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Failure of Common Glycation Assays to Detect Glycation by Fructose

Nessar Ahmed and Anna J. Furth¹

Serum albumin was modified by *in vitro* glycation with either fructose or glucose, to see whether the common clinical assays for glycation were able to detect both fructose- and glucose-induced changes in protein structure in diabetes. Although fluorescence measurements showed that fructose causes far more protein damage than glucose, neither serum fructosamine (SFA) nor phenylboronate affinity (PBA) glycation assays reflected these changes. The SFA method implied that fructose causes only about 5% of the glycation induced by glucose; with PBA the proportion was 25%. The thiobarbituric acid- and periodate-based assays also greatly underestimated the true extent of fructation. We discuss these discrepancies with respect to the underlying chemistry, emphasizing the difference between aldehydic and ketonic Amadori products (exemplified by fructose and glucose derivatives, respectively). The implications for detecting fructose-induced secondary diabetic complications are also discussed.

Additional Keyphrases: diabetes · glucose · aldehydes · ketones · variation, source of

In managing diabetes, the most pressing need is clearly to reduce hyperglycemia or, more specifically, hyperglucemia. However, protein glycation, which is thought to contribute to diabetic complications (1), can be caused by other dietary sugars, notably fructose. The first stable sugar-protein adduct formed by glycation is the Amadori product (AP); being a covalently modified form of the native protein, this would be expected to have modified structural and functional properties, and indeed several instances of altered biological activity have been reported for glycated proteins, *i.e.*, those in the AP form (2).² However, it is generally agreed that the accumulation of post-Amadori compounds or advanced glycation end products (AGEs) contributes more to diabetic complications than do the APs. These AGEs, usually detected fluorometrically, are thought to include cross-linked protein aggregates, as well as fluorophores and other undesirable structural modifications of the native protein (1, 2). *In vitro* studies show clearly that fructose is a more potent glycating agent than glucose (3) and is as much as 10-fold more efficient at forming AGEs (4-6). There being no simple assay for quantifying AGEs, the potential dangers of hyperglycemia are assessed by analyzing for AP. In practice, this

nearly always means analyzing for the glucose AP. The fructose AP has indeed been detected in diabetes (4, 7) and hereditary fructose intolerance (8) but only by using HPLC (4) or the charge-based assays possible with hemoglobin as analyte (7, 8).

Theoretical considerations of the chemistry of other glycation assays (reviewed in 9) suggest that neither the serum fructosamine nor the phenylboronate affinity (PBA) methods would detect *in vivo* fructation during routine diabetic monitoring. Both these assays were developed for the glucose AP of serum albumin, a ketone, whereas the fructose AP of any protein is an aldehyde (see Figure 1), and would be expected to react differently.

To investigate this possibility, we compared *in vitro* glucated and fructated serum albumin, looking for structural changes both by fluorescence measurements and by simple assays for quantifying glycation. Fluorescence changes are known to indicate cross-linking and AGE formation in a variety of proteins (2, 10), and have clearly shown that these changes are more rapidly induced by fructose than by glucose (4, 6, 11). However, to our knowledge, no one has yet made a comprehensive study to see whether simple glycation assays of these same glucated and fructated preparations would also reveal the greater dangers of fructose.

Materials and Methods

In vitro glycation was performed by incubating bovine serum albumin (Fraction V; BDH, Poole, UK) at a concentration of 10 g/L in a solution of sugar (0.5 mol/L), sodium phosphate buffer (0.05 mol/L), and sodium azide (3 mmol/L) at pH 7.4 and 37 °C. Samples were withdrawn after various intervals, and dialyzed exhaustively against distilled water or, for the PBA assay, against column starting buffer (see below); they were stored frozen before assaying for glycation or fluorescence.

Fluorescence emission was detected at 410 nm (excitation wavelength 350 nm) with an Aminco Bowman spectrophotofluorometer (Model J4-8960, Travenol Labs., MD).

The serum fructosamine assay was performed as described by Johnson *et al.* (12), the thiobarbituric acid (TBA) assay as described by Parker *et al.* (13), and the periodate assay as we described elsewhere (14). The PBA assay was adapted from that of Middle *et al.* (15): a 200- μ L sample containing 2 mg of albumin in starting buffer (0.25 mol/L ammonium acetate, pH 8.5, containing 50 mmol of magnesium acetate and 3 mmol of sodium azide per liter) was applied to a 1 × 2 cm column packed with Matrex PBA-10 (Amicon, Beverly, MA); after washing to remove unadsorbed protein, we eluted

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² Nonstandard abbreviations: AP, Amadori product; AGE, advanced glycation end products; PBA, phenylboronate affinity; and TBA, thiobarbituric acid.

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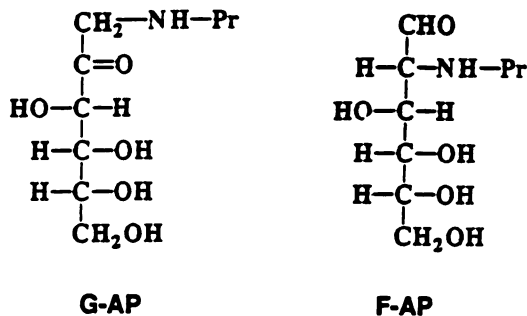


Fig. 1. Structures of glucose and fructose Amadori products (G-AP and F-AP, respectively); Pr represents the rest of the protein molecule

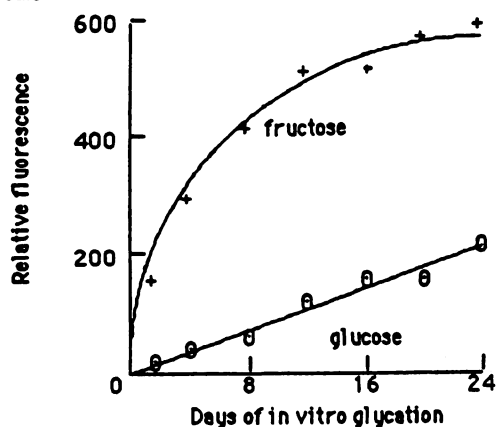


Fig. 2. Fluorescence changes during in vitro glycation of serum albumin by fructose or glucose

glycated albumin in starting buffer containing sorbitol, 0.25 mol/L, at a flow rate of 25 mL/h. The column was regenerated in acetic acid (0.1 mol/L).

