Glucosylated Albumin and Its Influence on Salicylate Binding

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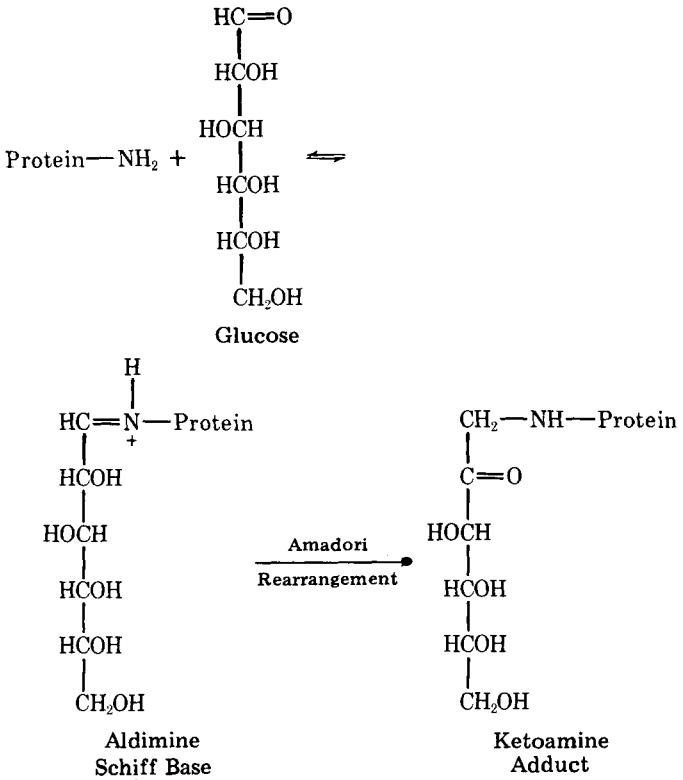
Abstract □ Human serum albumin was incubated at 37° in 0.01 *M* phosphate buffer (pH 7.4) under sterile conditions for up to 10 days with labeled [^{14}C]glucose (1–25 mg/ml). Glucose incorporated into albumin was calculated following extensive dialysis of the incubation mixture. The results indicated that glucose reacted with albumin by a nonenzymatic process involving Schiff base formation and Amadori rearrangement to a stable ketoamine derivative. The degree of glucosylation was dependent on the reaction time, glucose concentration, and pH. Glucosylation was enhanced when albumin was fatty acid free. Glucosylated albumin was separated from unmodified albumin by cation exchange chromatography on carboxymethylcellulose and quantitated colorimetrically with 2-thiobarbituric acid. Salicylate binding studies revealed that the glucosylated component had a decreased salicylate binding capacity accompanied by a reduction in the number of classes of binding sites.

Keyphrases □ Albumin, serum—effect of glucosylation on salicylate binding, human □ Glucosylation—human serum albumin, effect on salicylate binding □ Binding—salicylate, effect of glucosylation of human serum albumin

It is generally recognized that the major threat to life and function posed by diabetes mellitus is the insidious development of long-term complications such as atherosclerosis, microangiopathic vascular disease, and neuropathy (1, 2). Many investigators speculate that frequent lapses in diabetic control are associated with an increased incidence and rate of progression of the diabetic complications. Although the mechanism by which hyperglycemia may lead to these complications is unknown, recent attention has been focused on the nonenzymatic condensation of glucose with proteins to yield stable covalent adducts (3). Such nonenzymatic glucosylation could result in altered protein structure, function, stability, and immunological response (2).



Scheme I—Proposed nonenzymatic reaction sequence involved in the formation of glycosylated proteins.

