Chemistry of the Fructosamine Assay: D-Glucosone Is the Product of Oxidation of Amadori Compounds

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The chemistry of the fructosamine assay was studied by using the Amadori compound, Nα-formyl-Nα-fructoselysine (fFL), an analog of glycated lysine residues in protein. Previously (Clin Chem 1983;39:2460–5), we reported that free lysine was formed from fFL at 70% yield during incubation with alkaline nitroblue tetrazolium (NBT) under the conditions routinely used for the fructosamine assay (sodium carbonate buffer, pH 10.35 at 37°C). Here, we show that D-glucosone is the primary carbohydrate oxidation product formed from Amadori compounds in the fructosamine assay. Glucosone, which decomposes under alkaline assay conditions with a half-life of <30 min, reaches a maximum concentration of ~50% of the initial fFL concentration after 10 min of incubation. Like fFL, glucosone reduces NBT to the purple monoformaldahyde dye, but its decomposition is not accelerated by the presence of NBT. The dicarbonyl-trapping reagent, aminoguanidine, inhibits the fructosamine assay by ~25% when fFL is the substrate, but by nearly 100% with glucosone as substrate. Studies with serum samples from diabetics and nondiabetics indicate that glucosone formation does not have a significant effect on the clinical usefulness of the fructosamine assay; however, corrections for glucosone formation may be required when the assay is used for estimating the extent of glycation of proteins.

Indexing Terms: glycated proteins/diabetes mellitus/lysine

The fructosamine assay is a colorimetric test that determines the degree of glycation of serum proteins by measuring the reducing activity of serum in alkaline solution (1). The test relies on the ability of Amadori compounds to reduce nitroblue tetrazolium (NBT) to the tetrazinol radical NBT−, which disproportionates to yield a highly colored formazan dye (MF+) (2).5 In studies on the chemical mechanism of the fructosamine assay, using the model Amadori compound Nα-formyl-Nα-fructoselysine (fFL) (3), we observed that fFL degraded rapidly under assay conditions (pH 10.35 at 37°C), forming free lysine simultaneously with the reduction of NBT. We determined that the Amadori compound was the direct reductant of NBT, but the nature of the carbohydrate oxidation product(s) formed from the Amadori compound during the fructosamine assay remained unknown. Hodge (4) proposed that D-glucosone was the most likely oxidative degradation product of Amadori compounds in alkaline solution, on the basis of isolation of the phenylazone and tetrahydroxybutylquinazoline derivatives of 1-deoxy-1-p-toluindino-D-fructose. Kat (5) isolated 2,4-dinitrophenylhydrazine derivatives of 3-deoxy-D-glucosone and D-glucosone from browning reactions of N-D-glucosyl-n-butylamine in methanolic solution, and Kawakishi et al. (6) reported that glucosone was formed on reduction of cuprous ion at neutral pH. However, Ahmed et al. (7, 8) identified Nα-(carboxymethyl)lysine (CML) and Nα-(3-lactato)lysine (LL) as the principal products of autoxidation of fFL in aqueous buffers at neutral–alkaline pH. In the present work, we examined the degradation of fFL in the alkaline NBT reagent used in the fructosamine assay and identified glucosone as the carbohydrate oxidation product of fFL. We also examined the time course of the reaction and the contribution of D-glucosone to the absorbance change in the fructosamine assay, using the dicarbonyl trapping reagent, aminoguanidine (AG).

Materials and Methods

Materials. Blood samples were collected from 70 patients attending the Diabetic Clinic at Auckland Hospital. Procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983. Specimens were allowed to clot and serum was separated and stored at −20°C for later analysis. Bovine serum albumin (Cohn Fraction V), NBT, sodium borohydride (NaBH4), sodium borodeuteride (NaBD4), N-formylglycine, Nα-formyllysine, phytic acid, AG, EDTA, diethylenetriaminepentaaetic acid (DTPA), and methyglyoxal (400 mL/L solution, 6.6 mol/L) were from Sigma (St. Louis, MO). The Amadori compound, fFL, was synthesized from Nα-formyllysine and glucose by the method of Finot and Mauron (9), and D-glucosone from the phenylazone derivative of D-glucose (10, 11).

