# Glycosylated Serum Protein Levels Assayed With Highly Sensitive Immunoradiometric Assay Accurately Reflect Glycemic Control of Diabetic Patients

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**OBJECTIVE** — To develop a sensitive and reliable immunoradiometric assay to measure glycosylated lysine residues on serum proteins (GSP) and to evaluate its efficacy in monitoring glycemic control.

**RESEARCH DESIGN** — The effect of acute and chronic in vitro and in vivo changes in glucose levels on GSP concentration was evaluated. GSP determinations from insulin-dependent diabetic (IDDM) patients, non-insulin-dependent diabetic (NIDDM) patients, and control subjects were correlated with other indices of glycemic control.

**RESULTS** — The GSP levels were unaffected by acute glucose changes after food or intravenous glucose administration but increased during storage at −20°C due to in vitro glycosylation by endogenous glucose. Immediate acidification of the serum prevented this, permitting long-term storage despite high ambient glucose levels. In randomly selected diabetic patients, 96% of GSP values were greater than the mean +3SD of nondiabetic control subjects. In diabetic patients, GSP levels correlated with mean plasma glucose concentrations (Kendall correlation statistics 0.47, $P < 0.001$), fasting plasma glucose levels (Kendall statistics 0.42, $P < 0.001$), and glycosylated hemoglobin (GHb, Kendall statistics 0.30, $P < 0.005$). Induction of near-normal glycemia in poorly controlled NIDDM patients reduced GSP levels with a slope consistent with a half time of disappearance of 4.7 ± 0.4 days. GSP levels remained elevated in 6 of 10 well-controlled NIDDM patients, despite normal GHb concentrations. Chronic hypoglycemic states, like pregnancy and hyperinsulinemic hypoglycemia, were associated with significantly low GSP levels.

**CONCLUSION** — We describe a reproducible and sensitive immunoradiometric assay for GSP that closely reflects the degree of glycemic control in diabetic patients. Further studies are needed to determine whether this assay may be useful in screening for glucose intolerance or gestational diabetes.

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Convincing evidence suggests that most long-term complications of diabetes are caused or exacerbated by chronic hyperglycemia (1). Furthermore, good glucose control may prevent or delay the onset of most diabetic complications (2). For this reason, the approach to treating insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) has evolved considerably during the past decade, and great emphasis is being placed on early diagnosis and optimal glycemic control. Although the advent of home blood glucose monitoring has made a major contribution to the management of diabetes, a need still exists for methods that accurately monitor the quality of glycemic control over time. GHb determinations offer a partial answer because they reflect overall glycemic control over 2–3 mo (3,4). Although useful for monitoring long-term treatment in stable diabetic patients, GHb levels change too slowly to be useful in situations where closer monitoring is needed, as in patients undergoing major changes in treatment modality or in pregnant diabetic patients. Furthermore, the GHb assays currently available are not sensitive enough to differentiate between nondiabetic levels and levels of patients with minor hyperglycemia, making them of limited use in the follow-up of well-controlled patients and of no use at all in identifying patients with impaired glucose tolerance (IGT) or mild diabetes (5,6). In an effort to solve some of these problems, several assays have been developed that measure glycosylated serum protein (GSP) or albumin. We report an immunoradiometric assay (IRMA) based on an antibody directed against the glycosylated lysine residue. In this study, we evaluated the usefulness of this assay in the assessment of glycemic control in diabetic and nondiabetic subjects.

## RESEARCH DESIGN AND METHODS

— Blood samples and clinical information were obtained from pa-

tients of the diabetes clinics of the Hebrew University Hadassah Medical Center, Jerusalem, the General Health Fund of Israel, Netania, and the University of Modena Medical Center, Modena. The study was approved by the committee on human experimentation of these medical centers.

GHb levels were determined using the Glyc-Affin kit (Isolab, Akron, OH), which has intra- and interassay coefficients of variation (CV) of <3%. GSP was measured using an assay developed in our laboratory. Antibodies were raised in rabbits against in vitro glycosylated keyhole-limpet hemocyanine, and affinity purified on a column containing glucitollysine-human serum albumin (HSA) by the method of Jaton et al. (7). The antibody, which does not cross-react with nonreduced fructosyllysine HSA, was labeled with $^{125}$I to a specific activity of ~5000 cpm/ng (2.3 mCi/mg).