BACKGROUND

The demonstration of increased amounts of the glucosylated form of hemoglobin A (hemoglobin Alc) in diabetics was the first example of posttranslational protein modification correlated with elevated blood glucose concentrations (3). Evidence was recently published linking the development of corneal opalescence in diabetes with glucosylation of lens crystallins (4, 5). The nonenzymatic glucosylation of human serum proteins (6–8), insulin (9), and collagen (10) were also reported. Mechanistically, the nonenzymatic interaction of glucose and protein occurs by way of a ketoamine linkage involving the free amino group at the *N*-terminus or the ϵ-amino groups of lysine residues (Scheme I).

To establish a closer link between hyperglycemia and secondary complications of diabetes, experiments were initiated to compare the extent to which fatty acid-free albumin and albumin bound with fatty acids are susceptible to glucosylation *in vitro*. The hypothesis that enhanced rates of nonenzymatic protein glucosylation may contribute to the pathophysiology of diabetes is valid if such glucosylation is accompanied by altered protein function. To determine whether the glucosylation of albumin could result in altered protein function, salicylate binding to glucosylated, partially glucosylated, and nonglucosylated albumin was compared.

EXPERIMENTAL

Preparation of Glucosylated Albumin—Albumin[1] (Fraction V) solutions of 47.5 mg/ml were prepared in 0.01 *M* phosphate buffer, pH 7.4. Following the addition of D-[^{14}C]glucose[2] to final concentrations

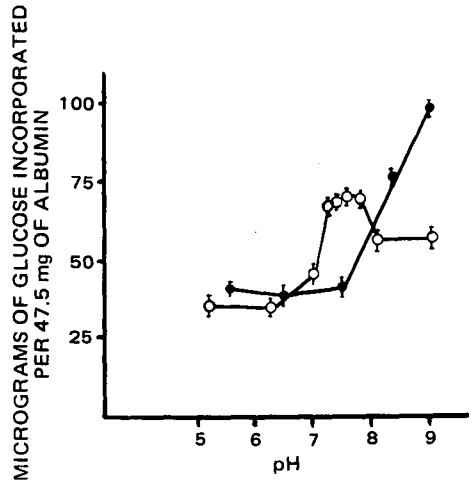


Figure 1—*Dependence of glucose incorporation into albumin on pH and composition of incubation buffer. Albumin (47.5 mg/ml) was incubated at 37° in 0.01 M phosphate buffer (○) and 0.1 M tromethamine buffer (●) containing 10 mg/ml (1 μCi) of [^{14}C]glucose. After 24 hr, samples with the indicated pH were dialyzed, lyophilized, and counted for radioactivity. Values are the means of four determinations ±SD.*

[1] Sigma Chemical Co., St. Louis, Mo.
[2] New England Nuclear, Boston, Mass.

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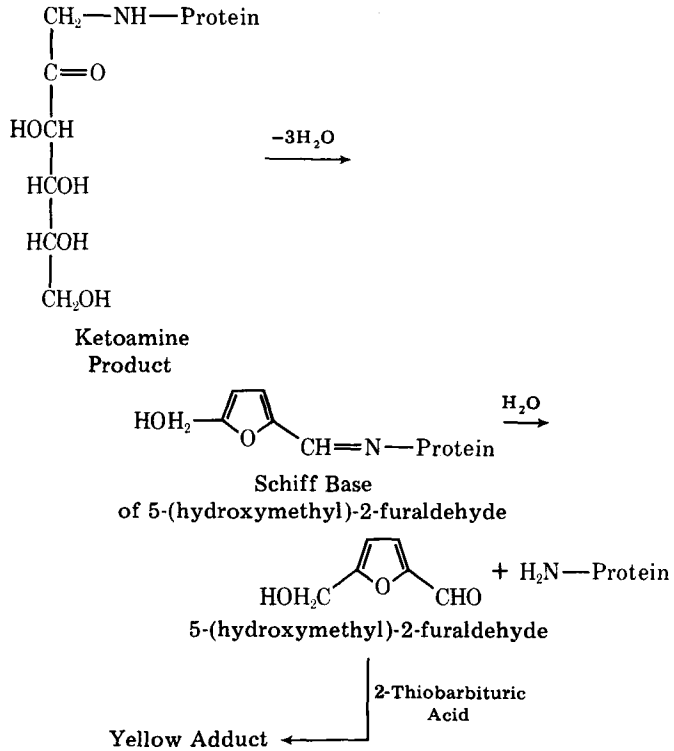

