The Alkaline Reducing Activity of Glycated Serum Proteins and Its Relevance to Diabetes Mellitus

Roger N. Johnson and John R. Baker

We investigated the ability of the fructosamine assay to detect glycation of serum proteins. We incubated both whole human serum and serum protein fractions in vitro with [14C]glucose, and analyzed for reducing activity and for uptake of 14C by protein. In all experiments, the reducing activity increased linearly with time for seven days and was correlated with 14C uptake (r = 0.94–0.98). Protein ketamines were about fivefold more actively reducing than equimolar concentrations of deoxymorpholinofructose, the fructosamine standard, which explains why values for fructosamine in serum are higher than the expected concentration of protein ketamines. We also used [14C, 2-3H]glucose to assess the contribution of the aldime component to 14C uptake. Whole human serum and albumin incubated with [14C, 2-3H]glucose showed little uptake of 3H in relation to 14C. We conclude that glycated protein can be simply and reliably quantified by the fructosamine assay, and we discuss the relevance of this conclusion to the monitoring of diabetes.

Additional Keyphrase: radiolabel uptake

We have proposed (1) that glycation of serum proteins might be quantified simply by measuring the reducing activity of serum in alkaline solution. The measurement of what is termed fructosamine, in recognition of the ketamine formed from glucose, correlates positively with other indices of glycemia (1, 2). However, the assay depends solely on an estimation of reducing activity and the nature of the active species has remained conjectural. Here, we present evidence that the fructosamine assay is a valid measure of glycation of serum proteins.

Materials and Methods

Materials: Penicillin and streptomycin were from Glaxo Laboratories, Greenford, U.K. [2-3H]glucose, [U-14C]glucose, and "NCS" solubilizing solution were from Amersham International, U.K. Albumin (containing a trace, by electrophoresis, of alpha-globulins) was from Commonwealth Serum Laboratories, Melbourne, Australia; Cohn Fraction II (containing, by electrophoresis, mainly gamma-globulin, with a trace of albumin) and Cohn Fraction IV (containing, by electrophoresis, approximately equal quantities of alpha- and beta-globulins, with some albumin) were from Serva Feinbiochemica, Heidelberg, F.R.G. 1-Deoxy-1-morpholinofructose for use as a standard was synthesized as described by Hodge (3). All other reagents were of the highest grade available.

Incubations: We adjusted 4 mL of pooled human serum to pH 7.4 with dilute (0.5 mol/L) HCl, then added, per liter, penicillin (106 int. units), streptomycin (0.1 g), glucose (50 mmol), and [U-14C]glucose (1-2 mCi). In some experiments, we also added [2-3H]glucose to give a final concentration of 5 mCi/L. These samples were incubated in stoppered tubes in the dark at 37 °C. At various intervals we removed 400-μL aliquots and dialyzed them overnight against isotonic saline. Duplicate 50-μL aliquots of the dialyzed material were mixed with 1 mL of a 0.5 mol/L solution of trichloroacetic acid, then centrifuged. The resulting precipitates were washed twice more with the trichloroacetic acid solution. The final pellets were dissolved in 0.5 mL of the NCS solubilizing solution and mixed with 10 mL of toluene-based scintillation fluid. To relate radioactivity with glucose concentration, we prepared standards in triplicate from 10-μL aliquots of incubation medium added directly to NCS solution plus scintillation fluid. The dialyzed material not treated in this way was stored frozen for fructosamine assay (see below).

We also isolated individual fractions of human serum protein (albumin, Cohn Fractions II and IV) in 4 mL of 0.1 mol of NaCl containing 50 mmol of NaPO4 per liter, pH 7.4, and processed these standards as we did the whole serum (above) except that we treated 100-μL aliquots with trichloroacetic acid. Radioactivity was quantified in a LS-7500 spectrometer (Beckman Instruments, Irvine, CA). Counts were corrected for background, quenching, and cross-contamination when both 14C and 3H were present.

Fructosamine assay: Fructosamine activity was measured as previously described (1, 4). When reacted with the nitroblue tetrazolium reagent, all protein factions examined showed spectral properties identical to those described for albumin (1).

Electrophoresis: Protein fractions were electrophoresed on cellulose acetate strips, with use of a sodium barbital buffer (90 mmol/L, pH 8.6). Protein was stained with Ponceau S dye.

Purification of [14C] and [2-3H]glucose: [14C]glucose (25–50 μCi) and [2-3H]glucose (100 μCi) were purified by two-dimensional thin-layer chromatography on cellulose plates (Cellulose F 254; E. Merck, Darmstadt, F.R.G.). The mobile phases were n-butanol/pyridine/water (in equal volumes; system 55 of Amersham International) and n-butanol/ethanol/water (52/33/15 by vol; system 102 of Amersham International). The major component identified by autoradiography was eluted and used immediately. Upon reaction with MgATP in the presence of hexokinase (EC 2.7.1.1), and chromatography of the mixture on Dowex-1 (Cl-), more than 98% of this material behaved like glucose.

Results

We found the fructosamine activity of whole serum incubated in vitro with glucose to increase linearly with time for seven days (Figure 1A) and to be linearly related to incorporation of [14C]glucose into protein (Figure 1B). On examining individual protein fractions at their respective concentrations in whole serum in the same manner, we found that the proteins differed markedly in their reactivity towards glucose (Figure 2) although the relationship between fructo-
Results and Comparisons

Fig. 1. Fructosamine activity after incubation of whole serum with [14C]glucose

Pooled normal serum was incubated at 37°C with [14C]glucose (50 mmol/L). Samples removed at the times indicated (A) were dialyzed, and analyzed for fructosamine activity and 14C incorporation. Glycoprotein concentration (B) was calculated as the concentration of [14C]glucose that was precipitated by trichloroacetic acid. The results are from duplicate incubations, mean values being shown in A, individual values in B.

