Nonenzymatic Glucosylation of Rat Albumin: Studies in Vitro and in Vivo

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Nonenzymatic Glucosylation of Rat Albumin
STUDIES IN VITRO AND IN VIVO*

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Incubation of rat serum with [6-3H]glucose in vitro resulted in nonenzymatic glucosylation of serum proteins. Analysis of freshly isolated rat albumin by ion exchange chromatography indicated that the glucosylated albumin accounts for 6.7 ± 0.9% of total albumin in normal rat serum. Glucosylation of rat albumin in vitro was first order with respect to glucose and albumin concentrations and occurs primarily (>90%) at intrachain lysine residues. Kinetic analysis and inhibition of glucosylation by aspirin suggest that 1 reactive lysine residue is the primary site of glucosylation. Less than 5% of the radioactivity from [6-3H]glucosyl-albumin was released as glucose or mannose by hydrolysis conditions normally used for the analysis of neutral sugars in glycoproteins. Studies in vivo demonstrated that the half-life of albumin in normal rats was unaffected by the addition of 1 mol of glucose/mol of albumin. In addition, glucosylation was found to be a stable modification since 151I-albumin isolated up to 3 days after injection of glucosylated 151I-albumin was recovered only in the glucosylated fraction. In contrast, following injection of unglucosylated 151I-albumin there was a gradual shift of 151I radioactivity to the glucosylated albumin fraction, as would be predicted for nonenzymatic glucosylation occurring in the circulation. Finally, levels of glucosylated albumin isolated from diabetic rats (alloxan induced) were significantly (4-fold) elevated 4 days after withdrawal from insulin therapy. These studies indicate that the rat should be a suitable animal model for in vivo studies on nonenzymatic glucosylation of albumin and other serum proteins in normal and diabetic metabolic states.

A general mechanism for nonenzymatic glucosylation of protein is outlined in Fig. 1. Free amino groups in protein react with the acyclic form of glucose to yield a Schiff-base intermediate which can undergo the Amadori Rearrangement to a stable ketoamine derivative, which then cyclizes to the hemiketal structure (2). Prolonged elevation of blood glucose in diabetes causes an increase in levels of nonenzymatically glucosylated hemoglobin, HbAlc, in erythrocytes (3); this post-translational modification affects hemoglobin's oxygen affinity (4, 5) and sensitivity to the allosteric effector, 2,3-diphosphoglycerate (5, 6). It has been hypothesized (7) that nonenzymatic glucosylation of other body proteins may also induce changes in their chemical, physical, and ultimately, biological properties, leading eventually to the pathological sequelae of diabetes. This hypothesis is supported by a growing body of evidence from clinical experience (8, 9) and animal model studies (10), which indicates that chronic, subclinical hyperglycemia, rather than the insulin deficiency itself, may be the major factor contributing to the progressive, secondary complications of diabetes. Stevens et al. (11) have, in fact, recently demonstrated that nonenzymatic glucosylation of α-crystallins in lens cultures in vitro causes aggregation and precipitation of the crystallins. Their observations suggest that a similar process occurring in vivo may contribute to the development of cataracts in diabetic patients.

In a recent communication (12) we reported that several classes of human serum proteins are also subject to nonenzymatic glucosylation in vivo, and that albumin isolated from normal human serum contains about 8% of a glucosylated derivative. Bailey et al. have also detected glucosylated lysine residues in albumin, erythrocyte membrane proteins (13), and collagens (14). Thus, it appears that numerous proteins in the body may be continuously modified by nonenzymatic glucosylation. By analogy to the observations with hemoglobin, the rates and extents of glucosylation of other proteins may also be enhanced in diabetic hyperglycemia.

Identification of specific proteins subject to nonenzymatic glucosylation and its effects on protein function and metabolism are essential for evaluating the hypothesized relationship between hyperglycemia and the chronic pathophysiology of diabetes. Since much of this work can be most readily carried out in animal model systems, we have undertaken a study of serum protein glucosylation in the rat, in order to establish that this animal will be a satisfactory model for the human system. We present here in vitro experiments parallel to those previously reported for glucosylation of human serum proteins (12) as well as studies on the physical and chemical parameters affecting the kinetics of glucosylation. In addition, in vivo studies are reported identifying the body compartment in which glucosylation occurs, and the effect of glucosylation on the survival of albumin in the circulation. Finally, we establish, for the first time, that the serum concentration of glucosylated albumin is significantly elevated in diabetic rats.