Scheme II—Detection of ketoamine adducts of proteins.

ranging from 1–25 mg/ml, the solutions were sterilized by ultrafiltration and incubated for up to 10 days in capped, sterile vials at 37°. At the end of each incubation period, 1-ml samples were removed and dialyzed against several 1000-fold volumes of distilled water for 24 hr at 4°. A portion of the dialysate was monitored for the presence of free glucose by the glucose oxidase method[3]. The remainder was lyophilized and counted for radioactivity. Incubated samples were compared to control samples which were prepared by dissolving identical quantities of albumin and [^{14}C]glucose in phosphate buffer and immediately dialyzing against distilled water for 24 hr.

Influence of pH on the Glucosylation of Albumin—Albumin (47.5 mg/ml) was incubated at 37° in 0.01 *M* phosphate buffer and 0.1 tromethamine buffer containing 10 mg/ml of D-[^{14}C]glucose (1 μCi). The pH of the reaction solutions was varied from 5.0–9.0. After 24 hr of incubation, the samples were dialyzed, lyophilized, and counted for radioactivity.

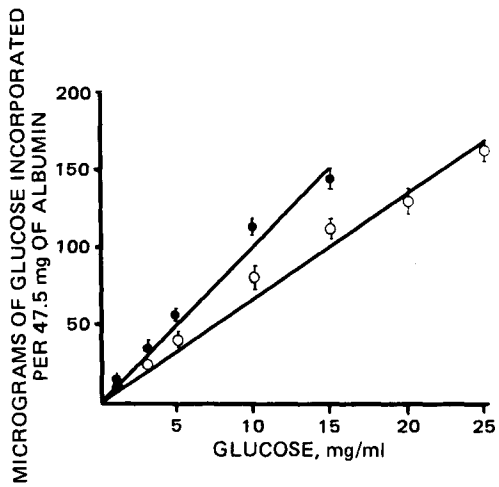

Figure 2—*Incorporation of glucose into albumin and fatty acid-free albumin as a function of glucose concentration. Albumin (○) and fatty acid-free albumin (●) were incubated at 37° in 0.01* M *phosphate buffer, pH 7.4, containing the indicated concentrations of [^{14}C]glucose. After 24 hr the incubated samples were dialyzed, lyophilized, and counted for radioactivity. Values are the means of four determinations* ±SD.

[3] Glucose oxidase kit, Sigma Chemical Co., St. Louis, Mo.

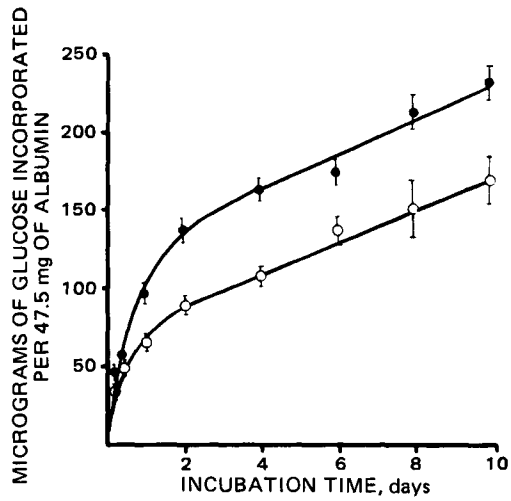

Figure 3—*Incorporation of glucose into albumin and fatty acid-free albumin as a function of incubation time. Albumin (○) and fatty acid-free albumin (●) were incubated at 37° in 0.01* M *phosphate buffer, pH 7.4, containing 10 mg/ml [^{14}C]glucose. Aliquots were removed at the indicated times, dialyzed, lyophilized, and counted for radioactivity. Values are the means of four determinations* ±SD.