The standard was prepared by in vitro glycosylation of crystalline HSA (Sigma, St. Louis, MO), which was incubated for 7 days at room temperature in the presence of 80 mM glucose, 30 mM $NaN_3$, 75 mM cyanoborohydrate, and 0.3 mM HSA. The standard curve was constructed by diluting this stock solution of maximally glycosylated albumin to concentrations ranging from 0.01 to 10 μg albumin/ml.

Except if otherwise indicated, all serum and plasma samples were immediately diluted with an equal volume of sodium acetate buffer (final pH 5) and stored at −20°C until assayed. This step was necessary to avoid in vitro glycosylation of serum (see below).

Thawed and centrifuged acidified serum or plasma was diluted 1:500 in phosphate buffer (pH 7.65). Serum proteins were then adsorbed on the walls of Nunc Star Tubes (Nunc, Kamstrup, Denmark) by overnight incubation at room temperature. The fructosyllysine residues on the adsorbed protein were reduced to glucitollysine using sodium borohydride in phosphate buffer. The adsorbed albumin was then incubated for 48 h in the presence of $^{125}$I-labeled anti-glucitollysine antibody prepared as described above. The supernatant was decanted, the tubes washed three times with distilled water, and then counted. The results were calculated by computer from a four-parameter curve-fitting program, as described by Rodbard and Hutt (8). To correct for interassay variation, the results were normalized to the value obtained with normal pooled human serum (NPHS; 100%), obtained from healthy volunteer blood donors. Mean plasma glucose (MPG) levels represent the average of seven glucose determinations (3 preprandial, 3 postprandial, and 1 at bedtime) with a portable reflectance meter.

Correlation between two measurements in the same population was evaluated with the Kendall correlation test. Differences between groups were tested with the Mann-Whitney *U* test. $P < 0.05$ was considered significant.

**RESULTS** — In 16 consecutive assays, NPHS contained the equivalent of 113.5 ± 28 (mean ± SD) ng/ml glycosylated human albumin standard (range 60–167, CV 28%). As described above, this variation was eliminated by normalizing all sample results in a given assay relative to the NPHS value for that assay (expressed as %). Dilution of the acidified serum preparations with buffer resulted in only a minor decrease in GSP level (at 1:1.5 dilution GSP 90 ± 1% of undiluted serum; at 1:2 dilution GSP 87 ± 2% of undiluted serum), indicating that binding sites of the Nunc Star Tubes are fully saturated even at protein levels one half of normal, and confirming that GSP levels are not affected by variations of protein concentrations within the physiological range.

GSP levels in 42 nondiabetic control subjects ranged from 67 to 132%, the mean ± SD being 100.1 ± 16%. Interassay CVs were 12, 8, and 7% at mean levels of 78, 288, and 561, respectively. The average minimal detectable dose for 14 assays was 8.2 ± 1.2%. Storage of unacidified plasma samples at −20°C resulted in a gradual increase of the GSP level that was dependent on the glucose concentration of the plasma (Fig. 1). Overnight storage of plasma or whole blood (mean glucose concentration 9.1 ± 0.9, range 4.4–18.3 mM) at 4°C resulted in a 25 ± 8% (range −14 to +127%, $P < 0.01$) increase of GSP levels. Once the sample was acidified to a pH of 4.5, however, GSP levels were stable for at least 170 days, even when stored in the presence of high glucose concentrations (Fig. 1). Similar studies conducted at 37°C demonstrated that in vitro glycosylation was temperature dependent (not shown).