MATERIALS AND METHODS
Rat serum albumin was prepared from fresh rat serum by affinity chromatography on Affi-Gel Blue (Bio-Rad) (15), followed by gel chromatography on Bio-Gel P-150 (Bio-Rad), or purchased from Miles Laboratories. D-[6-3H]Glucose (34 Ci/mmol), L-[3,4,5-3H]leucine (129.8 Ci/mmol) and L-[G-3H]serine (1 to 5 Ci/mmol) were purchased from New England Nuclear, and Na125I (carrier-free) from Amersham/Searle. Penicillin-streptomycin was obtained from Grand Island Biological Co.
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**Incubations**

Albumin solutions and rat serum containing 100 units/ml of penicillin and 100 µg/ml of streptomycin were incubated in the dark at desired temperatures. Albumin solutions (5 to 50 mg/ml) were prepared in Dulbecco's phosphate-buffered saline (16) containing 1 to 50 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 activities.

**Assays**

Acid-precipitable radioactivity was determined by adding 25-µl aliquots of incubation mixtures to 50 µl of a bovine serum albumin solution (10 mg/ml) and precipitating with 1 ml of cold 10% trichloroacetic acid. Pellets were washed twice by centrifugation and the final pellet was redissolved in 500 µl of H₂O and counted for radioactivity in Beckman Instruments Co. Bio-Solv EP.

Glucosylated protein was separated from nonglucosylated protein by carboxymethylcellulose chromatography at pH 4.65 as previously described (12). Glucosylated protein was detected using the thiobarbituric acid procedure of Fliickiger and Winterhalter (17) which measures 5-hydroxymethylfurfural released following hydrolysis of ketoamine adducts of protein. Hydroxymethylfurfural was used as the standard in these assays.

**Amino Acid Analyses**

Proteins in 6 N HCl were gassed with N₂ in a sealed tube and hydrolyzed at 100°C for 24 h, and amino acid analyses were carried out on a Beckman model 119-C analyzer. For identification of glucosylated amino acids, n-[6-³H]glucose-labeled albumin was dissolved in 0.1 M NaBH₄ in 0.1 M NaOH, to a final protein concentration of 7 mg/ml; the mixture was incubated with shaking at 25°C for 4 h and then dialyzed and lyophilized. Following hydrolysis of the reduced, glucosylated albumin in 6 N HCl as described above, the sample was divided in half. One portion was analyzed directly for ninhydrin-positive material. L-[³H]Leucine (70,000 cpm) and L-[³H]serine (20,000 cpm) were added as internal reference standards to the second portion, which was then applied to the analyzer column, but the effluent from the column was fed directly into a fraction collector; fractions were then counted for radioactivity. Glucitol-lysine was prepared from n-[6-³H]glucose and lysine as described by Bailey et al. (13), and purified by chromatography on Amberlite MB (Mallinckrodt).

**Carbohydrate Analyses**

Carbohydrates were separated by high pressure liquid chromatography using the procedure of Lee et al. (18, 19). For measurements of radioactivity in glucose or mannose following acid hydrolysis of n-[6-³H]glucose-labeled albumin, unlabeled glucose and mannose were added to samples at the end of the hydrolysis; the effluent from the column was fed into a fraction collector, and aliquots of the fractions were monitored for both carbohydrate, using the anthrone reagent (20), and radioactivity.

**In Vivo Experiments**

Half-life Studies—Unglucosylated albumin was iodinated using the lactoperoxidase method (21) and labeled, monomeric albumin was isolated by Bio-Gel P-150 chromatography; the final specific activity was 1.3 × 10⁶ cpm/mg. Two milligrams of this material were glucosylated in 20 mM glucose at 37°C for 3 days, and then separated into [125I]-glucosylated and [125I]-unglucosylated albumin by carboxymethylcellulose chromatography. Rats were injected intracardially with either 1 mg of [125I]-glucosylated or 0.85 mg of [125I]-unglucosylated albumin; radioactivity was determined in 50 µl of plasma samples obtained from lateral tail veins (22) over a 4-day period in order to compare the circulating half-lives of the two forms of albumin. In separate experiments, 1 ml of blood was removed by cardiac puncture at 1 and 3 days postinjection of each of the iodinated forms of albumin. Albumin was resiolated by affinity chromatography and then carboxymethylcellulose chromatography, for determination of recovery of [125I]radioactivity in glucosylated and unglucosylated albumins.

