Glycosylation of Plasma Protein and its Relation to Glycosylated Hemoglobin in Diabetes

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SUMMARY

Glycosylation of plasma proteins was studied in diabetic and normal subjects by an adaptation of a thiobarbituric acid method previously used for glycosylated hemoglobin. There was a highly significant correlation between the degree of glycosylation in vivo of plasma protein and hemoglobin. The process of glycosylation depended on time and temperature and was not mediated enzymatically. Plasma protein of all molecular sizes could be glycosylated. At 37°C, 4.6% of ^{14}C-glucose became attached to plasma protein after 24 h of incubation in vitro; no difference between normal and diabetic plasma protein could be demonstrated. After glycosylation, glucose dissociated from protein slowly; 72% remained attached after dialysis for 24 h. As an estimate of diabetic control, measurement of glycosylation of plasma protein is a suitable alternative to determination of glycosylated hemoglobin. Results of each method correlated equally well with the degree of diabetic control when this was assessed by calculation of the M-factor, mean plasma glucose concentrations, or variance of the plasma glucose determinations. In hemolytic anemia, hemoglobinopathy, and recent transfusion, measurement of glycosylation of plasma protein may be more accurate than that of glycosylated hemoglobin. It may also provide an estimate of diabetic control between that provided by short-term blood glucose determination and long-term glycosylated hemoglobin.

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The measurement of glycosylated hemoglobin (GHb) has improved significantly the objective assessment of diabetic control.[1] The glycosylation of hemoglobin depends on a nonenzymatic reaction occurring after the synthesis of hemoglobin. GHb is known to have an increased affinity for oxygen. The resultant tissue hypoxia has been incriminated as a cause of diabetic microangiopathy.[2] Glycosylation of rat and bovine lens crystallins has been reported and may be important in the genesis of cataracts in diabetes.[3] Glycosylation of serum albumin was also reported recently.[4] Glycosylation of other plasma proteins may likewise result in alterations of their functions. These may be important in producing the numerous metabolic and structural abnormalities known to occur in diabetes mellitus. This study was carried out to characterize further the process of glycosylation of plasma proteins.

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PATIENTS AND METHODS

Heparinized blood was obtained from 45 patients attending the Diabetic Clinic of the Royal Prince Alfred Hospital. Thirty-five patients were treated with insulin, seven patients by oral hypoglycemic agents, and three patients by dietary management. Routinely, plasma glucose levels were estimated at 10 a.m. and 2 p.m. during each clinic visit by the glucose-oxidase method. GHb was measured by cation-exchange chromatography using commercially available columns purchased from Isolab. The glycosylation of plasma protein was estimated by an adaptation of the thiobarbituric acid (TBA) method described by Flückiger et al.[5] for the measurement of GHb. The TBA reaction is specific for the detection of ketoamine-linked carbohydrate residues in proteins formed by the Amadori rearrangement[6] of protein-bound hexose. The 5-hydroxymethylfurfural (5-HMF) released by dehydration and hydrolysis of this linkage reacts with thiobarbituric acid to form a chromagen that has maximal absorbance at a wavelength of 443 nm. Plasma samples (2 ml) were separated from red cells immediately and free glucose was removed by dialysis for 12 h against 10 L of a buffer containing Na Hepes, 50 mmol/L, and NaCl, 0.15 mol/L, pH 7.4. Control experiments had shown that dialysis of this duration removed free glucose completely. To a 1-ml sample of dialyzed plasma was added 0.5 ml of oxalic acid (0.9 mol/L) and the mixture was boiled for 2 h in a water bath. After cooling to room temperature, 0.5 ml of 40% trichloroacetic acid (TCA) was added and immediately

vortexed before centrifugation at 1000 *g* for 10 min. One milliliter of the supernatant was removed and incubated with 0.25 ml of thiobarbituric acid (0.05 mol/L) at 40°C for 40 min, and the absorbance was read at 443 nm. All estimations were performed in duplicate, and only unhemolyzed plasma samples were used. Results were correlated with GHb determined from samples obtained on the same day.

