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Nonenzymatically Glucosylated Albumin

IN VITRO PREPARATION AND ISOLATION FROM NORMAL HUMAN SERUM*

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SUMMARY

Incubation of human serum with D-[6-³H]glucose resulted in the gradual accumulation of radioactivity in acid-precipitable material. Upon chromatography on Sephadex G-200, radioactivity was found associated with each of the major molecular weight classes of serum protein. Purified human serum albumin was also glucosylated *in vitro* upon exposure to D-[6-³H]glucose in phosphate-buffered saline. The glucosylated and unmodified albumins were separated by ion exchange chromatography. The physiological significance of these observations *in vitro* was confirmed by the isolation and quantitation of glucosylated albumin from normal human serum. Glucosylated albumin represents approximately 6 to 15% of total serum albumin in normal adults. The post-translational modification appears to occur by a nonenzymatic process analogous to that responsible for glucosylation of hemoglobin A to hemoglobin A_{1c}, i.e. through Schiff base formation and Amadori rearrangement to a ketoamine derivative.

While the acute manifestations of diabetes (e.g. hyperglycemia, glycosuria, ketoacidosis) can often be managed clinically by insulin therapy, progressive secondary complications are the major cause of the chronic illness and high mortality associated with this disease (1-3). These complications result primarily from severe, generalized vascular disease (microangiopathy and atherosclerosis), which is most apparent in critical organs such as the kidneys and eyes, and in the nervous system. There is an increasing body of evidence based on both clinical experience with patients (3) and animal model studies (4) that chronic, subclinical hyperglycemia is the major causative factor in the secondary complications of diabetes. The mechanism by which hyperglycemia may induce these vascular problems is presently unknown. It has been postulated, however, that glucose-dependent chemical alterations in body proteins could cause changes in protein structure, and hence, function, leading to the pathophysiology of diabetes (2). The demonstration in diabetics of increased amounts of hemoglobin A_{1c}, a nonenzymatically glucosylated form of hemoglobin A, was the first example of a protein modification correlated

with elevated blood glucose concentrations (5). More recently, Stevens *et al.* (6) have provided convincing evidence that glucosylation of lens crystallins may contribute to the development of corneal opacification in diabetics.

The chemical reactions leading to the nonenzymatic glucosylation of protein were originally described in studies on the browning reactions of food proteins (7). The interaction between glucose and protein occurs at free amino groups in the protein, and mechanistically, involves the free base form of the amine. In the case of hemoglobin, glucosylation occurs *in vivo* or *in vitro* by a condensation between glucose and the NH₂-terminal valine residues of the β chains. As shown in Fig. 1, the initial product, the Schiff base derivative of hemoglobin, undergoes an Amadori rearrangement to form an acid-stable ketoamine adduct (5). There is no *a priori* reason, however, why glucosylation should be unique to hemoglobin or crystallins, and we hypothesized that plasma proteins, which are normally bathed in a 5 mM glucose medium, should undergo similar modifications. We report here the *in vitro* glucosylation of albumin and several other serum proteins, and document, for the first time, that 6 to 15% of human albumin in serum exists naturally in a glucosylated form.

MATERIALS AND METHODS

Human serum albumin was purified from fresh human serum by affinity chromatography on Affi-Gel Blue (Bio-Rad) (8), followed by gel chromatography on Bio-Gel P-150 (Bio-Rad). D-[6-³H]Glucose (34 Ci/mmol) was purchased from New England Nuclear Co.

Incubations—Albumin solutions and human serum were sterilized by ultrafiltration, and incubated in the dark at room temperature. Albumin solutions were prepared in Dulbecco's phosphate-buffered saline (9), containing 5 mM glucose. Serum was incubated under an atmosphere of 95% O₂, 5% CO₂ to maintain pH 7.3 to 7.4. Trace amounts of [6-³H]glucose were added to incubation mixtures to obtain desired specific activity.