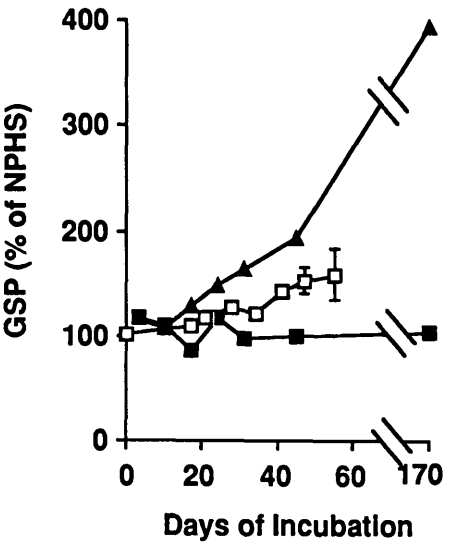


**Figure 1**—*Mean ± SE in vitro glycosylation of unacidified (pH 7.4) euglycemic serum at -20°C in the presence of 11.1 mM (□) or 27.8 mM (▲) added glucose and of acidified (pH 4.9) euglycemic serum in the presence of 27.8 mM added glucose (■). GSP, glycosylated serum protein; NPHS, normal pooled human serum. If not shown, standard error bars are smaller than the symbols.*

Figure 2 demonstrates that acute hyperglycemia induced by rapid intravenous glucose injection did not affect GSP levels in 10 nondiabetic subjects (0.5 g/kg glucose, *A*) or in 9 mild (fasting blood glucose 5.2 mM) NIDDM patients

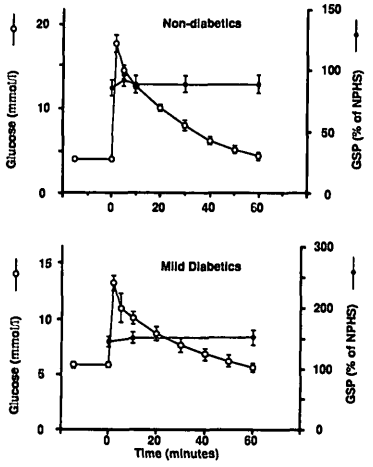


**Figure 2**—*Effect of acute hyperglycemia on glycosylated serum protein (GSP) levels in nondiabetic subjects (A) and mild non-insulin-dependent diabetic patients (B). Hyperglycemia was induced by injecting 0.5 (nondiabetics) or 0.3 (diabetics) g/kg glucose over 2 min. NPHS, normal pooled human serum.*

(0.3 g/kg glucose, *B*). In another group of more severe diabetic patients (fasting blood glucose 9.9 ± 1.6 mM) there was no diurnal variation in GSP levels nor was there a significant difference between pre- and postprandial GSP values (Fig. 3). The CV of glucose levels throughout the day was 29 ± 6.2%, consistent with expected meal-related glucose excursions. In contrast, the CV of GSP values during the day was 5.8 ± 1.2%, which is similar to the intra-assay variation and is consistent with stable GSP levels throughout the day.

In 70 randomly tested diabetic patients (57 NIDDM, 13 IDDM) mean ± SD GSP was 365 ± 198% (median 310%, range 103–859%), with 96% of the diabetics having levels >148%, which is the mean +3SD value of the healthy control group (Fig. 4). The three diabetic patients with normal GSP levels included a pregnant IDDM patient who was well controlled on continuous subcutaneous insulin infusion (CSII), and two mild NIDDM patients with fasting blood glucose values of 6.7 and 7.4 mM. There was no difference between NIDDM and IDDM patients in terms of mean GSP levels (373 ± 27% vs. 345 ± 53%) or range of GSP levels (103–859 vs. 123–740%).

Data from 43 diabetic patients (35 NIDDM, 8 IDDM), in whom simultaneous GHb, fasting plasma glucose (FPG), and MPG levels were also available, were analyzed separately. In these patients, GSP levels correlated significantly with mean plasma glucose concentrations, fasting plasma glucose concentrations, and GHb (Table 1). GHb also correlated significantly with fasting and mean blood glucose levels, but to a lesser degree.

