated with FPG in our group of maturity-onset, noninsulin-treated diabetic patients. These results extend previous observations of such a relationship in insulin-treated diabetics.[12] Moreover, a similar association

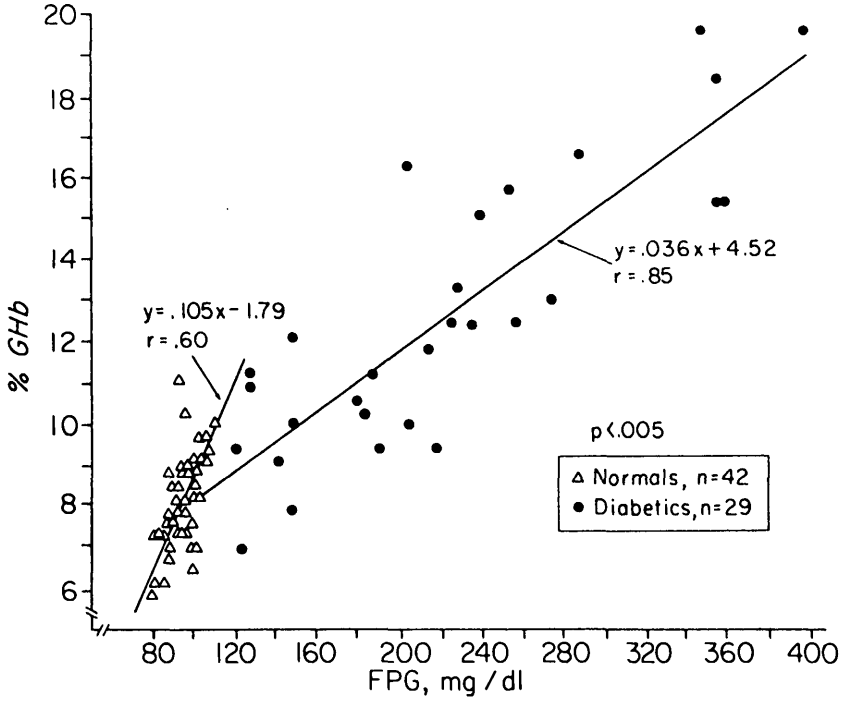


**FIGURE 3**

**Linear-regression analyses of GHb and FPG for both normals and diabetics. For both groups combined, a linear regression analysis is highly significant ($r = 0.91$, $p < 0.001$). Alternatively, the significant difference in slopes of normals and diabetics suggests the possible existence of a curvilinear system of glycosylation. Such a curve may be generated from the equation $y = x/(a+bx)$, the algebraic equivalent of the formula $1/y = a(1/x)+b$, where a = slope and b = intercept. This formula is analogous to that of the Lineweaver-Burke equation for saturable enzyme kinetics.[21] Such a curve fits all the data significantly ($r = 0.85$, $p < 0.001$) and is consistent with the hypothesis of a saturable model of glycosylation in man (see text).**

between GHb and FPG was seen in normal subjects. Thus, a relationship between glycosylated hemoglobin and FPG is maintained throughout the range of glycemia.

It has been proposed that the glycosylated hemoglobin concentration reflects the ambient glycemia to which the erythrocyte is exposed during its life cycle.[5] This hypothesis has not yet been confirmed by measurements of mean plasma glucose in a large number of subjects over a prolonged period of time. However, in a recent study, the mean of several daily glucose measurements and levels of glycosylated hemoglobin correlated significantly in a group of hospitalized insulin-treated diabetics.[12] Mean plasma glucose has been found to correlate significantly with FPG in both normals and diabetics.[17] Moreover, FPG is remarkably consistent from day to day in both normal and untreated adult diabetic subjects.[18] While continual monitoring of normal subjects has shown considerable stability of blood glucose for 48 hours,[17] no such studies have been performed in a group of stable untreated maturity-onset diabetics. Nevertheless, impaired but functional glucoregulatory mechanisms[19] in these patients may also maintain a consistent mean blood glucose over time despite large postprandial excursions from that mean. If relatively constant FPG and mean plasma glucose values persist in these two groups of subjects for the life cycle of the red blood cell, the strong correlation between FPG and glycosylated hemoglobin observed in the present study would be expected.

Previous studies of normal individuals have shown an increase of FPG[20] and a deterioration of glucose tolerance[21] with advancing age. Our finding of a positive correlation between FPG and age is in agreement with these observations. If higher levels of FPG and relative carbohydrate intolerance contribute to an elevated mean blood glucose in older subjects, increased levels of glycosylated hemoglobin might be anticipated in these individuals. Our demonstration of GHb increasing with age of normal subjects is consistent with this concept. Since no correlation between age and FPG was observed in the diabetics, an association between age and GHb would not be expected in this group. Our findings are in accord with this analysis.