In 24 patients, there were eight or more plasma glucose determinations made during visits to the Diabetic Clinic in the preceding 2 yr (14 with 20 determinations, one with 18 determinations, two each with 12 and 10 determinations, and five with eight determinations). Using these blood glucose results, the degree of diabetic control in these 24 patients was estimated by the calculation of M-factor,[7] mean plasma glucose concentration, and variance of the plasma glucose concentrations. The M-factor was derived according to Schlichtkrull,[7] except that 8–20 blood glucose determinations formed the basis of the calculation. Each of these parameters of diabetic control was correlated individually with results of glycosylation of plasma protein and GHb.

In six diabetic plasma samples the glycosylation of protein was studied over a fourfold dilution of plasma. Results at each dilution were expressed as a percentage of the absorbance at 443 nm of the undiluted sample.

Glycosylation of plasma protein was studied in vitro by incubating 0.5 ml of plasma with 50 μl of 6-^{14}C-glucose (New England Nuclear) containing 20–40 nCi. The reaction was carried out in glass tubes (0.8 × 5.5 cm). The temperature of incubation was varied from 4°C to 45°C, time of incubation from 1 h to 48 h, and glucose concentration from 0.1 mmol/L to 278 mmol/L. After incubation, plasma proteins were precipitated by 0.5 ml of 20% TCA and then washed twice with 2 ml of 2.5% TCA. The precipitate was dissolved in 2 ml Instagel (Packard Instruments) and counted for 5 min in a Wallac-LKB beta liquid scintillation counter. Nonspecific binding of glucose was determined by adding labeled glucose of the same specific activity to plasma, incubated under identical conditions, immediately before precipitation of the protein by TCA, as described. Specific binding was calculated by subtracting the nonspecific binding from the counts precipitated after appropriate incubation and was expressed as a percentage of the total radioactivity present. Glycosylation of protein was compared in samples obtained from 10 diabetic patients and 10 normal individuals. The incubation was carried out routinely at 37°C for 24 h.

The glycosylated components of plasma were identified by the following experiments. Plasma (5 ml) was fractionated on a Sephacryl S-300 (Pharmacia) column of 60 × 2 cm. Elution was carried out with Na Hepes–NaCl buffer and fractions of 4 ml were collected. Another 5 ml aliquot of the same plasma was incubated at 37°C for 24 h with ^{14}C-glucose at a final concentration of 16 mmol/L and 7 μCi/ml. The incubated sample was applied on to the same Sephacryl S-300 column and was eluted under identical conditions. The protein content of the eluate was estimated by measurement of absorbance at 280 nm. The glycosylation of protein in each fraction was analyzed by the thiobarbituric acid method. The radioactivity of ^{14}C-glucose bound to protein was determined by counting a 0.5 ml aliquot of each fraction in 10 ml of Instagel for 5 min. The elution position of alpha-2 macroglobulin, haptoglobin, IgG, and IgM was determined by single immunodiffusion using Tripartigen plates obtained from Behringwerke. The elution position of albumin was monitored by double immunodiffusion using the Ouchterlony technique.

