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# Rate of Formation of Glycated Albumin Revisited and Clinical Implications

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## Abstract

**Background:** Albumin modified by Amadori glucose adducts contributes to the pathogenesis of complications of diabetes and reducing its formation ameliorates their development, underscoring the need for accurate information on the rate of formation of this biologically active glycated protein. However, this subject has not been examined in over two decades, and there is reason to question data in older reports.

**Methods:** The present study used nonradioactive and nonreductive techniques to examine the rate of formation of glycated albumin and compare it to that of glycohemoglobin, using a sensitive and specific immunoassay for measurement of the stable glucose adduct formed after incubation of serum, plasma and purified albumin with glucose.

**Results and Conclusions:** We report that the rate of formation of glycated albumin parallels that of glycohemoglobin at approximately 0.005-0.008 percent per mM glucose per day, refuting values from the older literature and providing clinically relevant information concerning levels of glycated albumin in diabetes and of treatment directed at reducing its formation.

**Keywords:** Glycated albumin; Amadori glucose modification; Rate of formation of glycated albumin

## Introduction

Increased levels of albumin modified by Amadori glucose adducts, the principal form in which glycated albumin exists *in vivo*, associate independently with complications of diabetes [1-3] and contribute to the pathogenesis of diabetic nephropathy and retinopathy by influencing cell signaling pathways and molecular mediators known to be associated with the development of these complications [4-8]. Reducing the nonenzymatic glycation of albumin independent of glucose control has promise as a therapeutic strategy in diabetic nephropathy and retinopathy [9-12], underscoring a need for accurate information on the rate of formation of this biologically active glycated protein under clinically relevant conditions. This subject, however, has not been examined in over two decades and there is reason to believe that data from older studies are flawed. The issue has been confounded by the introduction of an enzymatic method (Lucica GA-L, Asahi Kasei, Tokyo, Japan) for measurement of glycated albumin that yields a normal range several fold greater than that of other methods [13] and that cites in support of these high values a report indicating that glycation of albumin proceeds at a rate 10-fold greater than that of hemoglobin [14]. Calculation of the rate of albumin glycation in that study employed unpurified radiolabeled glucose, which was shown in a subsequent publication to be contaminated with radioactive impurities that nonspecifically bind to the protein [15]. Another study conducted a few years later employed purified radioactive glucose, also reporting results with borohydride reduction that forces unphysiologic conversion of the glucosylamine to the Amadori product [16], and revised the estimated rate of albumin glycation to 5 times that of hemoglobin but contained a mathematical error (Baynes, personal communication).

The above considerations prompted the present study using nonradioactive and nonreductive techniques to examine the formation of albumin modified by Amadori-glucose adducts and to compare it to that of glycohemoglobin. Measurement of the amount of albumin containing glucose adducts was determined by affinity chromatography on phenylboronate agarose which, under alkaline conditions, complexes with compounds containing coplanar cis-hydroxyl groups that can be subsequently dissociated by lowering the pH or with a competing polyol [17]. The amount of albumin in the glycated fraction so eluted was measured with a sensitive and specific immunoassay for human albumin. We employed this methodology to quantify the rate of albumin glycation and to assess its comparability to glycohemoglobin formation.

## Materials and Methods

Purified human albumin (Octapharma, Centerville, VA) dissolved in phosphate buffered saline (PBS), pH 7.4, at a concentration of 40 mg/ml and aliquots (1 ml) of serum or plasma freshly collected from a normal volunteer were incubated with glucose (10,20,40 mM) in an atmosphere of 95% $O_2$/5% $CO_2$ at 37°C for 1,2,3,6 and 9 days. At the specified time points, samples were removed from incubation and immediately desalted on G-25 columns (GE Healthcare, Buckingham, UK) into the loading buffer (5 mM DL-asparagine, 8.5 mM L-methionine, 6 mM taurine, 50 mM $MgCl_2$, pH 9.0) for affinity chromatography on phenylboronate agarose (GlycoTek, Helena Labs, Beaumont, TX), eluting the bound (glycated) fraction with 0.1M Tris HCl, pH 7.0, containing 250 mM sorbitol and measuring albumin in the glycated fraction by immunoassay for human albumin (Exocell, Phila, PA) using standard curves controlled for the presence of sorbitol. The elution schedule provided optimum conditions, with recovery of >90% of applied glycated albumin, which did not appear in the non adsorbed fraction.