Analytical methods. Amino acid analyses were performed on a Waters (Milford, MA) HPLC system by cation-exchange chromatography with citrate buffers and a sodium chloride gradient, as previously described (7). The eluent was monitored for amino acids by post-column reaction with o-phthaldialdehyde and fluorometric detection. Gas chromatography–mass spectrometry (GC-MS) was performed on a Hewlett-Packard (Palo Alto, CA) Model 5890 gas chromatograph equipped with a Model 7673A autosampler and a Model...
5970 mass selective detector, and with a 30-m DB-5 capillary column (J&W Scientific, Folsom, CA).

Sample preparation. The fructosamine reagent contained 250 μmol/L NBT in 0.1 mol/L sodium carbonate buffer, pH 10.35. Samples contained 800 μmol/L fFL, plus 800 μmol/L N-formylglycine and 800 μmol/L myo-inositol as internal standards for amino acid and carbohydrate analysis, respectively. Sample solutions also contained 1 mmol/L phytic acid and 1 mmol/L DTPA to limit autoxidative degradation of fFL (7, 8). Sample and reagent were mixed in a 1:10 volume ratio for analysis (final sample concentration, 73 μmol/L) and incubated at 37°C under air, except as indicated in Table 1. Aliquots were removed at timed intervals and reduced by addition of 20 μL of 2.5 mol/L NaBH₄ or NaBD₄ in 0.1 mol/L NaOH. After 1 h at room temperature, excess reductant was discharged by addition of 80 μL of 6 mol/L acetic acid. Amino acids were deprotonated by hydrolysis in 2 mol/L HCl for 0.75 h at 95°C, concentrated by rotary evaporation, and redissolved in citrate buffer for amino acid analysis.

For GC-MS analysis, borate was removed from the reduced samples as the volatile methyl ester by making three separate additions of acidified anhydrous methanol, heating at 65°C for 30 s, and evaporating under nitrogen at room temperature. Alditol acetates were prepared by treating the samples with 0.5 mL of pyridine-acetic anhydride (3:2 by vol) for 30 min at 65°C, followed by evaporation of reagents under nitrogen; samples were redissolved in ethyl acetate for GC-MS analysis (12). Glucosone and myo-inositol concentrations were determined by selected ion monitoring GC-MS, quantifying the m/z 289 ion for reduced and acetylated glucosone and the m/z 210 ion for myo-inositol (13). For analysis of glycronic acids, samples were esterified by treatment with 2 mol/L HCl in methanol for 30 min at 65°C, converted to peracetylated derivatives as above, and assayed by GC-MS (14).

Fructosamine assay. Patients' specimens were assayed in a Cobas Bio (Hoffmann-LaRoche, Nutley, NJ) centrifugal analyzer as previously described (15) by using 250 μmol/L NBT in 0.1 mol/L sodium carbonate buffer, pH 10.35. Calibrators and controls were from either Hoffmann-LaRoche (Basel, Switzerland) or Boehringer Mannheim (Mannheim, Germany). AG was added to the NBT reagent, where indicated, before analysis.

Results

Measurement of fFL degradation. As reported previously (3), amino acid analysis of fFL samples incubated in alkaline NBT reagent showed the glycine internal standard, glucitol-lysine, mannitol-lysine, lysine, and traces of ammonia. There was no evidence of formation of CML or LL (<1% yield). About 70% of the fFL was degraded during the first 20 min of the reaction, and the sample was completely converted to free amino acid by 60 min (3). GC-MS analysis of carbohydrate products in NaBH₄-treated samples revealed only the internal standard, myo-inositol, and the epimeric hexitols glucitol and mannitol (Fig. 1). Comparison of the mass spectra of products formed on reduction with NaBD₄ and NaBH₄ was used to determine whether the parent compound was glucosone or a mixture of the two aldoses. For the NaBH₄-reduced samples (Fig. 2A), the presence of fragment ions in the mass spectrum at m/z 361, corresponding to the loss of a terminal CH₂OAc group, is consistent with either glucosone or the two aldoses as parent compounds. However, ion fragments at m/z 362 and m/z 363 in the mass spectra of NaBD₄-reduced samples (Fig. 2B), corresponding to the loss of terminal CH₂OAc or CHDOAc groups, could be obtained only from glucosone. The weak m/z 361 ion signal in the spectrum of the NaBH₄-reduced samples also excludes glucose or mannose as significant products of the reaction. Full-scan GC-MS chromatograms showed no evidence of 3-deoxyglucosone, pentoses, or tetrose, nor were their glycolic acid derivatives detected in separate analyses. Two- and three-carbon products of fFL or glucosone degradation