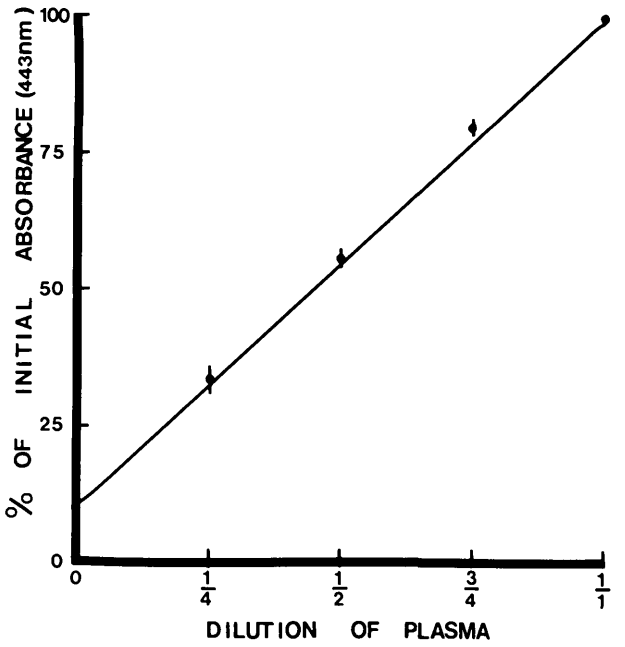


FIGURE 1. The relationship between measurement of glycosylation of plasma protein by the thiobarbituric acid method and dilution of plasma samples. The absorbance at 443 nm of each dilution is expressed as a percentage of the absorbance of the undiluted sample. Each point represents the $\bar{x}$ ± SEM of six plasma samples; 100% corresponds to an absorbance of 0.41 ± 0.04.

In 30 of the plasma samples obtained from diabetic patients the fibrinogen concentrations were measured by an ammonium sulfate precipitation method[8] and alpha-2 macroglobulin and haptoglobin by single immunodiffusion. The galactose and mannose contents of plasma protein were analyzed by the anthrone reaction.[9] Results were correlated with glycosylated plasma protein and GHb.

All results were expressed as $\bar{x}$ ± SEM. Linear correlations were calculated by the least squares' method.

RESULTS

The effect of serial dilution of plasma on measurement of glycosylation of plasma protein is shown in Figure 1. There is a linear relationship between absorbance at 443 nm and the amount of protein present over a fourfold dilution of the plasma samples. Eighty-five per cent (85%) of the specimens examined by the thiobarbituric acid method (38/45) had final optical density within this range. Serial dilution of a glucose solution (50 mmol/L) subjected to boiling in acid and analyzed by the thiobarbituric acid method also resulted in a proportional reduction in absorbance at 443 nm. The minimal glucose concentration tested that yielded a positive reading was 6.25 mmol/L, corresponding to an absorbance of 0.035 at 443 nm. The intra-assay coefficient of variation of the thiobarbituric acid method was 3.1%; the interassay coefficient of variation was 6.3%. The corresponding figures for the GHb measurements were 1.5% and 3.0%.

The highly significant correlation ($r = 0.74$, $p < 0.005$) between the degree of in vivo glycosylation of plasma protein and hemoglobin is shown in Figure 2. Each of these two

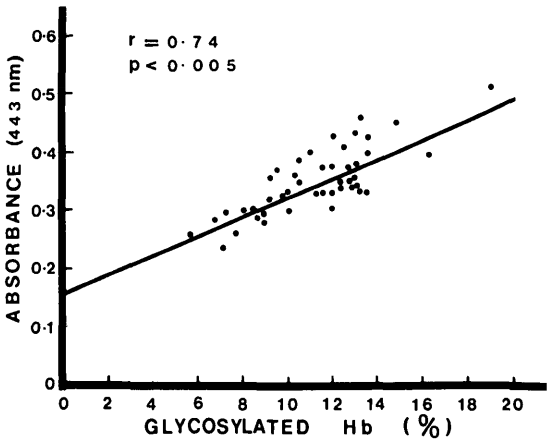
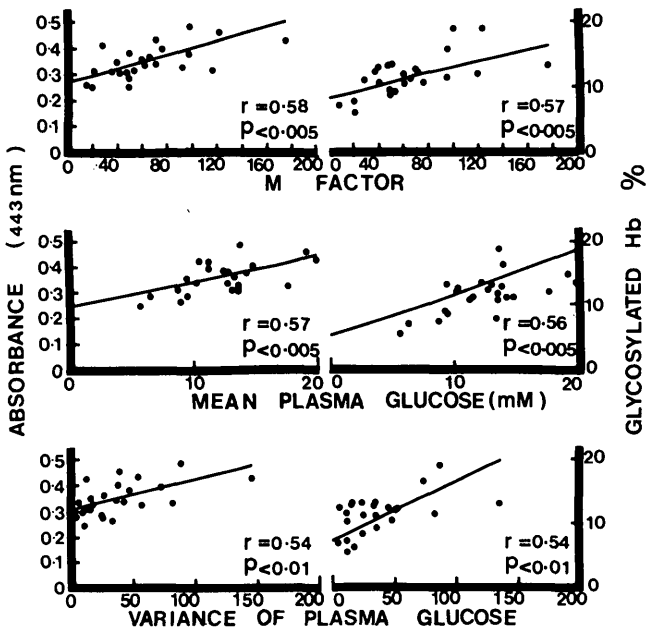