**Studies with Diabetic Rats**—Rats (male, Sprague-Dawley, 125 to 150 g) were made diabetic by the intravenous injection of 35 mg/kg of alloxan. Animals whose blood glucose exceeded 300 mg/lO0 ml at 2 days postinjection were classified as diabetic. These animals were then maintained with daily subcutaneous injections of 4 units of protamine zinc insulin for 2 weeks, when the therapy was stopped. Nineteen-six hours after the last insulin injection, the rats were anesthetized with ether and 1 to 2 ml of blood were removed by cardiac puncture. At the same time, blood was also obtained from a second set of alloxan-treated rats maintained on insulin therapy throughout, and from a sex-, age-, and weight-matched control group. Blood glucose was determined by a glucose oxidase-peroxidase method (Sigma), and serum albumin was purified and fractionated into unglucosylated and glucosylated albumin as described above.

**RESULTS**

**In Vitro Studies**—Radioactivity gradually accumulates in an acid-precipitable fraction during the incubation of rat serum with tracer amounts of [6-³H]glucose (Fig. 2). The reaction rate was linear for at least 200 h at room temperature by which time about 10% of glucose radioactivity was incorporated into protein. Based on the approximate specific activity of the [³H]glucose, 4 × 10⁶ cpm/µmol, an average molecular weight of serum proteins of 1 × 10⁵, and a serum protein concentration of approximately 70 mg/ml, the incorporation of 2 × 10⁶ cpm into protein in 1 ml of serum represents an average of 0.7 mol of glucose/mol of protein.

Following 200 h of incubation, reaction mixtures were dialyzed against pH 7.8, Tris-HCl at 4°C, overnight, with essentially quantitative recovery of acid-precipitable radioactivity. When the dialysate was chromatographed on Sephadex G-200...
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(Fig. 3), radioactivity was found associated with each of the major molecular weight classes of serum protein. Since the highest specific activity (counts per min/μmol) was observed in the region corresponding to serum albumin, we investigated nonenzymatic glucosylation of purified rat albumin in further detail.

Incorporation of [6-3H]glucose into albumin was also linear for at least 200 h at 22°C, as shown in Fig. 4. By 200 h, about 50% of the albumin had been converted to the glucosylated form. The rate of glucosylation was stimulated approximately 1.5-fold by the addition of 1 mg/ml of NaBH4CN. The stimulation by BH4CN probably results from selective reduction (23) of Schiff-base adducts formed between glucose and protein, pulling the initial equilibrium reaction to the right (Fig. 1). In contrast, aspirin inhibited the rate of glucosylation, suggesting that at least some glucose is being incorporated at the lysine residue in rat albumin, which is the site of acetylation by aspirin (see below).

The glucosylated rat albumin prepared in vitro was separable from unglucosylated albumin by chromatography on CM-cellulose (Fig. 5A). However, in contrast to our observations with human albumin (12), glucosylated rat albumin eluted from the column after the unglucosylated form. More than 95% of the protein-bound radioactivity from [6-3H]glucose was associated with the second protein peak. Peak II yielded a strong positive reaction in the thiobarbituric acid test for ketoamine derivatives of protein (17), while a weak but reproducible reaction was observed in Peak I (data not shown). When native albumin, freshly purified from rat serum by affinity chromatography, was chromatographed as above, two peaks of protein were observed (Fig. 5B), corresponding precisely to Peaks I and II obtained from the in vitro incubation (Fig. 5A); Peak II accounted for about 98% of the thiobarbituric acid reactivity. Amino acid analyses of Peaks I and II are essentially identical (Table I), and agree closely with results from hydrolysis of a commercial sample of purified rat albumin and published data (24). Based on relative areas under Peaks I and II, normal rat serum contains about 6.7 ± 0.9% (mean ± S.D., n = 8) glucosylated albumin.

