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Re-Evaluation of the Fructosamine Reaction

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The difference in spectral characteristics between 1-deoxy-1-morpholinofructose (DMF) and protein/plasma samples in the fructosamine reaction has been related to the solubility of the diformazan formed by reduction of nitro blue tetrazolium chloride. Addition of the surfactant Triton X-100 (20 g/L) to the reagent buffer not only corrects this anomaly but also enhances the absolute response. Detailed investigation of DMF and dihydroxyacetone as calibration standards for the reaction established a clear preference for the latter. Fundamental differences in reaction kinetics were also noted between the Amadori rearrangement products of glucose formed from morpholine (DMF) or the amino lysine groups of protein (glycated albumin). From the reactivity of dihydroxyacetone, as well as glyceraldehyde, observed in the fructosamine reaction, and the presence of this class of compounds (trioses) in human plasma, we infer that they may also contribute to the differentiation of diabetic and non-diabetic samples.

Additional Keyphrases: *diabetes · Amadori rearrangement · trioses · glycated proteins*

In 1982, Johnson et al. (*1*) reported that the extent of plasma protein glycosylation could be estimated by the reduction of nitro blue tetrazolium chloride (NBT) to its colored diformazan.[1] The actual reducing species is presumed to be the Amadori rearrangement product of the Schiff base that initially is formed between glucose and protein lysine amino groups (*2*).

It should be noted, however, that the base-catalyzed reduction of tetrazolium compounds has been extensively applied to the colorimetry of numerous reducing agents, particularly corticosteroids, since 1952 (*3*). The major modifications made to the tetrazolium reaction by Johnson et al. (*1*) were the use of an aqueous solvent, rather than ethanol, and a substantially lower pH. An apparent inconsistency, however, was that the chosen calibration standard, 1-deoxy-1-morpholinofructose (DMF), on reaction with NBT gave a visible spectrum clearly dissimilar from that obtained for protein or plasma samples. Addition of albumin to the DMF solution before reaction with NBT changed its spectral characteristics to mimic those observed for protein samples.

Consequently, albumin, in 40 g/L final concentration, was added to the DMF standard solutions, although the necessity for this has never been clearly elaborated. The use of the DMF/albumin calibration standard has several practical limitations and has in turn led to a plethora of publications addressing the question of assay standardization (*4–19*).

Unfortunately, the latter controversy has diverted attention from the basic anomaly of the reaction: the non-equivalent spectral properties of DMF and protein. Such a result implies a basic deficiency in our knowledge or assumptions concerning the underlying reaction mechanism, which requires elucidation before the question of assay standardization can be satisfactorily addressed. Accordingly, our aim here was to investigate experimental conditions that would allow equivalent reaction profiles for non-protein (e.g., DMF) and protein analytes.

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[1] Nonstandard abbreviations: NBT, nitro blue tetrazolium, which is 3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-bis[2-(*p*-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride; DMF, 1-deoxy-1-morpholinofructose; DHA, dihydroxyacetone; HSA, human serum albumin.

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Materials and Methods

Materials: Albumin (Cohn Fraction V), dihydroxyacetone (DHA), racemic glyceraldehyde, nitro blue tetrazolium chloride, *p*-iodonitrotetrazolium violet, iodonitrotetrazolium formazan, and nitro blue diformazan were all from Sigma Chemical Co., St. Louis, MO. DMF, prepared as described by Hodge and Rist (*20*), was purified by repeated recrystallization.

Instrumentation: For all spectral measurements we used a UV-Vis DMS 100/DS 15 spectrophotometer (Varian, Sunnyvale, CA).

Fructosamine assay: Mix 50 μL of the sample, 0.5 mL of carbonate buffer (0.1 mmol/L) containing 40 g of Triton X-100 per liter, and 0.5 mL of NBT (0.5 mmol/L in carbonate buffer), at pH 10.3, in a cuvette at 37 °C. Record the difference in absorbance at 525 nm 10 and 15 min later. Equilibrate the buffer and NBT solution at 37 °C before adding the sample. The concentrations of the calibration standard DHA are 0, 0.248, 0.503, 0.756, and 1.256 mmol/L. Prepare DHA standards in distilled water, seal them in glass ampules, and store at 0 °C, because DHA steadily decomposes in the carbonate buffer.

Within the time frame of the fructosamine reaction the instability of DHA is insignificant, because its rate of oxidation by NBT is appreciably faster than its rate of base-catalyzed decomposition.

In an attempt to gauge the rate of DHA decomposition in the absence of NBT, we allowed DHA (50 μL, 1 mmol/L) and 0.5 mL of carbonate (with 40 g of Triton X-100 per liter) to equilibrate at 37 °C for 0, 30, 60, 90, and 120 min before adding 0.5 mL of NBT (0.5 mmol/L, in carbonate buffer). The response recorded during the next 10 to 15 min was then related to the zero-time experiment. Respective decreases noted amounted to 15%, 40%, 60%, and 73%. In an identical experiment, DMF (2 mmol/L) showed no major change in its response.