Erythrocytes or hemoglobin prepared from red cells freshly collected from a normal volunteer were incubated with glucose

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(10,20,40 mM) in an atmosphere of 95% $O_2$/5% $CO_2$ at 37°C for 1-9 days. After collection by centrifugation, erythrocytes from the fresh blood were prepared for incubation by washing with PBS, pH 7.4, and adding PBS containing the indicated concentrations of glucose to bring the red cells to the initial volume of the blood sample. At the specified times the cells were removed from incubation, washed with PBS, again brought to the original volume of blood with PBS, centrifuged for one minute at 1500 x g, and aliquots were immediately assayed for glycohemoglobin on GlycoTek columns according to the manufacturer's instructions. Hemoglobin from the fresh erythrocytes was prepared for incubation by hypotonic lysis of the red cells in deionized water, with collection by centrifugation and solubilization in PBS, pH 7.4, containing 1 mM KCN for stabilization to prevent precipitation from solution during incubation. At the specified time points, samples were removed from incubation and immediately desalted on G-25 columns into the GlycoTek buffer for loading onto the glycated affinity columns.

Gel electrophoresis and Western blotting of samples before and after affinity chromatography was performed by application after reduction with 5% β-mercaptoethanol of triplicate aliquots each containing approximately 10 ug protein to SDS-PAGE (4-15% gradient gels). After electrophoretic transfer to nitrocellulose one of the transfers was stained for protein with Coomassie blue and the others were immunoblotted with either monoclonal antibody against Amadori-modified albumin [18] or anti-albumin polyclonal antibody (Sigma-Aldrich, St. Louis) followed by development with alkaline phosphatase-conjugated anti-mouse IgG (BioRad, Hercules, CA) and BCIP/NBT substrate (Promega, Madison, WI). The monoclonal antibody is site-specific for epitopes in albumin that contain an Amadori-glucose adduct whereas the polyclonal antibody recognizes albumin whether so modified or not; thus only glycated albumin is visualized on western blot with the anti-glycated albumin antibody whereas western blot with anti-albumin polyclonal antibody visualizes albumin whether glycated or not [18].

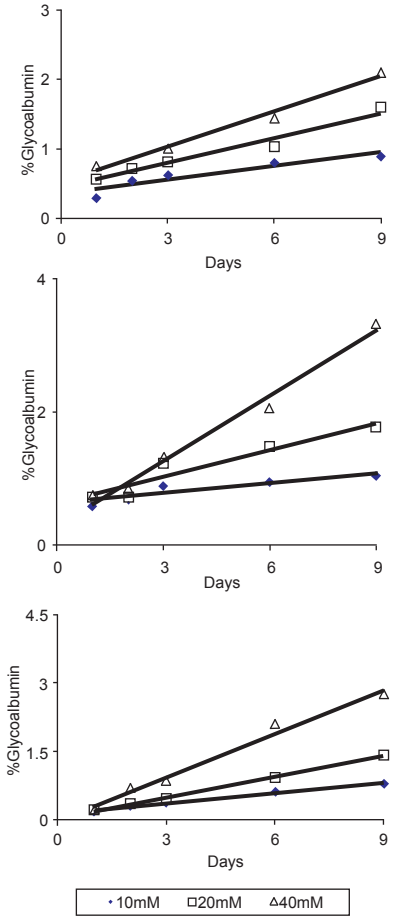


**Figure 1:** Formation of Amadori-modified glycated albumin in serum (Top Panel), plasma (Middle Panel) and purified albumin (Bottom Panel) in relation to time and glucose concentration. Each value represents mean of three determinations.

## Results

Incubation of serum, plasma or purified human albumin with glucose resulted in the formation of albumin modified by Amadori glucose adducts that increased in relation to time and glucose concentration (Figure 1). Baseline values were ≈ 0.6% in each instance and reached levels of 2.0%, 3.3% and 2.8% of total albumin in serum, plasma and purified albumin, respectively, after 9 days of incubation in 40mM glucose (Figure 1). Calculating from the slopes of the lines of the changes with time, glycated albumin formed at similar rates of approximately 0.005-0.008 percent per mM glucose per day in incubations of serum, plasma or albumin containing 10, 20 and 40 mM glucose (Table 1). Since the slope lines represent "best fit", the extrapolated y intercepts suggest some variance but the actual measured y intercepts at day zero in each series were within the narrow range indicated above. Formation of glycohemoglobin after incubation with glucose of erythrocytes or hemoglobin also increased in relation to time and glucose concentration (Figure 2). Baseline values were ≈ 5.5 % in both red cells and hemoglobin and reached levels of about 10.2% of total hemoglobin in erythrocytes and about 9.3% of total in incubations of hemoglobin after 9 days in 40 M glucose. Calculating from the slopes of the lines of the changes from baseline, glycohemoglobin formed at similar rates of approximately 0.010 percent per mM glucose per day in red cells or hemoglobin incubated with 10, 20 and 40 mM glucose (Table 2).