Verification that a lysine residue is the major site of glucosylation was obtained by amino acid analysis of an acid hydrolysate of NaBH4-reduced [6-3H]glucose-labeled albumin (Fig. 6). More than 90% of radioactivity obtained from hydrolysis of the protein co-chromatographed with the product obtained from a separate hydrolysis of glucitol-lysine in the presence of albumin (Peak II, Fig. 6). This product is not the hexitol-lysine itself (Peak I, Fig. 6), but probably an anhydro-derivative of hexitol-lysine produced during the hydrolysis (13). Derivatives of both mannitol- and glucitol-lysine should be obtained since the borohydride reduction of the ketoamine is not stereospecific, however these isomers are apparently not resolved in the standard elution scheme on the amino acid analyzer. The small amount of radioactive material appearing after Peak II has not been identified. From these results, we conclude that intrachain lysine residues, rather than the NH2-terminal glutamate, are the primary sites of glucosylation of rat albumin.

Table II summarizes the results from carbohydrate analyses of hydrolysates of [6-3H]glucose-labeled albumin (prepared nonenzymatically in vitro) carried out under conditions commonly used for the analysis of neutral sugars in glycoproteins.
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Hydrolysis for 2 or 4 h in 2 N trifluoroacetic acid, HCl, H2SO4, or 4 N acetic acid resulted in the recovery of no more than 5% of the starting radioactivity in glucose and mannose, under any of the conditions studied. More than 90% of the starting radioactivity was recovered as high molecular weight material, either excluded from or partially included in a Bio-Gel P-2 column. These data indicate that nonenzymatically attached glucose is likely to contribute only trace amounts of glucose or mannose to analyses of neutral sugars in glycoproteins.

The data in Figs. 7 to 9 summarize experiments describing some of the kinetic and thermodynamic properties of the glucosylation of albumin in vitro. The influence of glucose and albumin concentrations on the rate of glucosylation are shown in Fig. 7A and 8A, respectively. At higher glucose concentrations, the rate of glucosylation appeared to decline appreciably after the addition of 1 mol of glucose/mol of protein. The initial rate of glucosylation of albumin in vitro is first order with respect to both glucose (Fig. 7B) and albumin (Fig. 8B) concentration in the incubation mixture, and second order overall. As shown in Fig. 9, the rate of glucosylation is

<table>
<thead>
<tr>
<th>Amino acid*</th>
<th>Unglycosylated albumin§</th>
<th>Glycosylated albumin§</th>
<th>Commercial albumin§</th>
</tr>
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<tbody>
<tr>
<td>Asp</td>
<td>12.90</td>
<td>14.09</td>
<td>13.53</td>
</tr>
<tr>
<td>Thr</td>
<td>5.39</td>
<td>5.38</td>
<td>5.26</td>
</tr>
<tr>
<td>Ser</td>
<td>4.99</td>
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<td>4.94</td>
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<td>Glu</td>
<td>16.74</td>
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<td>Pro</td>
<td>5.24</td>
<td>6.10</td>
<td>4.01</td>
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<td>4.31</td>
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<tr>
<td>Ala</td>
<td>12.23</td>
<td>12.35</td>
<td>12.77</td>
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<td>Cys</td>
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<tr>
<td>Val</td>
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<tr>
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<td>0.77</td>
<td>0.82</td>
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<td>8.02</td>
</tr>
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<td>Tyr</td>
<td>4.81</td>
<td>4.89</td>
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<td>4.80</td>
<td>4.79</td>
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<tr>
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<td>2.39</td>
</tr>
<tr>
<td>Lys</td>
<td>8.14</td>
<td>8.19</td>
<td>8.16</td>
</tr>
<tr>
<td>Arg</td>
<td>3.60</td>
<td>3.70</td>
<td>3.69</td>
</tr>
</tbody>
</table>

* Amino acid analysis carried out following hydrolysis of protein (1 mg/ml) in 6 N HCl as described under "Materials and Methods."

§ Albumin was purified from rat serum by affinity chromatography on Affi-Gel Blue (15) and separated into glucosylated and unglycosylated fractions by carboxymethylcellulose chromatography as described for Fig. 4.

† Rat albumin was purchased from Miles Laboratories.

Fig. 6. Amino acid analysis of rat albumin after glucosylation with [6-3H]glucose and reduction with NaBH₄. Rat albumin was glucosylated with [6-3H]glucose, purified by carboxymethylcellulose chromatography, reduced with NaBH₄, and hydrolyzed for amino acid analysis as described under "Materials and Methods." Radioactivity at "L" represents added l-[3H]leucine marker, containing a trace contaminant of isoleucine. Peak I represents position of elution of authentic [3H]glucitol-lysine. Peak II represents position of elution of the H-labeled product obtained after hydrolysis of authentic [3H]glucitol-lysine at 100°C for 24 h in 6 N HCl in the presence of 1 mg/ml of albumin.