In 20 NIDDM patients hospitalized for tight glycemic control, GSP levels decreased slowly with a disappearance rate consistent with a half time of 4.7 ± 0.4 days (data not shown). Figure 5 shows the rate of decrease in glucose and GSP levels in 10 of these patients who participated in a strictly controlled protocol where near-euglycemia was induced within 2–3 days and then maintained for 14 more days with CSII. Seven of these patients, and three mildly diabetic patients not treated with CSII continued with sulfonylurea treatment (gliclazide) for 5–6 mo under careful monitoring of glycemic control. At the end of this period, the finding of near-normal fasting glucose levels (5.4 ± 0.3 mM) and mean daily plasma glucose concentrations (6.4 ± 0.2 mM) was consistent with excellent metabolic control. This was confirmed by GHb levels that were well within the normal range in most patients (6.6 ± 0.3%). GSP levels, however, were normal in four and abnormal in six patients (Fig. 6). There was no significant difference between patients with normal or elevated GSP in terms of mean plasma glucose or fasting plasma glucose (6.4 ± 0.3 vs. 6.3 ± 0.3 mM and 5.3 ± 0.3 vs. 5.5 ± 0.4 mM in normal and abnormal GSP patients, respectively).

Nineteen nondiabetic pregnant women, with normal (<7.8 mM) blood glucose levels 1 h after a 50 g oral glucose load, had GSP levels that were significantly lower than control subjects (78 ± 4%, $P < 0.001$). Two infants with hypoglycemia due to persistent hyperin-

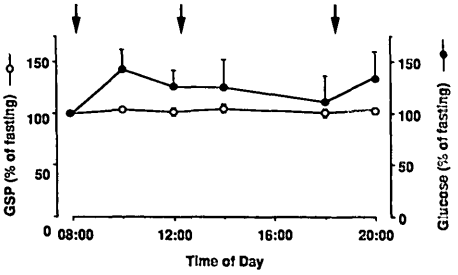


**Figure 3**—*Variation of plasma glucose and glycoylated serum protein (GSP) levels during the day in 7 diabetic patients. The values are given as percentage of the fasting level. Vertical bars denote SE; arrows indicate mealtimes.*

**Table 1**—***Correlation between glycosylated serum proteins and other measures of glycemic control***

| | *N* | τ* | *P* |
|---|---|---|---|
| GLYCOSYLATED SERUM PROTEINS | | | |
| FASTING BLOOD GLUCOSE | 41 | 0.42 | <0.001 |
| MEAN BLOOD GLUCOSE | 40 | 0.47 | <0.001 |
| GLYCOSYLATED HEMOGLOBIN | 38 | 0.30 | <0.005 |
| GLYCOSYLATED HEMOGLOBIN | | | |
| FASTING BLOOD GLUCOSE | 40 | 0.25 | <0.02 |
| MEAN BLOOD GLUCOSE | 39 | 0.24 | <0.02 |

*Kendall correlation statistic.