Corroboration that albumin in plasma and serum samples that complexed with and was eluted from phenylboronate agarose

| Glycated Albumin Formation | | | | | |
|---|---|---|---|---|---|
| | | | Extrapolated | | Rate |
| Sample | Glucose | Slope | y intercept | $R^2$ | (percent/mM gluc/day) |
| Serum | 10 mM | 0.0710 | 0.3257 | 0.9080 | 0.0071 |
| | 20 mM | 0.1302 | 0.3801 | 0.9608 | 0.0065 |
| | 40 mM | 0.1843 | 0.4168 | 0.9745 | 0.0046 |
| | | | | | |
| Plasma | 10 mM | 0.0521 | 0.6160 | 0.8663 | 0.0052 |
| | 20 mM | 0.1360 | 0.6713 | 0.9295 | 0.0068 |
| | 40 mM | 0.3070 | 0.4090 | 0.9756 | 0.0076 |
| | | | | | |
| Albumin | 10 mM | 0.0771 | 0.1294 | 0.9905 | 0.0077 |
| | 20 mM | 0.1470 | 0.0687 | 0.9967 | 0.0074 |
| | 40 mM | 0.3137 | 0.0352 | 0.9869 | 0.0078 |

**Table 1:**

| Glycated Hemoglobin Formation | | | | | |
|---|---|---|---|---|---|
| | | | Extrapolated | | Rate |
| Sample | Glucose | Slope | y intercept | $R^2$ | percent/mM gluc/day |
| Erythrocyte | 10 mM | 0.1026 | 5.81 | 0.8487 | 0.0103 |
| | 20 mM | 0.2061 | 6.09 | 0.7337 | 0.0103 |
| | 40 mM | 0.4513 | 6.27 | 0.9139 | 0.0113 |
| | | | | | |
| Hemoglobin | 10 mM | 0.1209 | 5.30 | 0.8500 | 0.0121 |
| | 20 mM | 0.1983 | 5.60 | 0.7705 | 0.0096 |
| | 40 mM | 0.4139 | 6.05 | 0.9196 | 0.0103 |

**Table 2:**

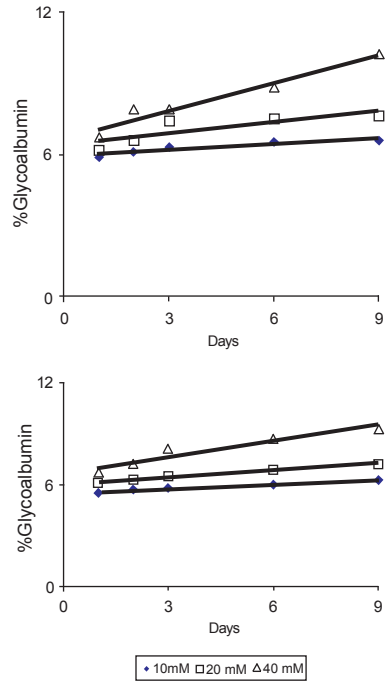


**Figure 2:** Formation of glycohemoglobin in erythrocytes (Top Panel) or hemoglobin (Bottom Panel) in relation to time and glucose concentration. Each value represents the mean of three determinations.

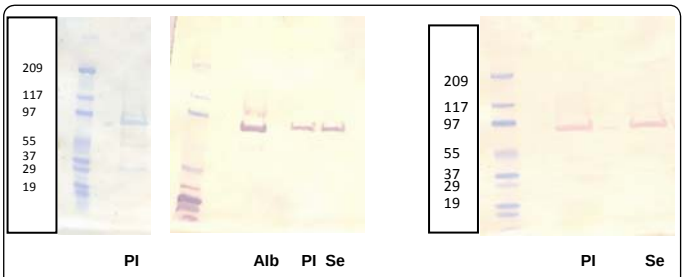


**Figure 3:** Glycated albumin in plasma and serum. Left panel=Coomassie blue staining of plasma proteins (Pl) that complexed with phenylboronate and were eluted with 250 mM sorbitol. Middle panel=immunoblotting with anti-albumin antibodies of albumin (Alb) and of plasma (Pl) and serum (Se) proteins that were eluted from phenylboronate. Right panel=immunoblotting with anti-glycated albumin antibodies of plasma (Pl) and serum (Se) proteins that were eluted from phenylboronate. Molecular weight markers are indicated.

contained the glycated species was obtained by subjecting aliquots to SDS-PAGE and immunoblotting with monoclonal antibody specifically reactive with Amadori modified glycated albumin or with anti-albumin polyclonal antibody. Although several bands were seen on Coomassie blue staining of plasma protein complexing with PBA, only one protein band reacted with anti-glycated albumin antibody (Figure 3). This ≈ 68,000 kDa protein band, which was also observed in unfractionated plasma, co-located with the protein band in albumin, plasma and serum that reacted with anti-albumin antibody (Figure 3). The unbound fraction collected after application to the affinity column showed no reactivity on immunoblotting with the anti-glycated albumin antibodies.