The relationship between FPG and glycosylated hemoglobin in a combined group of normals and diabetics is highly significant when described by linear regression analysis. However, this type of analysis fails to account for the difference in slopes seen between the two groups (figure 3). This difference may represent, in part, a sampling artifact, since the distribution of FPG in normal subjects is relatively narrow in contrast with the wide range of hyperglycemia in the diabetics. On the other hand, if the observation is valid, this difference between euglycemic and hyperglycemic subjects suggests that the relationship between FPG and GHb may be curvilinear. Although glycosylation of hemoglobin is believed to be a nonenzymatic process,[5] it is possible to derive an equation analogous to the Michaelis-Menten model of saturable enzyme kinetics[22] and, from such an equation, to generate a curve that fits our data (see legend to figure 3). This curve describes a saturable, though nonenzymatic, system of glycosylation in man. This analysis predicts a concentration of FPG of 180 mg. per deciliter at which hemoglobin will be half-maximally glycosylated, analogous to Km, and suggests a theoretic limit to the percentage of hemoglobin that is glycosylatable (23.1 per cent), analogous to Vmax. Previous studies have found levels of GHb that approach, but do not exceed, this predicted maximum.[7-12]

Explanations for possible saturability of this system are not clear, but may include the following. Studies in vitro have shown a reduced rate of glycosylation of hemoglobin in the presence of oxygen or 2,3 DPG,[4] possibly as a result of steric hindrance or competitive inhibition at the binding site, which may contribute to a saturable system. Similarly, prior glycosylation of one hemoglobin dimer may interfere with subsequent glycosylation of its paired dimer through conformational changes in the hemoglobin molecule. Alternatively, saturation of an enzyme of glucose metabolism might be offered as an explanation. Glucose-6-phosphate linked to hemoglobin has been proposed as a precursor of GHb,[4] and enzymatic production of this compound could limit glycosylation. However, the kinetics of erythrocyte hexokinase, an enzyme that is saturated at a low concentration of glucose,[23] would not fit a curve with a Km of 180 mg. per deciliter. Other precursors have been suggested,[24] and the possibility that saturable enzyme systems involved in their formation (or formation of as yet unsuspected intermediates) are responsible for a saturable system of glycosylation awaits further investigation. On the other hand, the slightly shortened erythrocyte life cycle observed in diabetics,[25] with subsequent decreased time of red cell exposure to ambient glucose levels, may suggest a slower rate of glycosylation in hyperglycemic subjects when, in fact, no difference from normals exists.

Because other studies have suggested that deficiency of insulin, as seen in diabetics and in infants with cystic fibrosis,[11] and of insulin resistance, as seen in pregnancy,[26] might be responsible for the high levels of glycosylated hemoglobin observed in these conditions, we investigated the relationship of GHb to basal IRI. Basal IRI secretion was comparable in our normal and diabetic subjects and did not correlate with GHb. Nevertheless, since insulin response to a glucose challenge is impaired uniformly in subjects with FPG exceeding 115 mg. per deciliter and is virtually absent when FPG is greater than 150 mg. per deciliter,[18] it is still possible that the elevations of GHb observed in diabetics represent increased glycosylation due to insulinopenia. Although there is no available evidence to support the hypothesis that insulin influences glycosylation in vitro or in vivo, we cannot entirely exclude this explanation for our findings.

Whether our data describe a linear or a saturable system of glycosylation in man, it is evident that levels of glycosylated hemoglobin are influenced by fasting plasma glucose concentrations in normal as well as in hyperglycemic subjects. These findings suggest that a convenient, rapid, and inexpensive determination of FPG should provide an accurate index of past and present glucose homeostasis for stable noninsulin-dependent diabetics. On the other hand, in insulin-dependent diabetics or those in whom dietary or drug treatment is changing, levels of fasting and mean plasma glucose are subject to day-to-day variability. In these patients, periodic measurement of GHb concentrations may prove to be of valuable assistance.[9,12]

In addition, the findings of this study may have some bearing on studies of diabetic complications. Glycosylated hemoglobin has been shown to be a reversible metabolic consequence of hyperglycemia.[8] The glycosylation of hemoglobin may be analogous to the formation of other glycoproteins thought to be implicated in the vascular complications of diabetes.[27] If the relationship between FPG and GHb is, in fact, curvilinear, partial lowering of the FPG (corresponding to the flatter part of a saturable curve) would be expected to have a relatively small effect on GHb levels. Whether a linear or a saturable relationship between FPG and GHb concentrations exists, the reversal of elevated levels of GHb in a diabetic would require attainment of virtually normal levels of FPG. Thus, the achievement and maintenance of euglycemia, impractical with present diabetes treatment modalities,[28] may be required to determine whether or not a direct relationship exists between hyperglycemia and the occurrence and progression of diabetic complications.

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