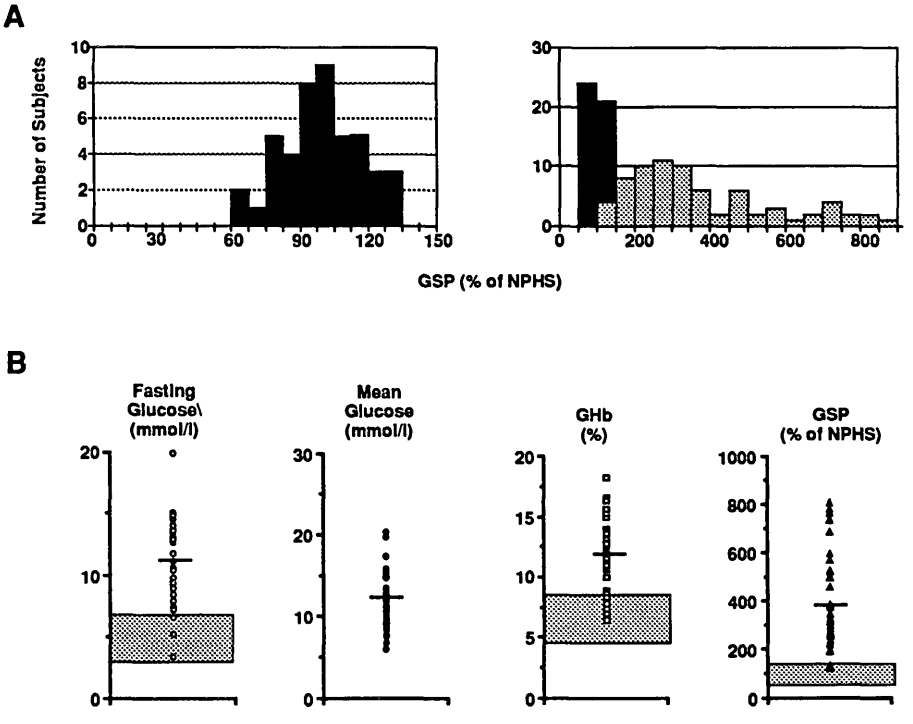


**Figure 4**—*A: distribution of glycosylated serum protein (GSP) levels in diabetic and nondiabetic subjects. The left panel shows the distribution among the nondiabetic control subjects. The right panel shows the distribution of both the normal control group (stippled bars) and the diabetic patients (shaded bars). Note the change in scales in the right histogram. Only 3 diabetic patients had GSP levels in the normal range. B: Fasting and mean glucose levels, glycosylated hemoglobin, and GSP levels in 43 diabetic patients (35 non-insulin-dependent diabetes mellitus, 8 insulin-dependent diabetes mellitus). The stippled areas represent the normal range. The horizontal bar represents the mean for the diabetic group. Note that the overlap with the normal range is smaller for GSP than for any of the other parameters. NPHS, normal pooled human serum.*

sulinemic hypoglycemia of infancy had GSP levels that were below the normal range (65 and 40%).

**CONCLUSIONS**— A new assay for GSP is described that has several advantages over previous methods. Being an IRMA with no extraction or chromatography steps, it is readily adaptable to high-volume laboratories. Although in its current configuration this is a slow assay, with a total of 66 h incubation time, the amount of incubation time can be reduced by further modifications. As an example, the first step during which the protein coats the plastic tube can be reduced from the current 18 to 2 h without loss of accuracy. The possibility of reducing the time allotted for antibody binding has not been critically evaluated. The small volume of plasma needed (5 μl for sample run in duplicate or triplicate) is a major advantage, permitting the adaptation of the assay for use on capillary blood blotted and stored on filter paper. The potential usefulness of filter paper GSP determinations has been stressed (9).

Our assay was calibrated against in vitro glycosylated HSA, and results are expressed relative to a normal serum pool. The use of albumin standard in an assay that measures glycosylation sites in whole serum can be justified on the basis of the findings of Dolhofer and Wieland (10), who reported that albumin accounts for 90% of the glycosylated protein in diabetic patients and nondiabetic control subjects. Furthermore, they demonstrated that serum glycosylprotein correlated well with glycosylalbumin ($r$ = 0.956, $y$ = 1.03$x$ + 0.086), making the two determinations essentially interchangable. Albumin, being much better defined than serum protein, can be glycosylated in vitro to make a standard solution that is reproducible over time.

Our method of expressing GSP differs from that used in affinity chromatography assays, where the percent of albumin molecules containing at least one glycated moiety is determined (11), and from that used by Yamamoto et al. (12) and Schleicher et al. (13), where results were expressed as the total number of glycosylated lysine residues per milligram protein. Because it is not known which if either of these two measurements is clinically more relevant, we feel that neither has any intrinsic advantage over our simpler method, which compares the patient's serum to that of nondiabetic subjects in terms of total number of glucitollysine residues available for antibody binding. This contention is confirmed by our data, which clearly show that this assay provides as good or better correlation with actual glycemic control in diabetic and nondi-

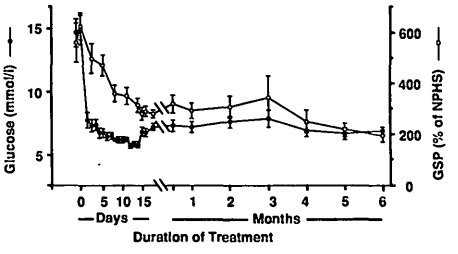


**Figure 5**—*Mean daily plasma glucose and glycosylated serum protein (GSP) levels in 10 non-insulin-dependent diabetic patients in whom euglycemia was rapidly induced and maintained for 14 days with continuous subcutaneous insulin infusion. Thereafter, the patients were controlled on maximum tolerated dosages of gliclazide. NPHS, normal pooled human serum.*