FIGURE 2. The correlation between glycosylation of plasma protein measured by the thiobarbituric acid method and glycosylated hemoglobin measured by cation-exchange chromatography.

parameters is correlated with three different estimates of diabetic control and the results are shown in Figure 3. There is a significant and similar correlation of each with M-factor, mean blood glucose levels, and variance of the blood glucose determinations. For each assay the correlations with M-factor and mean blood glucose concentrations are slightly better than that with the variance of blood glucose determinations.

The effects of temperature and duration of incubation on the in vitro glycosylation of protein is shown in Figure 4. The degree of glycosylation depends on time and temperature. At 37°C, glycosylation is detectable by 1 h, and it continues to rise slowly up to 48 h. After incubation with ^{14}C-glucose for 24 h at 37°C, the mean in vitro glycosylation in diabetic plasma was 4.6 ± 0.3% of the total radioactivity present, not significantly different from that in normal individuals of 4.5 ± 0.6%. There was no evidence of saturation of the glucose binding onto plasma protein, even at glucose concentrations of 278 mmol/L.

FIGURE 3. The correlation of glycosylation of plasma protein and glycosylated hemoglobin with M-factor, mean plasma glucose concentrations, and variance of the plasma glucose determinations in 24 diabetic subjects.



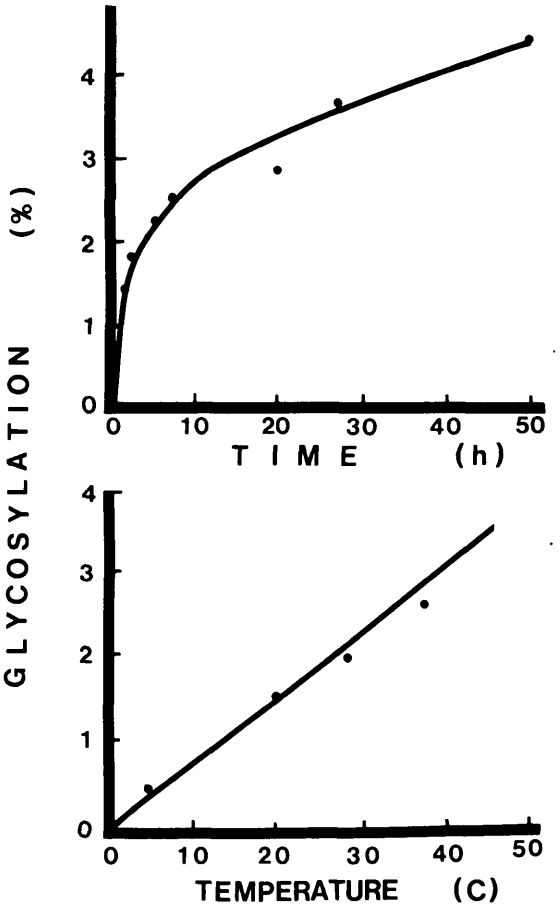


FIGURE 4. The in vitro glycosylation of plasma protein at different time and temperature of incubation. ^{14}C-glucose was present at a concentration of 0.1 mmol/L and 40–80 nCi/ml.

The elution pattern of plasma proteins on Sephacryl S-300 chromatography is shown in Figure 5. When plasma was incubated with glucose (16 mmol/L), increased glycosylation of proteins of all molecular sizes could be demonstrated by the thiobarbituric acid method. ^{14}C-labeled glucose was shown to bind to all protein components, but the major peak corresponded to the position of albumin. The radioactivity bound to protein was 2.2 ± 0.1% (mean of four experiments) of the total radioactivity present.

Fractions 25, 26, and 27 of the above experiment, containing the albumin peak, were pooled and dialyzed against Na Hepes–NaCl buffer at 4°C and 37°C. No difference in the rate of disappearance of 6-^{14}C-glucose from plasma proteins could be detected at these temperatures. In three experiments the mean radioactivity remaining attached to plasma protein after 6 h of dialysis was 87%; the corresponding figure of 24 h was 72%.