## Discussion

The foregoing results demonstrate that the rate of formation of glycated albumin parallels that of glycohemoglobin, contradicting reports in the older literature that albumin is nonenzymatically glycated at a rate 10-fold greater than that of hemoglobin [14,19,20]. In such studies, nonspecific binding of contaminants in the labeled glucose preparations artificially elevated values for albumin glycation [15]. Further, removal of hemoglobin by precipitation out of solution during incubation artificially lowered values for the rate of formation of glycohemoglobin (Baynes, personal communication), a problem not encountered in the present study by including KCN in the incubations. In a subsequent study employing purified radiolabeled glucose [16] and in a review article [21], the investigators concluded that glucose condenses with albumin about 5 times faster than with hemoglobin, but the report contained a mathematical error resulting in an overestimation of the rate of albumin glycation by at least two-fold (Baynes, personal communication). In the present study, the rate of formation of glycated albumin when serum, plasma or purified albumin was incubated with glucose showed relatively consistent values for percent per mM glucose per day across different glucose concentrations, the exception being serum incubated with 40 mM glucose. A lower rate of glycation *in vitro* with serum compared to plasma at higher glucose concentration, which drives the reaction, is consistent with changes in the local milieu with loss of 15-20% volume attendant to removal of coagulation factors. HbA1c, which is modified by glucose at the N-terminal valine, is estimated to form at a rate of 0.009 percent per mM glucose/day *in vitro* [22] and 0.018 percent per mM glucose/day *in vivo* [23], the faster reaction *in vivo* being ascribed to the presence of effectors in the erythrocyte milieu [24] which may also promote glycation of hemoglobin at lysine residues, referred to as glycohemoglobin, at a higher rate [21,25]. The present data indicating a rate of formation of glycohemoglobin of ≈ 0.010 percent per mM glucose/day *in vitro* agree with the above estimate.

Given that normal values for glycated albumin using affinity chromatography methods range from ≈ 0.6 to 1.4-3% [26,27] and that similar reference range values (0.5-1.5%) are reported with an immunoassay that employs the monoclonal antibody [18] site specific for albumin modified by Amadori glucose adducts (Exocell, Phila), it is unclear why the enzymatic assay yields values higher than those observed with such methods [26,27] but methodologic aspects are relevant. Complexation with boronate or monoclonal antibody may be restricted to one cis-hydroxyl group per albumin molecule, the resin may require two glucose groups per molecule to effectuate binding [28], and/or the number of binding sites in the resin be inadequate for the number of glucose-modified sites. On the other hand, the enzymatic assay uses protease digestion to hydrolyze albumin to glycated amino acids followed by oxidation with ketoamine oxidase to convert the glycated amino acids to glucosone and generate hydrogen peroxide which is coupled to a dye yielding a purple color. Specificity for glycated amino acids arising from albumin is claimed by the manufacturer to be conferred by a unique protease digesting only albumin, but information regarding the source or documentation of the specificity of the enzyme is not provided and it is possible that glycated amino acids liberated from other serum proteins contribute to peroxide formation. Referring to a study that reports heterogeneity of albumin glycation [29], the manufacturer's description also implies that glycation at multiple sites within the albumin molecule could explain values greater than those of other methods, but nonenzymatic glycation of albumin occurs predominantly at the lysine 525 residue [30,31] and is governed by the time-averaged glucose concentration, the rate of glycation, the half life at which the protein population disappears and is replenished, the accessibility of the free amino group, the

presence of other ligands, and the microenvironment including pKa and proximity to catalytic groups [24,30-32]. Although three other sites may become glycated, their total contribution is relatively minor [30,31].

The present findings have clinical relevance with respect to reducing the formation of glycated albumin as a therapeutic strategy for complications of diabetes by providing useful information for addressing drug-to-target stoichiometric relationships. Albumin newly released into the circulation does not contain glucose adducts, but forms Amadori products at a constant rate over a range of glucose concentrations to which it is exposed and that remains until removal. At a steady-state albumin concentration of ≈ 3.5 gm/dL and half-life of 17-21 days, which is unaffected by glycation [19], the approximately 4 gms (60 μmoles) of albumin released per day represent the most susceptible population requiring protection from the hyperglycemic milieu that results in a level of glycated albumin that is 1.5-3 fold greater in patients with diabetes than in nondiabetic subjects.

In summary, we provide evidence that the rate of formation of albumin modified by Amadori glucose adducts is similar to that of hemoglobin modified by Amadori glucose adducts at ≈ 0.005-0.008 percent per mM glucose/day, refuting values from older literature as well as the hypothesis that the high values observed with a recently introduced enzymatic method are consistent with a rate of albumin glycation that is 10-fold greater than that of hemoglobin.

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