Galactosylation of Albumin—Albumin (47.5 mg/ml) was incubated with 1 μCi of D-[6-^{3}H]galactose (1 mg/ml) in 0.01 *M* phosphate buffer, pH 7.4, at 37°. After 36 hr the reaction samples were treated as described previously.

Glucosylation of Albumin with D-[2-^{3}H] and D-[5-^{3}H]glucose—Albumin (47.5) mg/ml) was incubated with 1 μCi D-[2-^{3}H]glucose (10 mg/ml) in 0.01 *M* phosphate buffer, pH 7.4 at 37°. In an identical incubation, albumin was reacted with 1μCi D-[5-^{3}H]glucose (10 mg/ml). After 36 hr of incubation, the reaction samples were treated as described previously.

Thiobarbituric Acid Test for Glucosylated Protein—Glucosylated protein was detected using a modification of the thiobarbituric acid procedure of Flückiger and Winterhalter (11). This assay measures 5-(hydroxymethyl)-2-furaldehyde released upon hydrolysis of ketoamine adducts of proteins (Scheme II).

Samples of glucosylated albumin (5 mg) were placed in 15-ml culture tubes and dissolved in distilled water (1 ml). After the addition of 1.0 *N* oxalic acid (0.5 ml), the tubes were gently shaken, capped, and placed in a heating block at 100° for 5 hr. The tubes were subsequently cooled and

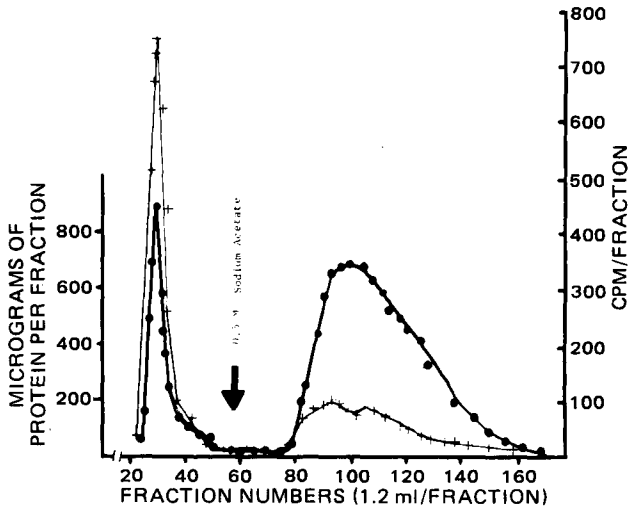

Figure 4—*Separation of human serum albumin into glucosylated and nonglucosylated components by chromatography on carboxymethylcellulose. Samples in 0.01* M *sodium acetate, pH 4.65, were applied to a column (17 × 2 cm) of carboxymethylcellulose and eluted using a 200-ml gradient of 0.01–0.5* M *sodium acetate, pH 4.65. Elution with 0.5* M *sodium acetate is indicated by the arrow.*

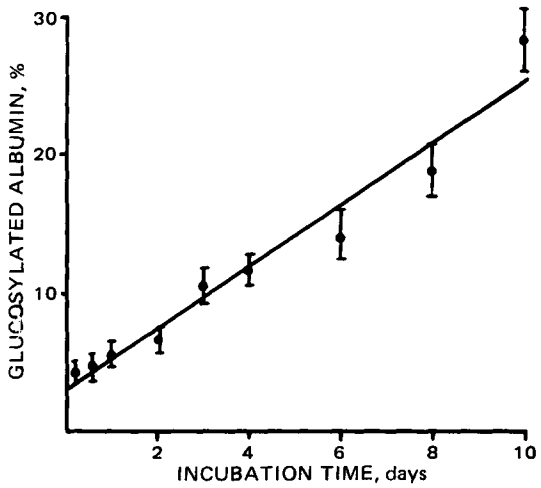

Figure 5—*Percentage glucosylated albumin as a function of incubation time. Albumin (47.5 mg/ml) was incubated at 37° in 0.01* M *phosphate buffer, pH 7.4, containing 10 mg/ml glucose. Aliquots were removed at the indicated times, dialyzed, lyophilized, and 10 mg was subjected to carboxymethylcellulose chromatography to separate the glucosylated fraction. Values are the means of four determinations* ±SD.