Assays—Acid-precipitable radioactivity was determined by adding 25- μ l aliquots of incubation mixtures to 50 μ l of a bovine serum albumin solution (10 mg/ml) and precipitating with 1 ml of cold 10% trichloroacetic acid. Precipitates were washed twice by dissolution in 100 μ l of 0.1 N NaOH and reprecipitated with acid. The washed precipitates were dissolved in 500 μ l of H₂O and counted for radioactivity in Beckman Instruments Co. Bio-Solv EP.

Glucosylated protein was detected using the thiobarbituric acid procedure of Flückiger and Winterhalter (10) which measures 5-hydroxymethylfurfural released upon hydrolysis of ketoamine adducts of protein. All samples were scanned in the 400 to 500 nm region, yielding a λ_{\max} at 443 nm, characteristic of the reaction of thiobarbituric acid with 5-hydroxymethylfurfural.

RESULTS

When human serum was incubated with tracer amounts of [6-³H]glucose under sterile conditions, radioactivity gradually accumulated in an acid-precipitable fraction (Fig. 2). Accumulation of radioactivity was linear with time for at least 200 h, and, by the end of this time, about 10% of the starting glucose had been transferred to acid-precipitable material. As a first step in the characterization of the reaction product, the remainder of the incubation mixture at 200 h was dialyzed. Essentially all of the initial acid-precipitable radioactivity in the incubation mixture was stable to dialysis and was nondialyzable. Upon chromatography of the dialysate on Sephadex G-200 (Fig. 3), radioactivity was associated with each of the three major molecular weight species of serum proteins. The

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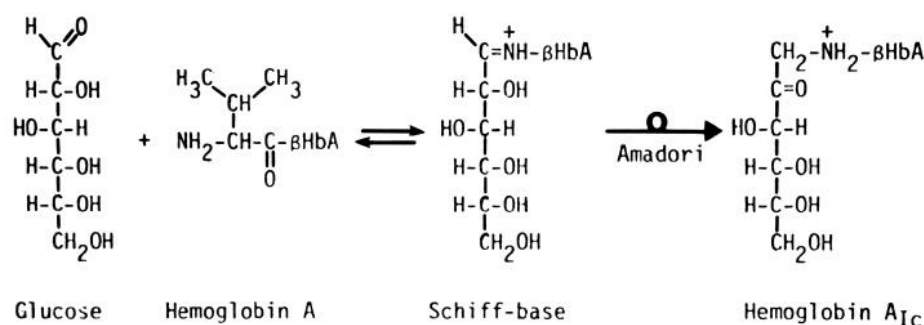


FIG. 1. Formation of hemoglobin A_{1c} from glucose and hemoglobin A.