There was no significant correlation between the plasma concentration of haptoglobin, alpha-2 macroglobulin, and fibrinogen with glycosylation of plasma protein. The correlation coefficients were 0.10, 0.04, and 0.08, respectively. The total mannose and galactose concentrations of plasma protein also showed no correlation with glycosylation of plasma protein (r = 0.08). Similarly, no correlation between any of these measurements with GHb could be demonstrated. The

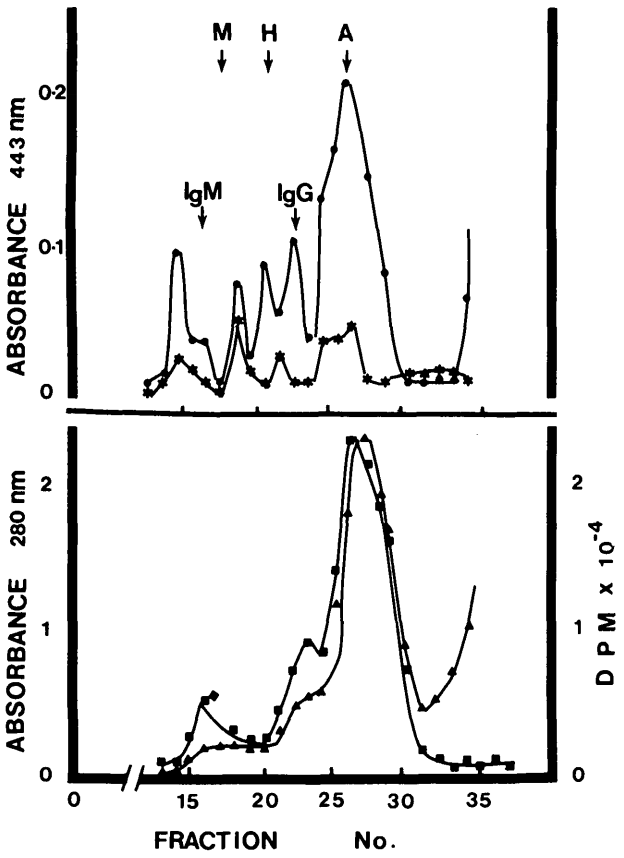


FIGURE 5. The elution profile of plasma protein on Sephacryl S-300 chromatography. The elution positions of IgM, IgG, alpha-2 macroglobulin (M), haptoglobin (H), and albumin (A) are indicated by arrows. Glycosylation of plasma protein with (●) and without (*) preincubation with glucose (16 mmol/L) is shown in the top panel. The radioactivity of ^{14}C-labeled glucose (▲) bound to plasma protein preincubated with radioactive glucose (7 μCi/ml) and the absorbance of eluate at 280 nm (■) are shown in the bottom panel.

corresponding correlation coefficients were 0.12, − 0.17, and 0.06, respectively.

DISCUSSION

The formation of GHb by a post-translational attachment of glucose to hemoglobin is now well established. Glycosylation of plasma proteins by similar mechanisms has been demonstrated in this study. The glycosylation process depends on temperature and time of incubation. Hyperglycemia, lasting for only a few hours at physiologic temperature, would result in readily detectable glycosylation. Similarly to the formation of GHb, glycosylation of protein appears not to be mediated by an enzymatic mechanism, as the process is relatively slow, nonsaturable even at high glucose concentrations, and is not reduced by incubation temperature higher than 37°C. The glycosylation of protein is only slowly reversible; 72% of glucose remained attached to protein after dialysis at physiologic temperature for 24 h. In vivo the net dissociation of glucose from protein could be expected to be even slower, as plasma always contains free glucose.