cold 40% trichloroacetic acid (0.25 ml) was added to precipitate the protein. The contents were then centrifuged at 2000×*g* for 10 min. An aliquot (1 ml) of the supernate was removed and added to a solution of 0.05 *M* aqueous 2-thiobarbituric acid (0.5 ml). After mixing and an incubation period of 15 min at 37°, the absorbance of each sample was measured at 443 nm.

Chromatographic Separation of Glucosylated and Nonglucosylated Albumin—Glucosylated and nonglucosylated albumin were separated using cation exchange chromatography on carboxymethylcellulose (6). Carboxymethylcellulose was suspended in 0.01 *M* sodium acetate buffer, pH 4.65, and packed in a 30 × 1.7-cm column to a length of 20 cm. Samples (50 mg) in the sodium acetate buffer were applied to the column and eluted with a 200-ml gradient of 0.01–0.5 *M* sodium acetate buffer, pH 4.65. Fractions (1 ml) were collected and assayed for protein content by an earlier method (12). An aliquot (0.1 ml) of each fraction was monitored for radioactivity.

Utilizing larger columns, 1-g quantities of glucosylated and nonglucosylated albumin were separated for use in the salicylate binding studies. Separated fractions were dialyzed against water for 24 hr and then lyophilized prior to their use in salicylate binding studies.

Salicylate Binding Capacity of Glucosylated and Nonglucosylated Albumin—The salicylate binding properties of nonglucosylated, 5–10% glucosylated, 25–30% glucosylated, and 100% glucosylated albumin were compared by the equilibrium dialysis method[4]. Albumin (47.5 mg/ml) was dialyzed against 1.2, 1.0, 0.8, 0.6, 0.5, 0.4, 0.25, and 0.125 mg/ml sodium salicylate. After 2.5 hr of dialysis, the solutions were acidified and extracted with 1 ml of chloroform. The concentration of salicylate in the chloroform extracts was assayed at 310 nm (13).

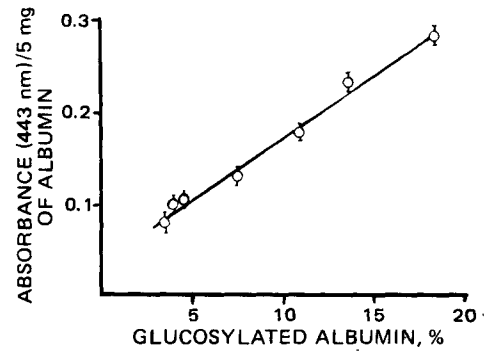

Figure 6—*Formation of 5-(hydroxymethyl)-2-furaldehyde from glucosylated albumin. Albumin (47.5 mg/ml) was incubated at 37° in 0.01* M *phosphate buffer, pH 7.4, containing 10 mg/ml of glucose. Aliquots were removed at different times, dialyzed and lyophilized. Ten milligrams was subjected to carboxymethylcellulose chromatography to determine the percentage of glucosylated albumin formed. Another 5 mg was subjected to the 2-thiobarbituric acid test. Values are the means of four determinations* ±SD.

[4] Dianorm multiple dialyzing system, Medizinisch-Wissenschaffliche Institute, Zurich, Switzerland.