Table II

<table>
<thead>
<tr>
<th>Hydrolysis conditions</th>
<th>% recovery</th>
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<tbody>
<tr>
<td>Acid</td>
<td>Time (h)</td>
</tr>
<tr>
<td>2 N Trifluoroacetic</td>
<td>2</td>
</tr>
<tr>
<td>2 N HCl</td>
<td>4</td>
</tr>
<tr>
<td>2 N H₂SO₄</td>
<td>2</td>
</tr>
<tr>
<td>4 N Acetic</td>
<td>2</td>
</tr>
</tbody>
</table>

Table I

<table>
<thead>
<tr>
<th>Amino acid analyses of rat albums</th>
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<tbody>
<tr>
<td>Amino acid*</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>Asp</td>
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<tr>
<td>Thr</td>
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<td>Glu</td>
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<tr>
<td>His</td>
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<tr>
<td>Lys</td>
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<tr>
<td>Arg</td>
</tr>
</tbody>
</table>

* [6-3H]Glucose-labeled albumin (1 mol of glucose/mol of albumin), 0.25 mg/ml, containing ~40,000 cpm was hydrolyzed in acids at 100°C for various times.

© Carbohydrates were separated by ion exchange chromatography (18, 19), and recovery of radioactivity in individual sugars was determined as described under "Materials and Methods."
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Fig. 8. Rate of glucosylation of rat albumin as a function of albumin concentration. Rat albumin at indicated concentrations was incubated in phosphate-buffered saline containing 5 mM glucose (4 \times 10^6 cpm/\mu mol). A, acid-precipitable radioactivity in aliquots removed at indicated times was determined as described under "Materials and Methods." B, initial reaction rates shown in A are plotted as a function of albumin concentration.

Fig. 9. Rate of glucosylation of rat albumin as a function of temperature. Rat albumin, 25 mg/ml, was incubated in phosphate-buffered saline containing 5.0 mM glucose (4 \times 10^6 cpm/\mu mol) and acid-precipitable radioactivity in aliquots removed at indicated times was determined as described under "Materials and Methods." Temperature-dependent, but an Arrhenius plot of the data has a convex curvature, prohibiting a straightforward determination of the activation energy of the glucosylation reaction. The extent of reaction was negligible during 120 days storage in the freezer at -20°C.

In Vivo Studies—In order to estimate the rate of formation of glucosylated albumin in vivo, and to evaluate its in vivo stability, animals were injected with either unglucosylated or glucosylated 125I-albumin. Carboxymethylcellulose chromatography of the albumin reisolated at various times postinjection was used to monitor any shifts of 125I radioactivity from one form of the albumin to the other. At 1 and 3 days postinjection of 125I-unglucosylated albumin, 3.0 and 7.9%, respectively, of the recovered 125I-albumin was found in the glucosylated fraction. These results document that glucosylation is a post-translational modification occurring in the circulation. Glucosylation is apparently an irreversible process in vivo since no 125I radioactivity was recovered from the unglucosylated region of the carboxymethylcellulose column for as long as 3 days (1.5 half-lives) after injection of glucosylated 125I-albumin.

The structure of carbohydrate side chains are known to influence profoundly the metabolism of plasma proteins. Therefore, we investigated the possible role of glucosylation in regulating albumin clearance from the circulation, by studying the half-lives of glucosylated and unglucosylated 125I-albumins from the rat circulation. Albumin was iodinated, glucosylated, and separated into glucosylated and unglucosylated components by carboxymethylcellulose chromatography, all as described under "Materials and Methods." Animals (two/experiment) were injected with one or the other form of the albumin, and the disappearance of 125I radioactivity from the circulation monitored in blood samples taken at indicated times. Time of equilibration of albumin into extravascular space indicated by --.--.

The structure of carbohydrate side chains are known to influence profoundly the metabolism of plasma proteins. Therefore, we investigated the possible role of glucosylation in regulating albumin clearance from the circulation, by studying the half-lives of glucosylated and unglucosylated 125I-albumin. Iodination was carried out prior to glucosylation to avoid the possibility that the conditions of iodination might modify the nature of the glucosyl adduct. The circulating half-life of both iodinated forms of albumin (Fig. 10) was found to be 48 h, indicating that glucosylation does not affect the fractional catabolic rate for albumin in the circulation.

