Nonenzymatically Glycosylated Serum Protein: Spurious Elevation Due to Free Glucose in Serum

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SUMMARY

The presence of free glucose in serum was found to interfere with accurate measurement, by a colorimetric method, of nonenzymatically glycosylated serum proteins. A mean elevation to 241% of basal levels was observed in the serum of 11 nondiabetic subjects to which glucose, in a concentration of 300 mg/dl, had been added immediately before assay. After dialysis of serum samples to remove glucose, levels of nonenzymatically glycosylated serum proteins were 0.27 ± 0.11 and 0.79 ± 0.24 nmol 5-hydroxymethylfurfural/mg protein (mean ± SD), respectively, in 57 nondiabetic and 62 type I diabetic subjects. Levels observed before dialysis of serum were approximately two to three times higher. These studies indicate that removal of free sugar from serum is necessary for accurate measurement of glycosylated protein by the colorimetric method, and this can be achieved by overnight dialysis of serum against normal saline.

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The recognition that hemoglobin undergoes postsynthetic, nonenzymatic glycosylation in vivo led to speculation that other proteins may be similarly affected.[1] Subsequently, glycosylation of lens crystallins,[2] collagen,[3] erythrocyte membrane proteins,[4] albumin, and other serum proteins [5,6] has been shown to occur in vivo.

It is known that glycosylated hemoglobin reflects the degree of glycemia in the preceding weeks to months, since erythrocytes have a half-life of approximately 60 days.[1] It has been postulated that nonenzymatic glycosylated serum protein (N.G.S.P.) levels may indicate the status of glycemia during the 1–2-wk period before measurement because serum proteins have a much shorter half-life than erythrocytes.[5] If measurement of N.G.S.P. is to be useful clinically, it is obviously important that the prevailing level of serum glucose should not interfere with its accurate measurement.

We have investigated the effect of artificially elevating the serum glucose level, just before assay, on the measurement of N.G.S.P. by a recently described method.[5] We have also investigated the effect on the measurement, in diabetic and control subjects, of removing glucose from serum by dialysis before assay. Our results show that free glucose in serum leads to spurious elevation of N.G.S.P. and that previously reported levels in diabetic and control subjects are falsely high.

METHODS

Serum was separated from venous blood samples obtained from 62 ambulatory, active, type I (juvenile-onset) diabetic and 57 normal control subjects after an overnight fast. N.G.S.P. was measured in each serum sample before and after dialysis against normal saline (18 h, serum/saline ratio of 1/350). Serum protein, both before and after dialysis, was measured by the Biuret method. Glucose was measured by a standard glucose oxidase method. Glucose was added, in increments of up to 300 mg/dl, to 11 control sera just before assay to determine whether the presence of free glucose would influence the measurement of N.G.S.P.

The method used for determinating N.G.S.P. was the one recently described by McFarland et al.[5] Glucose bound nonenzymatically to serum protein is released, by hydrolysis with 1 M oxalic acid at 100°C for 5 h, as 5-hydroxymethylfurfural (HMF), which is quantified colorimetrically by measuring the absorbance at 443 nm after reaction with thiobarbituric acid. Every sample was run in duplicate and assayed against its own blank in which the ketoamine protein derivative was reduced to a nonreactive form by $NaBH_4$.[7] The N.G.S.P. was calculated as nmol HMF/mg protein using pure 5-hydroxymethylfurfuraldehyde (Sigma Chemical Company) as standard, whereas McFarland et al. used glycosylated human albumin as standard.

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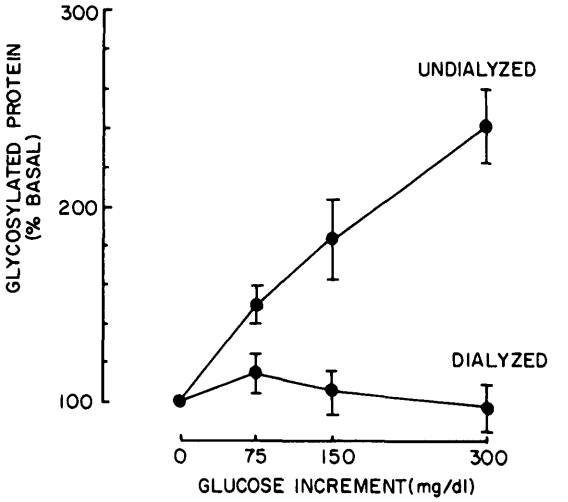

FIGURE 1. Effect on measurement of N.G.S.P. of adding glucose, in increasing concentration immediately before assay, to serum from nondiabetic control subjects. The points represent the mean values, as percent basal (no added glucose), in 11 subjects. The bars represent SEM. The lower line shows the effect of dialyzing the serum against normal saline to remove the added glucose before assay.