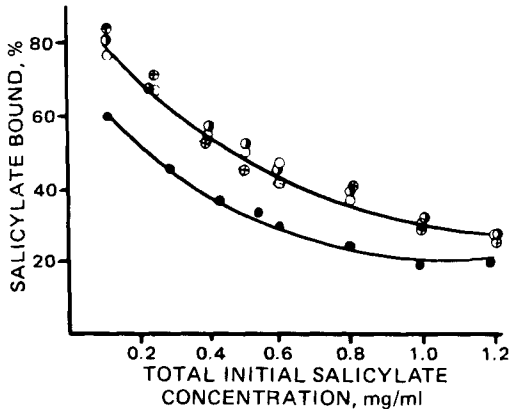

Figure 7—*Salicylate bound to nonglucosylated, partially glucosylated, and completely glucosylated human serum albumin. Percent salicylate bound to partially glucosylated (5–10%, ⊕; 25–35%, ◑), nonglucosylated (○), and completely glucosylated (●) human serum albumin.*

RESULTS AND DISCUSSION

Incubation of [^{14}C]glucose with human serum albumin at physiological pH, temperature, and albumin concentration resulted in the incorporation of labeled glucose into protein. This radioactivity was not removed by extensive dialysis against distilled water, suggesting a covalent linkage between glucose and albumin. To determine the nature of the linkage between glucose and albumin, the glucosylated albumin was treated with oxalic acid, resulting in the release of 5-(hydroxymethyl)-2-furaldehyde, which was detected by the characteristic colored adduct formed with 2-thiobarbituric acid (Scheme II). Formation of 5-(hydroxymethyl)-2-furaldehyde is characteristic of monosaccharides bound to protein by a ketoamine linkage (3).

Additional evidence for the ketoamine linkage was provided by studies in which albumin was separately incubated with 1 μCi of D-[2-^{3}H]glucose and D-[5-^{3}H]glucose having identical specific activities. In the case of glucose labeled in the 5-position, the glucosylated albumin had a specific activity of 670 cpm/mg of albumin. In contrast, albumin incubated with glucose labeled in the 2-position showed a specific activity of 267 cpm/mg of albumin. These results indicated loss of the label from the 2-position of the sugar, thus implying the occurrence of an Amadori rearrangement during the reaction. Obviously, glucosylation of albumin occurred by the same mechanism as in the formation of hemoglobin Alc; that is, glucose reacted with the amino groups of albumin to form aldimine linkages, which subsequently underwent an Amadori rearrangement to form the more stable ketoamine linkages (Scheme I).

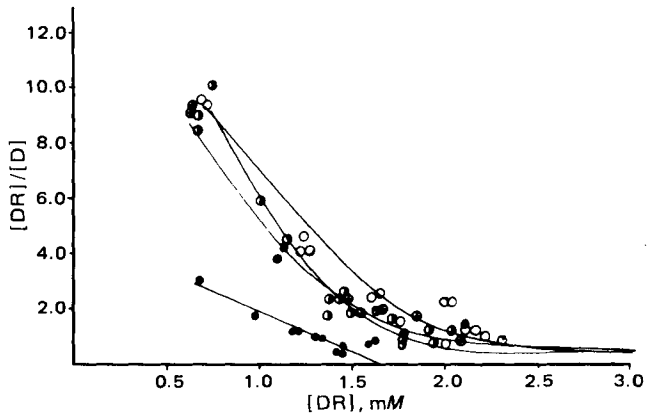

Figure 8—*Rosenthal plot for salicylate bound to nonglucosylated, partially glucosylated, and completely glucosylated human serum albumin. [DR], bound salicylate (*m*M); [D], unbound salicylate (*m*M). Key: ⊕, 5–10% glucosylated albumin; ◑, 25–30% glucosylated albumin; ○, nonglucosylated albumin; and ●, completely glucosylated albumin.*

The degree of nonenzymatic glucosylation of human serum albumin was dependent on the pH and composition of the incubation buffer (Fig. 1). The reaction between glucose and albumin most likely occurs by means of a nucleophilic attack by an unprotonated amino group on the glucose molecule. At physiological pH, few of the primary groups exist in the unprotonated state. This situation may account for the relatively slow rate of the glucosylation reaction.