Since the rate of glucosylation in vitro is first order with respect to glucose concentration (Fig. 7), higher rates of glu-
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TABLE III

<table>
<thead>
<tr>
<th>Assay</th>
<th>Metabolic condition*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Blood glucose mg/100 mlb</td>
<td>70, 73.5</td>
</tr>
<tr>
<td>% Glucosylated albuminc</td>
<td>7.2, 8.1</td>
</tr>
</tbody>
</table>

a Data are for two rats maintained under each set of conditions described under "Materials and Methods."
b Measured by glucose-oxidase peroxidase procedure.
c Determined by CM-cellulose chromatography as described for Fig. 5.

cosylation of albumin might be expected to occur during diabetic hyperglycemia in vivo. In addition, because glucosylation does not affect the half-life of albumin in the circulation (Fig. 10), higher steady state levels of glucosylated albumin should be observed in diabetic animals. This prediction was confirmed by measurements of per cent glucosylated albumin in normal and diabetic rats (Table III). Highest levels of glucosylated albumin are observed in untreated, diabetic animals, and the 4-fold elevation of glucosylated albumin is comparable to the 6-fold increase in blood sugar. A significant, 2.5-fold, elevation in glucosylated albumin is also observed in insulin-treated diabetic animals, suggesting that overall control of hyperglycemia is suboptimal although fasting blood glucose is near normal.

DISCUSSION

The studies presented here, along with our previous report (12), indicate that nonenzymatic glucosylation is a common post-translational modification of serum proteins. The observation that albumin is particularly reactive to nonenzymatic glucosylation, its ease of purification, and the fact that albumin is a well characterized plasma protein led us to investigate nonenzymatic glucosylation of this protein as a model for other plasma proteins.

The rate of glucosylation of rat albumin in vitro appears to be first order with respect to glucose concentration, but slows down abruptly after transfer of approximately 1 mol of glucose/mol of protein (Fig. 7A), suggesting the presence of a single, rapidly modified amino acid residue, and other less reactive secondary sites. From studies of glucosylated albumin prepared in vitro, we have identified a lysine residue(s) as the major site(s) of glucosylation of albumin (Fig. 6). The inhibition of glucosylation by aspirin (acetylsalicylic acid) suggests that this is the same low pK lysine residue which is acetylated (25). Studies are in progress to verify this assignment.

Variations in oxygen binding equilibria for several HbA1 subfractions have been documented (4, 5) suggesting long range conformational alterations in hemoglobin's tertiary or quaternary structure upon glucosylation. Similarly, glucosylation of albumin might also affect its affinity for a variety of ligands, including fatty acids, bilirubin, clinical dyes, and pharmacological agents. As a minimum, glucosylation undoubtedly contributes to the well known structural microheterogeneity of albumin (26), and may be responsible for some of the multiple electrophoretic forms observed for this and other proteins by high resolution techniques.

While the actual mechanisms regulating plasma protein catabolism are poorly understood, there is good evidence that albumin is catabolized, at least in part, directly from the vascular compartment (27). Foster has suggested that albumin's catabolism may be the end result of a series of chemical or enzymatic insults to the protein, which take place in the circulation, and contribute to its gradual denaturation (26). Since variations in the structure of carbohydrate side chains are known to have a critical role in regulating the uptake and catabolism of circulating glycoproteins (28, 29), we hypothesized that glucosylation might be a chemical insult initiating albumin's catabolism. Our data, however, do not support this hypothesis (Fig. 10), but suggest that glucose attached nonenzymatically to albumin does not serve as a recognition marker triggering specific removal of the glycoprotein from the circulation in normal rats.