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**Table 1. Effect of pH on degradation of d-glucosone under anaerobic conditions.**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% degradation*</th>
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<tbody>
<tr>
<td>Sodium phosphate buffer, pH 7.4</td>
<td>14.1 ± 9.6</td>
</tr>
<tr>
<td>Sodium carbonate buffer, pH 10.3</td>
<td>66.4 ± 1.0</td>
</tr>
<tr>
<td>Sodium carbonate buffer, pH 10.3 + 250 μmol/L NBT</td>
<td>75.7 ± 1.1</td>
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Assays were carried out for 20 min at 37°C under nitrogen. Traces of oxygen were removed from the nitrogen by bubbling through 1 mol/L sodium dithionite solution. Reaction mixture contained 80 μmol/L d-glucosone, 80 μmol/L myo-inositol, 0.1 mol/L DTPA, and 0.1 mol/L phytic acid in 0.1 mol/L sodium phosphate buffer or 0.1 mol/L sodium carbonate buffer.

* Results are expressed as percent of d-glucosone concentration in each sample at 0 min (mean ± SD of 4 replicates).

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![Fig. 1. Identification of carbohydrate products in fructosamine assay mixture by GC-MS.](image-url)
Differences appeared, and Fig. 10 showed that glucosone, fructosamine, and lysine had been degraded, with LR values reaching 95% at 0-100 mm, and lysine accounted for an independent rate of glucosone production. As shown in Table 1, the glucosone concentration reached a maximum of ~50% yield at 10 min, then gradually decreased, with an apparent half-life of <30 min. As shown in Table 1, glucosone is unstable in neutral and alkaline buffers. It is more rapidly degraded in sodium carbonate buffer, pH 10.35, than in neutral buffer, but the presence of NBT did not markedly accelerate the loss of glucosone. GC-MS analysis of NaBH₄-reduced samples showed traces of a five-carbon product, but no evidence of glycanic acids. As with fFL, two- and three-carbon products may have been formed, but would not be detected by our analytical procedure.

Relative response of fFL and glucosone in the fructosamine assay. To assess the effect of glucosone after the results of the fructosamine assay, we compared the color yield from a commercial control serum (Precipath Fructosamin; Boehringer Mannheim) reported to contain 540 μmol/L fructosamine (final assay concentration, 49 μmol/L) with that from a theoretically equivalent solution containing 540 μmol/L fFL. These samples yielded a mean (± SD) MF⁺ of 4.77 ± 0.03 and 5.43 ± 0.04 μmol/L, respectively (Fig. 4, zero AG concentration). The same concentration of glucosone yielded 2.82 ± 0.04 μmol/L MF⁺, i.e., about half the yield obtained from fFL. Methylglyoxal was even less reactive, yielding 1.00 ± 0.11 μmol/L MF⁺. The lesser reactivity of glucosone, compared with fFL, in the assay suggests alternative modes of degradation of glucosone, independent of reduction of NBT—which is consistent with the data in Table 1. The experiments also indicate that glucosone production may contribute to the color yield in the fructosamine assay.

To confirm the contribution of glucosone to assay results, we included an excess of AG in the fructosamine reagent to trap the dicarbonyl sugar and limit its contribution in the assay. AG is a hydrazine compound that forms stable triazine derivatives with dicarbonyl sugars (14). As shown in Fig. 4, the added AG caused a concentration-dependent decrease in yield of MF⁺ from fFL, control serum, glucosone, and methylglyoxal. Maximum
inhibition was achieved at 10 mmol/L AG: ~25% and 14% inhibition for fFL and control sera, respectively. At this concentration of AG, fFL and the control serum, which theoretically contained similar amounts of fructosamine, yielded similar assay results; at higher concentrations, AG had an anomalous stimulatory effect on assay results with plasma samples. The yields of MF⁺ from glucosone and methylglyoxal were decreased by 86% and 76%, respectively, at 10 mmol/L AG, and the reaction was essentially quantitatively inhibited at higher AG concentrations. These results suggest that glucosone could make some contribution to MF⁺ formation in a standard 15-min fructosamine assay, and that, in fact, the fructosamine assay may overestimate the extent of glycation of plasma proteins by ~10–25%, given the extent of AG inhibition of assays of fFL and control serum.