The GHb level is determined primarily by the mean blood glucose concentration in the preceding 2–3 mo.[1] The highly significant correlation between the in vivo glycosylation of plasma protein and hemoglobin observed in this study suggests that the mean glucose concentration is also the most important parameter in determining the extent of protein glycosylation. This is supported by the good correlation observed between glycosylation of plasma protein and mean blood glucose levels in diabetic patients. Plasma proteins of all molecular sizes can be glycosylated, but albumin was quantitatively the most important, presumably because of its high concentration in plasma. It is unlikely that the correlation between glycosylation of protein and hemoglobin can be explained by an increased de novo synthesis in glycoprotein in poorly controlled diabetes. When concentrations of such individual glycoproteins as fibrinogen, alpha-2 macroglobulin, and haptoglobin were analyzed, no correlation with glycosylation of plasma protein or GHb was observed. There is no evidence that plasma proteins from diabetic individuals are more likely to be glycosylated; we could not demonstrate any difference in the in vitro incorporation of ^{14}C-labeled glucose to plasma proteins from diabetic or normal subjects. Mannose and galactose are quantitatively the most important hexose constituents of plasma glycoprotein. As mannose and galactose can also be converted to 5-HMF and react with thiobarbituric acid, it is possible that the observed correlation between glycosylation of protein and hemoglobin is due to an increased mannose and galactose content of plasma glycoproteins. However, when the content of these two hexoses in plasma proteins was measured, no correlation with either glycosylation of plasma protein or GHb could be detected. Thus, the most likely explanation for the increased protein glycosylation in poorly controlled diabetes is a post-translational incorporation of glucose as a consequence of hyperglycemia. This would explain the detection of carbohydrate on albumin, which is known not to be a glycoprotein.[10,11] Many substances are transported while bound to albumin, and binding of certain drugs to alpha-1 acid glycoprotein has been reported.[12]

Our observation that albumin was quantitatively the most important glycosylated plasma component supports the finding of Day et al.[4] Our results also agreed well with a recent report by Kennedy et al.[13] that protein-bound hexose concentration was correlated with GHb level. In addition, we were able to demonstrate a correlation between the degree of protein glycosylation with M-factor, mean glucose levels, and variance of blood glucose concentrations, while they were unable to show such a relationship between protein-bound hexose with fasting glucose level, further evidence that a single glucose determination is not a good index of overall diabetic control. We were unable to show the correlation between haptoglobin concentration and the degree of glycosylation of either plasma protein or hemoglobin reported by Kennedy et al.[13]

The measurement of glycosylated plasma protein by the thiobarbituric acid method is reproducible, technically easy, and requires only readily obtainable chemicals. It can provide the same information as the measurement of GHb. The almost identical correlation of glycosylated protein and glycosylated hemoglobin with M-factor and with mean and variance of blood sugar levels indicate that these two methods are equally suitable as assessments of diabetic control. The better correlations of each with M-factor and mean blood glucose concentrations than with variance of blood glucose determinations suggest that they reflect more accurately persistent hyperglycemia rather than instability of diabetes. In such clinical conditions as hemolytic ane-

mia, hemoglobinopathy, and recent transfusion the measurement of GHb is unreliable.[14] In these situations the assessment of diabetic control can be made by measuring the glycosylation of plasma protein. As the circulating half-lives of plasma proteins are only 2–3 days the monitoring of glycosylation of protein may represent a temporal assessment of diabetic control intermediate to that of blood sugar and GHb measurements.[10] It may provide an objective index of the efficacy of treatment in diabetes without waiting for the slow changes in GHb levels. It is possible that the binding and subsequent dissociation of glucose from plasma proteins may serve as a buffer against major fluctuations in blood sugar concentrations. However, in view of the slow nature of both the forward and reverse reactions, it seems unlikely that this will be of great physiologic significance in the minute to minute regulation of blood sugar concentration.

The affinity of GHb for oxygen is known to be greater than that of the native hemoglobin. This phenomenon is attributed to the steric hindrance of the additional glucose for the binding of 2,3-diphosphoglycerate onto the hemoglobin molecule. The glycosylation of plasma protein as a result of persistent hyperglycemia may similarly affect the functions of other plasma protein components. These possibilities remain to be explored. Glycosylation of tissue proteins may also account for such phenomena as basement membrane thickening, receptor dysfunction, and metabolic abnormalities, which are sequelae of long term diabetes mellitus.[15,16]

ACKNOWLEDGMENTS

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