Fig. 2. Fructosamine activity after incubation of serum protein fractions with [14C]glucose (50 mmol/L) at 37°C for various periods, dialyzes, and analysis for fructosamine activity and 14C incorporation

The protein concentrations were: albumin (control), 40 g/L; Cohn Fraction II (C), 16 g/L; Cohn Fraction IV (D), 18 g/L.

The slope and 14C uptake was similar in each case (Table 1).

We examined the assumption that 14C uptake represents ketoamine formation by using [2-3H]glucose as an index of Schiff base formation (5, 6). We first confirmed that 3H in the 2 position was present in an authentic aldime, glucosylpiperidine, and absent in an authentic ketoamine, deoxymorpholinofructose (Table 2).

Incubation of whole serum with [14C, 2-3H]glucose showed

Table 2. Incorporation of [14C, 2-3H]Glucose into Aldimine and Ketoamine

<table>
<thead>
<tr>
<th>Compound</th>
<th>3H (mCi/mol)</th>
<th>14C (mCi/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosylpiperidine</td>
<td>133</td>
<td>132</td>
</tr>
<tr>
<td>Deoxymorpholinofructose</td>
<td>5</td>
<td>141</td>
</tr>
</tbody>
</table>

Glucosylpiperidine (7) and deoxymorpholinofructose (3) were synthesized in the presence of [2-3H]glucose (0.2 mCi/mmol) and [14C]glucose (0.04 mCi/mmol). We dissolved 35 mg (about 140 µmCi) of each crystalline product in water and counted the radioactivity in a scintillation fluid consisting of toluene and Triton X-100. The specific radioactivity of the glucose was estimated from the radioactivity of the commercial preparation and the weighed quantity of glucose.

That uptake of 3H was minor relative to that of 14C (Figure 3). A similar result was obtained with albumin (not shown). Fructosamine activity correlated equally well with 14C uptake and with the difference between 14C and 3H uptakes.

Fig. 3. Time-course of 14C uptake (■) and 3H uptake (○) from [14C, 2-3H]glucose by serum protein

Conditions as in Fig. 1. Results, expressed as glucose concentrations, are means of three incubations.

Table 1. Relationship between Fructosamine Activity and 14C Incorporation

<table>
<thead>
<tr>
<th>Material</th>
<th>Slopes of linear regression</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>4.9</td>
<td>0.98</td>
</tr>
<tr>
<td>Fraction II</td>
<td>4.9</td>
<td>0.98</td>
</tr>
<tr>
<td>Fraction IV</td>
<td>6.4</td>
<td>0.94</td>
</tr>
<tr>
<td>Albumin</td>
<td>4.9</td>
<td>0.98</td>
</tr>
</tbody>
</table>

For incubation conditions see Figure 1 and Figure 2. The slopes are for comparisons of the type shown in Figure 1B.

CLINICAL CHEMISTRY, Vol. 32, No. 2, 1986 399
however, the slopes of linear regression increased from 4.7 to 5.6 when allowance was made for $^3$H uptake, as the aldime (exemplified by glycosylpiperidine) is not reducing (1).

**Discussion**

We proposed the fructosamine assay as a measure of glycosylprotein concentration based on the alkaline reducing activity of nonprotein ketoamines (3). In this study, the reducing activity of serum and serum protein fractions correlated very significantly ($p < 0.001$) with $[^14C]$glucose uptake, of which aldime represented only a minor component (Figure 3). By these criteria, the fructosamine assay measures protein glycation.

We and others have observed that serum specimens and deoxymorpholinofructose, used as a standard, differ in their reactivity depending on reaction conditions (1, 4, 8). Under our conditions of assay, protein fructosamines were five- or sixfold more reactive than equimolar concentrations of deoxymorpholinofructose (Table 1). The same degree of difference in reactivity was observed whether we used whole serum with a protein concentration of 70 g/L or individual serum protein fractions in concentrations ranging from 16 to 40 g/L.

This difference in reactivity can explain the relatively high fructosamine values (1, 2, 9) compared both with the molarity of serum proteins and with the values from other procedures for estimating serum protein glycation. For example, furosine derived from glycated proteins by acid hydrolysis is found, 3 nmol/mg of protein, when normal serum is analyzed (10). This value approximates 210 mmol/L in serum containing 70 g of protein per liter and represents 700 mmol of glycated protein per liter when one considers that the yield of furosine is 30% (11). The equivalent value in fructosamine units is therefore 3.5 nmol/L, which is close to our current reference interval of 2.0–2.7 mmol/L (4). Identical arithmetic applies to hydroxymethylfurfural, because a normal value of 0.3 nmol/mg of protein (12) represents only 10% of the yield of furosine (11).

Albumin incubated with glucose gained reducing activity more rapidly than did other serum protein fractions (Figure 2). This behavior is likely to be reproduced in vivo, because albumin is present in serum in high concentration, has ample amino groups (58 lysine residues per molecule, ref. 13), and, compared with other serum proteins, has a relatively long biological half-life (14). Clinically, this finding suggests that the measurement should prove more responsive than hemoglobin $A_1c$ as an index of glycaemia, in keeping with other estimates of serum protein glycation (12, 15). Such an expectation is borne out in practice (2, 9).

**References**