Since glucosylation does not affect albumin's circulating half-life, it is possible, by a relatively straightforward kinetic analysis, to predict the steady state level of glucosylated albumin in vivo from knowledge of the kinetics of glucosylation and catabolism of albumin in vivo. The relationship may be written:

\[
\frac{d[GA]}{dt} = k'[A] - k_2[GA]
\]

where \( GA \) = glucosylated albumin, \( A \) = unglucosylated albumin, and \( k' \) and \( k_2 \) are rate constants for glucosylation and catabolism of albumin. (For convenience \( k' \) is treated here as a pseudo-first order rate constant, although the synthetic reaction is second order overall, i.e. \( k' = k_1 [glucose] \).) Then, at steady state,

\[
\frac{d[GA]}{dt} = 0
\]

and

\[
\frac{k_2}{k_1} = \frac{[GA]}{[A]}
\]

The in vivo rate constant, \( k_1 \), can be estimated from the observation that about 7.9% of injected, unglucosylated 125I-albumin isolated at 3 days postinjection was recovered as the glucosylated form. Thus, \( k_1 = 0.026 \text{ day}^{-1} = 0.0011 \text{ h}^{-1} \). Similarly, from the half-life of glucosylated albumin shown in Fig. 10,

\[
k_2 = \frac{0.693}{48 \text{ h}} = 0.014 \text{ h}^{-1}.
\]

These estimates of rate constants are, within experimental error, consistent with the observed steady state level of glucosylated albumin in rat serum, as predicted in Equation I, i.e.

\[
k_2 = 0.0011 \text{ h}^{-1}
\]

and, since approximately 6.7% of albumin exists normally in the glucosylated form,

\[
\frac{[GA]}{[A]} = 0.067 = 0.072.
\]

The agreement between these two ratios indicates that the rate of nonenzymatic glucosylation in the extracellular compartment in vivo is adequate to explain the steady state level of glucosylated albumin in the circulation, and argues that glucosylation occurs as a postsecretory, rather than an intracellular, event. The rate of glucosylation of albumin observed in serum at 37°C in vitro, 37% in 200 h, \( k' = 0.0018 \text{ h}^{-1} \) (data not shown) is reasonably close to the value observed in vivo. However, the rate observed with purified albumin at 37°C in vitro was faster, 61% in 200 h, \( k' = 0.0031 \text{ h}^{-1} \); thus, the glucosylation reaction proceeds more rapidly with the pure protein in vitro, suggesting that other ligands bound to albumin may inhibit the rate of glucosylation in serum.
In the past, the detection of low levels of glucose in non-collagen glycoproteins has often been attributed to trace contamination by cellulosic materials. Within the last few years, however, glucose has been identified as a component of the oligosaccharide of both lysosomal enzymes (30) and intracellular intermediates in the processing of secreted and membrane glycoproteins (31). It was important, therefore, to determine the extent to which nonenzymatically bound glucose could interfere with analyses for glucose in glycosidic linkage to protein. From the data in Table II, however, it is clear that nonenzymatically bound glucose is not likely to affect carbohydrate analyses on glycoproteins. Only trace amounts (≤5%) of free glucose or mannose were released under standard conditions for hydrolysis of neutral sugars. Since more than 75% of the radioactivity in the hydrolysates was partially or completely excluded from Bio-Gel P-2, and adsorbed to mixed bed resins, it is likely that the glucose residue is released from the protein in a peptide-bound form. Based on the model studies of Heyns et al. (2, 32), the glucose may be found in the peptide as an ε-N-(2-hydroxymethyl)- derivative of lysine.

In the preliminary experiments reported here, we observed that untreated diabetic hyperglycemia in the rat results in about a 4-fold increase in per cent glucosylated albumin, while insulin-treated diabetic rats have about a 2-fold elevation. It should be emphasized that the insulin regimen used in these experiments is designed both to provide for the convenient, daily administration of a long acting insulin, and to be adequate to prevent glucosuria and ketoacidosis. However, the regimen is not optimized for the individual animals, and should be emphasized that the insulin regimen used in these experiments is designed both to provide for the convenient, daily administration of a long acting insulin, and to be adequate to prevent glucosuria and ketoacidosis. However, the regimen is not optimized for the individual animals, and variations in blood glucose do occur during the daily cycle. In fact, the elevated level of glucosylated albumin in the insulin-treated animals may be a better indicator of a mild hyperglycemic state than the single fasting blood glucose measurements. Further studies in rats which monitor changes in per cent glucosylated albumin during both uncontrolled hyperglycemia and therapeutic management with insulin should permit an evaluation of the sensitivity of glucosylated albumin to average blood glucose levels. The enhanced levels of glucosylated albumin detected in diabetic rats suggest that measurements of albumin glucosylation in man may also be useful in the detection and management of diabetes.

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