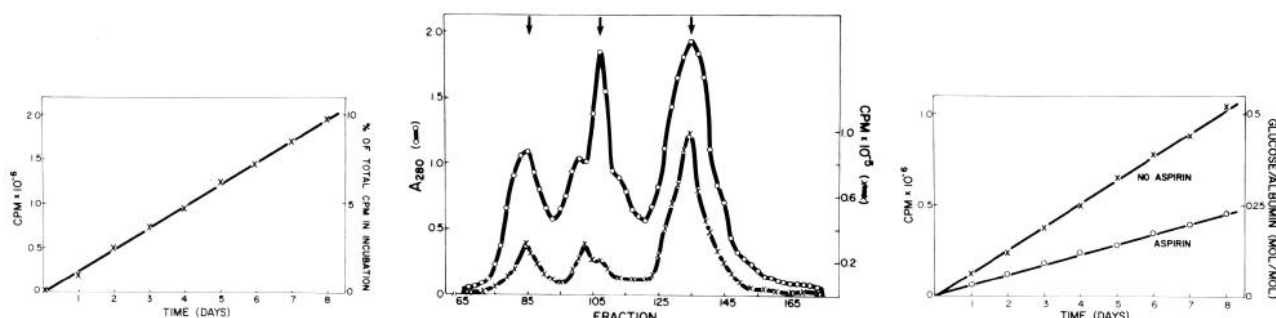


FIG. 2 (left). *In vitro* incorporation of acid-precipitable radioactivity into human serum incubated with [6-³H]glucose. One milliliter of human serum was incubated with 2×10^7 cpm ($\sim 1 \mu\text{g}$) of [6-³H]glucose at 25°C; aliquots were removed at indicated times and radioactivity precipitable by trichloroacetic acid was determined as described under "Materials and Methods". Data are expressed as precipitable counts per min in total incubation.

FIG. 3 (center). Sephadex G-200 chromatographic profile of human serum after 8 days of incubation with [6-³H]glucose. Human serum was incubated with [6-³H]glucose for 200 h, as in Fig. 2, dialyzed overnight at 4°C against two changes (1000 volumes) of 0.1 M Tris-HCl, pH 7.8, 1.0 M NaCl, and chromatographed on a G-200 column (1.5×63 cm, 1 ml/fraction). The column was eluted with the same

buffer, and the effluent was monitored for both protein and glucose radioactivity. Arrows indicate position of elution of IgM, IgG, and albumin, respectively.

FIG. 4 (right). *In vitro* incorporation of [6-³H]glucose into human serum albumin in the presence and absence of aspirin. One hundred three nmol (7 mg) of human serum albumin were incubated in 1 ml of Dulbecco's phosphate-buffered saline (9) containing 5 mM glucose, 2×10^7 cpm of [6-³H]glucose, with 0.5 mM aspirin (○—○) or without aspirin (×—×). Aliquots were removed at indicated times and acid-precipitable radioactivity was determined as described under "Materials and Methods." Data are expressed as radioactivity in total incubation.

highest specific activity was found in the region corresponding to authentic human albumin. For this reason, we decided to study the glucosylation of serum albumin itself, at physiological pH and electrolyte concentration *in vitro*.

Monomeric human serum albumin was prepared as described under "Materials and Methods" and incubated with [6-³H]glucose. As shown in Fig. 4, incorporation of glucose into albumin (by acid precipitation) was linear for at least 8 days, at which time 0.5 mol of glucose were incorporated per mol of albumin. This experiment with purified albumin indicates that glucosylation can proceed by a nonenzymatic mechanism. Since the nucleophilic amino group of lysine 189 ($\text{pK} \approx 7.9$) of human serum albumin is known to be reactive to acetylation by aspirin (acetyl salicylate) (11), a competition experiment was carried out to evaluate the possibility that glucosylation could also occur at that site. Addition of aspirin to incubations at one-tenth the concentration of glucose (Fig. 4) resulted in a 50% inhibition of the rate of incorporation of [6-³H]glucose into albumin, suggesting that at least some glucose is being incorporated at lysine 189.

The tentative identification of lysine 189 as one site for glucosylation *in vitro*, and the acid stability of the glucose linkage to albumin, suggested that albumin was being modified by a mechanism similar to that proposed for the formation of hemoglobin A_{1c} (Fig. 1). Because the Amadori rearrangement to the ketoamine adduct eliminates the chiral center at C-2 of glucose, both glucose and mannose are obtained upon hydrolysis of hemoglobin A_{1c}. Similarly, radioactivity was recovered in both glucose and mannose following chromatography of a hydrolysate of albumin obtained at 200 h from the

incubation described in Fig. 3. The ratio of glucose to mannose, $\sim 4:1$, was comparable to that described for hemoglobin A_{1c}, $\sim 3:1$ (12).