**Effect of AG on assay of diabetic and non diabetic sera.**

To determine the contribution of glucosone formation to fructosamine assay results for diabetic and nondiabetic patients, the fructosamine assay was performed on patients' sera with and without added AG (10 mmol/L) in the NBT reagent. Comparison of assay results (Fig. 5) shows a significant shift from slope = 1 and intercept = 0 for the correlation analysis. The addition of AG caused a slight but statistically significant increase in assay results for 19 samples for which fructosamine concentrations were in the nondiabetic range (252.4 ± 38.5 vs 280.4 ± 34.6 μmol/L; P < 0.001), but no significant change in fructosamine results for 51 sera with fructosamine concentrations in the diabetic range (457.5 ± 99.8 vs 455.7 ± 86.7 μmol/L; P > 0.05). The effects of AG in the assay of clinical specimens are difficult to interpret in a straightforward manner; however, the data suggest that, despite potential interference, glucosone formation does not significantly affect the results of the fructosamine assay of patients' sera.

**Discussion**

**Formation of D-glucosone.** In previous work (3) we identified the Amadori compound as the direct reducing agent of NBT in the fructosamine assay. In the present study we have shown that glucosone is the primary carbohydrate oxidation product formed in the reaction and also contributes to the color yield in the assay. Both Kato (5) and Kawakiishi et al. (6) have identified glucosone as a product of autoxidation of Amadori compounds. However, both groups carried out their reactions at physiological pH, and in both instances the reaction was favored by strong oxidizing conditions. Thus, Kato (5) reported a twofold increase in glucosone yield by bubbling oxygen through the reaction mixture, and a considerable decrease in yield by bubbling nitrogen. Kawakiishi et al. (6) also reported that Amadori compounds were completely converted to glucosone in 24 h at 40°C in the presence of stoichiometric amounts of cupric ion, and that the reaction was markedly inhibited by chelating agents such as EDTA and DTPA. In contrast, formation of MF⁺ in the fructosamine assay is stimulated under anaerobic conditions and is not inhibited by chelators (3), indicating that autoxidation of fFL is not a significant source of glucosone in the fructosamine assay. These data are also consistent with results of Ahmed et al. (7, 8), who identified CML and LL as major products of autoxidation of fFL at pH 7–10.

Overall, the most likely source of glucosone in the fructosamine reaction is its direct formation on reduction of NBT by the Amadori compound. A reaction mechanism for the formation of glucosone from fFL in alkaline NBT solution is shown in Fig. 6. The first step, which is rate-limiting, is the enediol rearrangement of fFL, forming a C-1–C-2 enaminol (17, 18). This is followed by the loss of a proton to yield the enaminol anion and transfer of an electron from the C-2 oxygen atom to NBT, yielding NBT⁺. In the process, fFL is converted to an enaminol radical, which then contributes a second electron for stoichiometric reduction of NBT, followed by hydrolysis of the ketoimine adduct to form glucosone. Further enolization and oxidation of glucosone may then contribute to an enhanced color yield in the fructosamine assay.

**Kinetics of degradation of fFL and glucosone formation.** In this study we observed the complete degradation of fFL in alkaline NBT solution at 37°C within 60 min (Fig. 3). The rapid degradation of fFL was also catalyzed by NBT and by trace amounts of hydrogen peroxide and hydroxyl radicals (3). Although Cheng et al. (19) reported gradual accumulation of dicarbonyl compounds in a reaction mixture on oxidation of glycated polysaccharides at neutral pH, glucosone was rapidly degraded at alkaline pH after reaching maximal concentrations in the fructosamine reaction mixture at 10 min. This finding is consistent with Hodge's finding that dicarbonyl sugars react readily in alkali to form α-enediol compounds, which are strongly reducing even in acidic solution (4).