Protein was assayed by the bicinchoninic acid method (16).

Results and Discussion

Figure 2 shows the effects of in vitro fructation and glucation on the fluorescence of serum albumin and clearly suggests that fructose has a greater effect on protein structure than does glucose. However, the results of common clinical assays (Table 1) suggest the opposite. Glucose appears to produce four- to 20-fold more AP than fructose, at different stages of the in vitro glycation. The more sensitive assays, performed in research laboratories (TBA and periodate assays), give the same misleading information.

There may be two reasons for this apparent anomaly. First, steady-state concentrations of AP will depend on the rate at which AP is converted to AGE; this reaction, as in the initial conversion of protein to Schiff base, will be faster for sugars such as fructose, which has a high proportion of acyclic form (3). (The ring form cannot react with proteins because it has no free carbonyl group). Second, the underlying chemistry suggests that many glycation assays will fail to respond to the fructose AP. For example, because fructose AP is an aldehyde, with carbonyl on C-1 (Figure 1), it is unable to cyclize to form the hydroxymethylfuran that reacts in the TBA assay. Similarly, aldehydic APs do not readily dehydrogenate (17) and so do not react with redox dyes such as

nitrotetrazolium blue in the serum fructosamine assay. In the periodate assay, the aldehydic fructose AP does not liberate the formaldehyde from which the chromophore is generated, and in the PBA chromatography assay, its conformation lacks the *cis*-diols needed for strong adsorption and hence a high "percentage glycation" reading. This is because, rather confusingly, sugars adopt a glucose-like conformation in the fructose AP, and a fructose-like conformation in the glucose AP; fructose is well known to bind more strongly to phenylboronate resins than glucose does (18, 19).

The PBA method was included in this study not only because it is a common clinical assay but also because it quantifies a rather different variable. Percentage glycation in the PBA assay is the proportion of albumin molecules that are carrying one or more sugar residues, whereas the colorimetric assays give an average value of moles of bound sugar per mole of protein. So, for example, taking just the glucation figures after five days (top line of Table 1), there is no reason to expect absolute values with the PBA method to be the same as those obtained colorimetrically. Discrepancies between the colorimetric assays themselves reflect the different yields of the various chromophores from AP.

It is perhaps surprising to find any contribution from the fructose AP in the three colorimetric assays in Table 1. However, McPherson et al. (4) found that, by mechanisms unknown, some 15% of the fructose-protein adduct appears as a compound identical to the glucose AP, i.e., a derivative of *N*-(1-deoxyhexos-1-yl); this compound could be responsible for the small positive reactions in TBA and serum fructosamine assays. The periodate assay may reflect an additional contribution from another AP isomer, generated from the fructose Schiff base during the Amadori rearrangement, as suggested by Suarez et al. (5).

Whatever the reasons, it is clear that the simple glycation assays in Table 1 fail to reveal the full extent of fructose-induced changes in protein structure, despite the fluorescence evidence in Figure 2. This is presumably why in vivo fructation has so far been detected only by the charge-based glycation assays possible with hemoglobin (7, 8). In subjects monitored only by PBA or serum fructosamine assays of serum albumin, any in vivo fructation would be greatly underestimated. This could be relevant clinically, because diabetics can convert glucose to fructose via the sorbitol pathway (20), and tissue concentrations as great as 12 mmol/L have been reported (Table 1 in reference 20). Furthermore, dietary fructose is a permitted sweetener for diabetics and a major component of the nondiabetic diet (e.g., combined with equimolar quantities of glucose, in sucrose); even a moderate intake of apple juice can raise serum concentrations to 0.44 mmol/L (21). The greater reactivity of fructose could compensate for its comparatively rapid clearance from the bloodstream, particularly because any fructose AP formed would be more reactive than the corresponding glucose AP in producing AGEs.

Table 1. Amadori Product Resulting from In Vitro Glucation (G-AP) and Fructation (F-AP), as Detected by Various Glycation Assays^a

Days of in vitro glycation	SFA assay ^b			TBA assay ^c			Periodate assay ^d			PBA assay ^d		
	G-AP	F-AP	% ^e	G-AP	F-AP	%	G-AP	F-AP	%	G-AP	F-AP	%
5	3.9	0.3	7.7	2.67	0.07	2.6	3.12	0.78	25	79	20	25
10	5.8	0.2	3.4	3.70	0.14	3.7	3.46	0.92	26	85	25	29
16	7.9	0.4	5	4.40	0.29	6.5	4.50	1.20	27	84	28	33

SFA, serum fructosamine.

^a All results are for the net increase in glycation, obtained by subtracting the values for unincubated protein to discount any in vivo glycation in the starting sample.

^b Bound sugar, mmol/L of protein solution.

^c Bound sugar, mol/mol of protein.

^d Percentage glycation: $100 \times (\text{absorbed protein}/\text{total protein})$.

^e Apparent F-AP as a percent of G-AP.

In conclusion, dietary fructose could be causing structural damage that at present goes undetected. There is a pressing need for a simple assay of fructose-modified proteins that could be used to investigate this possibility.

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