We next attempted to separate the *in vitro* glucosylated albumin from unmodified albumin by chromatographic methods. Following dialysis of albumin incubated with [6-³H]glucose for 130 h, under conditions described in Fig. 4, the albumin was resolved into two distinct fractions upon chromatography on carboxymethylcellulose (Fig. 5A). The fast moving fraction (I) contained 30% of the protein and more than 95% of the radioactivity, while the second fraction (II) contained the remaining 70% of protein, but only trace amounts of radioactivity. These two fractions together accounted for 100% of the applied protein (A_{280}) and radioactivity.

The glucosylated albumin in Peak I of Fig. 5A accounted for about 108 nmol out of the original 368 nmol, based on absorbance measurements. However, based on the specific activity of the glucose used (4×10^3 cpm/nmol), only 46 nmol of sugar were present in Peak I. Assuming 1 nmol of glucose/nmol of albumin, there is an apparent discrepancy of about 62 nmol, or 17% of the total albumin. This discrepancy suggested either that there was a major nonglucosylated contaminant in Peak I, or that the starting material contained approximately 17% unlabeled, glucosylated albumin. The former possibility was unlikely since polyacrylamide gel electrophoresis of the original albumin in both denaturing and nondenaturing systems revealed less than 1% contaminating protein (data not shown). However, chromatography of this albumin preparation on carboxymethylcellulose, shown in Fig. 5B, clearly

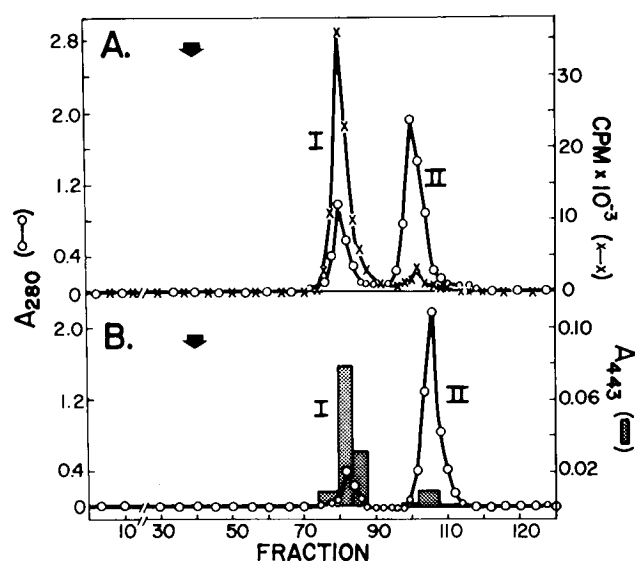


FIG. 5. Separation of human serum albumin into glucosylated and nonglucosylated components by chromatography on carboxymethylcellulose. Samples in 0.01 M sodium acetate, pH 4.65, were applied to a column (17.5 × 2 cm) of carboxymethylcellulose and washed with 10 column volumes of starting buffer. The column was eluted using a 150-ml gradient of 0.01 to 0.5 M sodium acetate, pH 4.65. The start of the gradient elution is indicated by the arrow. Fraction size = 1 ml. A, chromatography of albumin following *in vitro* glucosylation with [6-³H]glucose. B, chromatography of albumin isolated from human serum. Peaks I and II were each divided into three equal pools (by volume) and tested for ketoamine structures (A_{443}) by the thiobarbituric acid assay (9).

revealed two peaks of protein, one running exactly where radioactivity was recovered from *in vitro* incubations using [6-³H]glucose. The material in Peak I of Fig. 5B represented 13% of the total absorbance, very close to the 17% predicted from the previous experiment. In addition, when subjected to the thiobarbituric acid assay for ketoamine adducts of protein, a strong positive test was obtained across Peak I, while Peak II yielded a very low, but reproducible response.

We have also examined purified albumin from frozen pooled human serum, as well as albumin prepared from fresh serum of six healthy donors. In all cases, the glucosylated albumin was detected chromatographically, and identification was confirmed by the thiobarbituric acid assay (10). Glucosylated albumin accounted for approximately 6 to 15% of the total albumin in all samples.