RESULTS

The effect of acutely increasing the glucose concentration on the measurement of N.G.S.P. in control serum is illustrated in Figure 1. There was a clear increase with increasing glucose concentration, so that in the presence of an added 300 mg/dl of glucose, artifactual elevations of 200–300% were observed. After dialysis to remove the added glucose no such increase was observed.

Serum glucose levels in the controls ranged from 51 to 108 mg/dl and in the diabetics from 27 to 538 mg/dl with a median value of 180 mg/dl. After overnight dialysis against normal saline, all serum glucose values were less than 12 mg/dl, and there was no significant difference between the levels in controls and diabetics. Mean serum protein levels before and after dialysis did not differ significantly. Figure 2 shows the levels of N.G.S.P. measured in the serum of control and diabetic subjects before and after dialysis against normal saline. Before dialysis, the mean ± SD N.G.S.P. was 0.93 ± 0.26 nmol HMF/mg protein in controls and 1.80 ± 0.63 nmol HMF/mg protein in diabetic subjects. After dialysis of serum, these values were 0.27 ± 0.11 and 0.79 ± 0.24 nmol HMF/mg protein, respectively, in control and diabetic subjects.

The intra-assay coefficient of variation for the complete N.G.S.P. assay procedure, including dialysis of serum, was 11%. The interassay coefficient of variation was 13%.

DISCUSSION

We have demonstrated that free glucose in the serum at the time of assay will cause spurious elevation of the level of N.G.S.P. as measured by the current method. It has been shown that nonenzymatic glycosylation of protein in vitro is dependent on the glucose concentration to which the protein is exposed, the length of time of exposure, and the temperature of incubation.[8] During the assay for N.G.S.P., serum and oxalic acid are incubated together for 5 h at 100°C in order to release the nonenzymatically bound glucose as 5-HMF. We would postulate that at this temperature

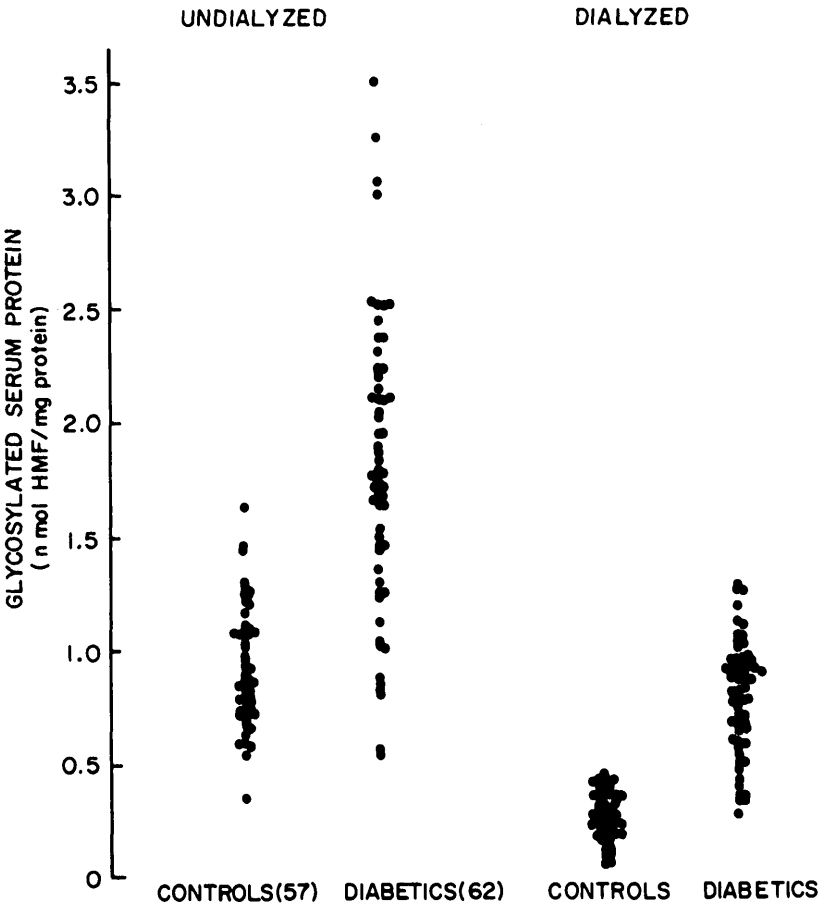

FIGURE 2. N.G.S.P. levels in 62 type I diabetic subjects and 57 age-matched controls. Sera were assayed before (left) and after (right) dialysis against normal saline.

further rapid glycosylation of protein by free sugar in the serum is enhanced, with subsequent hydrolysis and release of 5-HMF. This error can be circumvented by first dialyzing the serum to remove free glucose (Figure 1).