At physiological pH and temperature, human serum albumin as well as fatty acid-free albumin incorporated labeled glucose in a concentration-dependent manner (Fig. 2). However, at every glucose concentration employed, glucose incorporation into fatty acid-free albumin was significantly greater than that incorporated by albumin bound with fatty acids. Perhaps common sites were utilized in the covalent binding of glucose and the ionic binding of fatty acids. In diabetes, the increased levels of albumin glucosylation may adversely affect fatty acid transport and, thus, may contribute to the pathogenesis of the disease.

The rate of glucose incorporation into albumin and fatty acid-free albumin was also dependent on the reaction time (Fig. 3). In both cases, during the initial 2 days of incubation the rate of glucose uptake was faster than in the succeeding days. This indicated that some sites on the albumin molecule were more easily glucosylated than others. The initial rate of glucose incorporation into fatty acid-free albumin was considerably faster than into albumin. This suggests that the process of defatting the albumin resulted in the release of additional sites that were capable of being glucosylated. However, the preparation of fatty acid-free albumin was accomplished at a pH below 3.0 for a period of not less than 8 hr (14, 15). This treatment may have caused the hydrolysis of glucose molecules already covalently bound to albumin, thereby releasing additional sites for glucosylation.

Nonenzymatic glycosylation of albumin by means of a ketoamine linkage can occur with hexoses other than glucose. Calculations based on the amount of radioactivity incorporated into albumin revealed that 9.95 μg of galactose and 8.8 μg of glucose were bound to albumin following identical incubations in which galactose and glucose (1 mg/ml) were reacted separately with albumin (47.5 mg/ml) under physiological conditions for 36 hr. This incorporation of galactose into albumin is not surprising in view of the reported glycosylation of hemoglobin with other sugars and sugar–phosphates (3).

Glucosylated albumin was readily separated from unmodified albumin by cation exchange chromatography on carboxymethylcellulose (Fig. 4). Prior to the *in vitro* incubation with glucose, albumin was found to be 5–10% glucosylated as determined by the chromatographic separation of the glucosylated from nonglucosylated components. In addition, the percentage of glucosylated albumin increased proportionally with time (Fig. 5).

Figure 6 illustrates the results obtained when glucosylated albumin is subjected to the 2-thiobarbituric acid test. It is evident that 5-(hydroxymethyl)-2-furaldehyde generation is directly dependent on the percentage of glucosylated albumin formed, suggesting the basis for a practical colorimetric assay to monitor the degree of diabetic control. Until now, only hemoglobin Alc has been considered as a diagnostic indicator for the long-term control of diabetes. Measurement of glucosylated albumin may be more advantageous, since the half-life for albumin is only 20 days (16) compared with 120 days for erythrocytes. Assuming that glucosylated and nonglucosylated albumin have the same turnover rate, one would expect fluctuations in blood glucose to be detected more sensitively by changes in glucosylated albumin than in glucosylated hemoglobin. Moreover, unlike hemoglobin Alc values, the amount of glucosylated albumin is not influenced by hemolytic disorders.

The results obtained from the salicylate binding studies indicate that the percentage of salicylate bound to totally glucosylated albumin is significantly lower than that for nonglucosylated or partially glucosylated albumin (Fig. 7). Analysis of the data by means of a Rosenthal plot (17) suggests that nonglucosylated and partially glucosylated albumin possess more than one class of binding sites, whereas 100% glucosylated albumin appears to possess only one class of binding sites (Fig. 8). The fact that the glucosylated component of albumin has a significantly decreased capacity to bind salicylate may have little significance for the diabetic, since the glucosylated fraction comprises a relatively small proportion of total albumin. Even at 25–30% glucosylation, there is no significant decrease in the salicylate binding capacity.

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