DISCUSSION

These experiments were initiated in order to provide insight into the relationship between hyperglycemia and the pathophysiology of diabetes. Beginning with the hypothesis that enhanced rates of protein glucosylation in diabetes might contribute to this pathophysiology, we decided first to determine the extent to which plasma proteins are susceptible to glucosylation *in vitro*. Our finding that several classes of plasma proteins were, in fact, glucosylated *in vitro* was not unexpected since the generality of the reaction between glucose and amino groups of proteins is well documented (7).

The observation that the albumin-containing molecular weight fraction of serum proteins was most highly glucosylated *in vitro* was also consistent with the known reactivity of a few low pK lysine residues in albumin (11).

The *in vitro* observations are physiologically relevant, however, only if glucosylated albumin (and other serum proteins) can be demonstrated in normal serum. The development of a chromatographic procedure which reliably separated the *in vitro* glucosylated from unmodified albumin permitted us to detect and quantitate the naturally glucosylated albumin derivative in normal serum. The detection of glucosylated albumin *in vivo* suggests that other plasma proteins glucosylated *in vitro* will also be found as glucosylated derivatives *in vivo*. The electrophoretic and isoelectric heterogeneity of albumin and the other plasma proteins undoubtedly depend to some extent on this post-translational modification. The previous characterization of glucosylated hemoglobin and the findings described here suggest that the already extensive list of stable post-translational modifications of proteins, recently reviewed by Uy and Wold (13), should be expanded to include the ketoamine derivatives of proteins at lysine and NH₂-terminal residues.

Glucosylation, as a chemical insult to protein, may be physiologically significant in determining the basal rates of catabolism of plasma proteins. The resultant disturbance in the homeostatic equilibria between protein synthesis and catabolism may be a factor in the gradual development of the pathophysiology of diabetes. Glucosylation could also affect the function, and possibly the turnover, of other proteins exposed to the high glucose concentration of the extracellular fluid, including proteins in basement membranes, as well as plasma membranes of endothelial cells, blood cells, and platelets.

REFERENCES

1. Marble, A., White, P., Bradley, R. F., and Krall, L. P., eds (1971) *Joslin's Diabetes Mellitus*, 11th Ed, Lea and Febiger, Philadelphia
2. Renold, A. E., Mintz, D. H., Muller, W. A., and Cahill, G. F., Jr. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., and Fredrickson, D. S., eds) pp. 80-109, McGraw-Hill Books Co., New York
3. West, K. M. (1978) *Epidemiology of Diabetes and Its Vascular Lesions*, Chap. 6, American Elsevier Publishing Co., New York
4. Fox, C. J., Darby, S. C., Ireland, J. T., and Sonksen, P. H. (1977) *Br. Med. J.* **2**, 605-607
5. Bunn, H. F., Gabbay, K. H., and Gallop, P. M. (1978) *Science* **200**, 21-27
6. Stevens, V. J., Rouzer, C. A., Monnier, V. M., and Cerami, A. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 2918-2922
7. Gottschalk, A. (1972) in *Glycoproteins* (Gottschalk, A., ed) pp. 141-157, American Elsevier Publishing Co., New York
8. Travis, J., and Pannell, R. (1973) *Clin. Chim. Acta* **49**, 49-52
9. Dulbecco, R., and Vogt, M. J. (1954) *J. Exp. Med.* **98**, 167-173
10. Flückiger, R., and Winterhalter, K. H. (1976) *FEBS Lett.* **71**, 356-360
11. Taylor, R. P. (1977) in *Albumin, Structure, Function and Uses* (Rosenoer V. M., Oratz, M., and Rothschild, M. A., eds) pp. 183-202, Pergamon Press, New York
12. Bunn, H. F., Haney, D. N., Gabbay, K. H., and Gallop, P. M. (1975) *Biochem. Biophys. Res. Commun.* **67**, 103-109
13. Uy, R., and Wold, F. (1978) *Science* **198**, 890-896