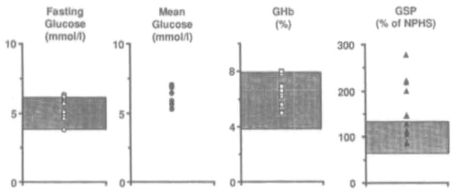


**Figure 6**—*Fasting plasma glucose, mean plasma glucose, GHb, and glycoylated serum protein (GSP) levels in 10 well-controlled non-insulin-dependent diabetic patients. Fasting glucose and GHb levels were normal in all patients, but GSP levels were elevated in 6 of 10 patients. The shaded areas represent the normal ranges of each parameter. NPHS, normal pooled serum protein.*

abetic subjects when compared with other glycosylated protein assays.

The small overlap in GSP values between diabetic and control populations in our assay (5%) contrasts with the larger overlap reported by Yamamoto et al. (12) with an assay based on a similar principle, or by Winocour et al. (14) with a colorimetric fructosamine assay. It is unlikely that this difference can be explained by differences in the degree of glycemic control because the patients presented by Yamamoto and Winocour had fasting plasma glucose levels that were the same as or greater than our patient population. Shima et al. (15), studying another group of patients with similar fasting plasma glucose levels, reported excellent separation between diabetic and control GSP levels with a high-performance liquid chromatography system, confirming that when a sensitive and specific assay is used, virtually all diabetic patients show elevated GSP levels.

We have clearly shown that GSP levels measured in our assay are not affected by time of day, acute changes in blood glucose, or meals. GSP levels are, however, affected by continued in vitro glycosylation of proteins in serum kept under normal storage conditions. This in vitro glycosylation is temperature and glucose concentration dependent, and is quite significant during storage for a few days at −20°C, a condition common to most laboratories. Schäffer et al. (16) reported a similar phenomenon with a high-performance liquid chromatography system to measure furosine in hydrolyzed serum. Despite this, to the best of our knowledge, other investigators have not called attention to this storage effect, which may seriously reduce the reliability of GSP determinations, particularly in long-term studies where samples are stored and then run together to avoid interassay variation. Immediate acidification of serum prevents any further in vitro glycosylation, and properly prepared serum can be kept at −20°C indefinitely without alteration of GSP levels.

Our GSP assay is quite sensitive in and around the normal range of glucose levels. In pregnant nondiabetic women, the low GSP levels we found are consistent with the known lower mean glucose concentrations during pregnancy (17). Two patients with chronic hypoglycemia, due to persistent hyperinsulinemic hypoglycemia of infancy, also had low GSP levels. NIDDM patients, who were well controlled on oral hypoglycemic agents, and who had GHb levels well within the normal range, frequently had GSP levels that were above normal. This is consistent with the known fact that even in the most stringently controlled diabetic patient, postprandial hyperglycemia is still common (18). A more detailed study of the effect of minor alterations in glucose homeostasis on GSP in treated diabetic subjects is needed.

The clinical sensitivity of this assay suggests that it could be helpful for screening patients for glucose intolerance. Previous attempts to screen patients with conventional GHb assays have been futile because GHb levels increase above the normal range only in the face of markedly abnormal glucose homeostasis. However, Makita et al. (19) report significantly elevated GHb levels in patients with IGT with a sensitive radioimmunoassay, similar in principle to the assay reported herein. This suggests that patients with IGT do have mildly increased glycosylation of blood proteins, and this increase can be detected if the method used is sufficiently sensitive. Two of our patients with mild NIDDM and several diabetic patients, well controlled on CSII, had GSP levels within the normal range, suggesting that mild glucose intolerance may not consistently give GSP levels above the normal range as defined herein (mean +3SD). Therefore, further studies are needed to determine whether a less stringent cutoff point may make the test sufficiently sensitive and specific to use as a screening procedure for subjects with IGT.

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