![Graph](image-url)

**Fig. 5.** Effect of 10 mmol/L AG on serum fructosamine results from diabetics and nondiabetics.

Samples were from the diabetes clinic and from routine specimens presented to the laboratory but were not classified with respect to presence of diabetes. Quantification of fructosamine was based on a commercial assay standard (Precath Fructosamin; Boehringer Mannheim) having a calibrated fructosamine value of 415 μmol/L. Reference interval for nondiabetics; dashed line, theoretical line for perfect correlation between the assays; a solid line, least-squares fit to the data.
Under fructosamine assay conditions at alkaline pH, glucosone was rapidly degraded, irrespective of the presence of the reducible substrate, NBT (Table 1). This observation is consistent with those of Namiki and Hayashi (20), who reported maximal free-radical formation in mixtures of sugars and amino acids at pH 10 and no significant difference between aerobic and anaerobic reactions.

Alkaline conditions would also favor C-2–C-3 enediol rearrangement of glucosone and, in the absence of a reducible substrate, reverse aldol fission reactions would probably occur, forming C2 and C4 fragments (4). Although we did not pursue the identification of products containing fewer than four carbon atoms, Namiki and Hayashi (21) isolated the C2 fragment, glycaldehyde, from mixtures of glucose and 4-butylamine heated in ethanol and showed that formations free radicals, indicative of oxidation chemistry, coincided with formation of glycaldehyde. As shown in Fig. 4, smaller dicarbonyl sugars formed in the reaction, if any, might, like glucosone, make a small contribution to the color yield in the fructosamine assay.

An alternative reaction mechanism for glucosone degradation, the oxidation of the C-1 carbon to form 2-keto-gluconic acid, seems less likely. Mashino and Fridovich (22) had proposed such a mechanism for the alkaline degradation of α-ketoaldehydes, based on their observations with the cyanide-catalyzed divalent oxidation of phenylglyoxal. In the present study, GC-MS analysis of glucosone reaction mixtures revealed traces of lower-order carbohydrates, but no evidence of glyconic acids.

**Contribution of glucosone to fructosamine assay results.** Unlike the inhibitory effects of AG on assays of fFL and α-dicarbonyl species, AG had a nonlinear effect on assay results for a control serum sample (Fig. 4). Maximum inhibition was observed at ~10 mmol/L AG, but higher AG concentrations actually led to an increase in fructosamine yield. These concentration-dependent effects are difficult to interpret in a straightforward way, given that AG may affect the fructosamine assay by activities other than its action as a dicarbonyl trap. Ou and Wolff showed recently (23), for example, that AG has prooxidant activity since autoxidation of AG in physiological buffers generates both superoxide and hydrogen peroxide, compounds known to increase the color yield in the fructosamine assay (3). Thus, the nonlinear response of the assay to AG may result from its primary activity in trapping glucosone at low concentrations and then from its predominantly prooxidant activity at higher concentrations in assays of control sera. In assays of patients' samples AG, at 10 mmol/L, tended to enhance the response for samples in the normal range, but had a negligible effect on samples in the diabetic range. These differences may result from the balance between the carbonyl trapping and prooxidant activity of AG; i.e., the prooxidant activity and enhancement of color yield may be observable in samples with lower proportions of protein glycation, whereas only the inhibitory effect is observed in samples within the diabetic range. It is still not clear, however, why similar nonlinear effects are not observed with model compounds (Fig. 4). Possibly the release of metal ions, resulting from denaturation of serum proteins during the assay, mediates the prooxidant effect of AG.

We conclude that the unmodified amino acid, lysine, and the oxidized carbohydrate, glucosone, are the primary products formed from Amadori adducts on glycated proteins during the fructosamine assay; secondary reduction of NBT by glucosone may contribute to the color yield. Although this activity of glucosone affects the accuracy of the fructosamine assay of model compounds and serum samples, it does not significantly affect the clinical utility of the assay for assessing the status of glycemic control in diabetes.

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References