The ranges of values for N.G.S.P. we observed in the undialyzed serum of control and diabetic subjects were remarkably similar to those found by McFarland et al., despite the use of a different standard. However, whereas they found no overlap between diabetic and control values, 20 (32%) of our diabetics had N.G.S.P. values for undialyzed serum that were less than the highest value seen in controls. The explanation for this difference probably lies in the fact that only 3 (10%) of the South Carolina group's diabetic patients had serum glucose values of <100 mg/dl compared with 17 (27%) of ours. Therefore, there was probably less artifactual elevation of N.G.S.P. levels in those diabetic patients with low blood sugar levels. It is interesting that after dialysis of the serum to remove glucose, we found much less overlap between our control and diabetic subjects, with only 7 diabetics (11%) having values lower than the highest value in controls.

We do not dispute the conclusions of McFarland et al. that N.G.S.P. levels probably reflect the level of glycemia in the preceding 1–2 wk, as we have found a good correlation between dialyzed N.G.S.P. levels and glycosylated hemoglobin and fasting serum glucose (unpublished data). However, we believe that the actual ranges of values that we have observed in dialyzed serum are more correct. In support of this claim are the findings of Pecoraro et al.,[9] using a similar colorimetric method and 5-HMF as standard, that glycosylated hemoglobin levels range from approximately 1.3 to 5.5 nmol HMF/mg Hb in diabetic subjects. That is approximately a fourfold increase in the range that we found for glycosylated serum protein in diabetics, which would be expected since the half-life (and hence exposure to glucose) of hemoglobin is approximately four times that of serum proteins.

The main significance of our findings relates to the potential use of N.G.S.P. measurements as another index of glycemia, analogous to glycosylated hemoglobin, but reflecting glycemia over a shorter time. If the measurement is subject to error due to the prevailing level of glucose at the time the sample is drawn, then it would be of no value in many diabetic patients, particularly those with type I diabetes, where considerable perturbations of serum glucose levels may occur in minutes to hours and where successive fasting glucose levels may vary considerably without necessarily any real difference in the overall glycemia of the preceding 24 h. This problem can be overcome by dialyzing the serum before assay, a step that unfortunately, but unavoidably, lengthens the complete assay procedure. On the other hand, since many samples can be processed in one assay, measurement of N.G.S.P. has definite clinical and research potentials.

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REFERENCES

[1] Peterson, C. M., and Jones, R. L.: Minor hemoglobins, diabetic "control" and diseases of postsynthetic protein modification. Ann. Intern. Med. *87*:489–91, 1977.

[2] Stevens, V. J., Rouzer, C. A., Monnier, V. M., and Cerami, A.: Diabetic cataract formation: potential role of glycosylation of lens crystallins. Proc. Natl. Acad. Sci. USA *75*:2918–22, 1978.

[3] Robins, S. P., and Bailey, A. J.: Age related changes in collagen: the identification of reducible lysine-carbohydrate condensation products. Biochem. Biophys. Res. Commun. *48*:76–84, 1972.

[4] Bailey, A. J., Robins, S. P., and Tanner, M. J. A.: Reducible components in the proteins of human erythrocyte membrane. Biochem. Biophys. Acta *434*:51–57, 1976.

[5] McFarland, K. F., Catalano, E. W., Day, J. F., Thorpe, S. R., and Baynes, J. W.: Nonenzymatic glucosylation of serum proteins in diabetes mellitus. Diabetes *28*:1011–14, 1979.

[6] Dolhofer, R., and Wieland, O. H.: Glycosylation of serum albumin: elevated glycosyl-albumin in diabetic patients. FEBS Lett. *103*:282–86, 1979.

[7] Holmquist, W. R., and Schroeder, W. A.: A new terminal blocking group involving a Schiff base in hemoglobin A_{1c}. Biochemistry *5*:2489–2503, 1966.

[8] Day, J. F., Thornburg, R. W., Thorpe, S. R., and Baynes, J. W.: Nonenzymatic glucosylation of rat albumin. J. Biol. Chem. *254*:9394–9400, 1979.

[9] Pecoraro, R. E., Graf, R. J., Halter, J. B., Beiter, H., and Porte, D., Jr.: Comparison of a colorimetric assay for glycosylated hemoglobin with ion-exchange chromatography. Diabetes *28*:1120–25, 1979.