Contour plots: Under the general experimental conditions noted above, calibration curves were determined for both DMF and DHA at specific pH/NBT combinations. Based on the regression curves obtained, the concentration of a reference HSA sample (40 g/L) was calculated in duplicate. From a series of nine such measurements we established a relationship between the calculated HSA concentration and the respective values for pH and NBT (mmol/L), using the technique of iterative multidimensional least-squares curve fitting (*21*). The equations so derived, based on DMF [1] and DHA [2] as the respective calibrants, are (NBT in mmol/L final concentration):

$$\text{HSA (mmol/L)} = 18.92 - 1.703 \cdot \text{pH} - (24.11 + 2.23 \cdot \text{pH}) \cdot \text{NBT} \quad [1]$$

$$\text{HSA (mmol/L)} = 0.613 - 0.0365 \cdot \text{pH} - (2.83 + 0.306 \cdot \text{pH}) \cdot \text{NBT} \quad [2]$$

The contour plots then generated for both DMF and DHA, based on the algorithm reported by Simons (*22*), are shown below in Figures 4 and 5. Interpretation of contour plots relies on noting the change in the response (dashed lines, values indicated) as the pH is increased and the NBT concentration is kept constant, or vice versa.

Results and Discussion

It is well established that, when DMF or HSA is allowed to react with excess NBT, the final spectra are clearly different (Figure 1), although the same product, the diformazan of NBT, is assumed to form. NBT is a dimeric tetrazolium salt, so a possible explanation is that the reducing agent, DMF or protein, directly influences the respective formation of either a mono- or diformazan. We tested this latter assumption by reacting DMF and HSA with the monotetrazolium salt, 3-(*p*-iodophenyl)-2-(*p*-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride. Results similar to those noted for NBT were obtained; therefore, the differential formation of mono- or diformazans could be discounted.

As an extension of the mono-/diformazan study we also investigated the spectral characteristics of formazans in general. We found that these compounds are practically insoluble in aqueous solvent and only slightly soluble in ethanol. The spectral characteristics of the formazans in chloroform were as reported (*23*), but scanning slightly turbid ethanol solutions revealed spectral features similar to those observed in the DMF reduction of NBT. Furthermore, it was also observed that in many instances the color formed in the reaction between NBT and DMF gradually precipitated on standing.

The latter observations made it apparent that the difference between DMF and protein in their reactions with NBT may be related to solubility of the nitro blue diformazan. Accordingly, we tested various surfactants as dispersing agents in the reaction. As a consequence, Triton X-100, at a final concentration of 20 g/L, was added to the reagent buffer. The reaction of DMF, HSA, or plasma with NBT then gave solutions with identical spectral characteristics and absorbance maxima at 525 nm (Figure 2). Concomitantly, the absolute response for DMF increased by approximately 250%, while that for a 40 g/L HSA reference solution was about 160%. The response characteristics for DMF, with and without Triton X-100, are shown in Table 1.

During our studies we also evaluated dihydroxyacetone (DHA) as a calibration standard. Although it is not structurally equivalent to the Amadori-rearrangement product, its reaction profile is identical to that observed for both protein and DMF. Furthermore, DHA is readily available from numerous commercial sources at low cost. DHA is considerably more reactive towards NBT than DMF, as shown in Table 1. Glyceraldehyde, which, like DHA, belongs to the class of compounds known as *trioses*, is also quite reactive in the fructosamine reaction (Table 1).

It is noteworthy that Fantl et al. (*24*) have shown that glyceraldehyde reacts with hemoglobin to form a ketoamine, and therefore the presence of equivalent protein analogs cannot be discounted.

When the long-term reaction profile is compared for HSA, DHA, and DMF (Figure 3), it is quite evident that the DMF reaction does not obey a simple pseudo-first-order mechanism, but is more representative of a complex reaction presumably involving consecutive first-order processes. While such a result may be initially rationalized on the basis that DMF contains a tertiary amine, whereas that for

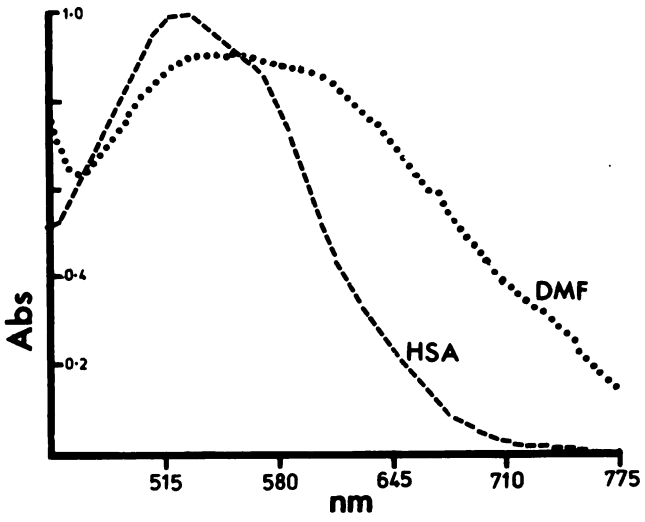

Fig. 1. Spectra of DMF and HSA generated by reduction of NBT under standard published (*1*) conditions

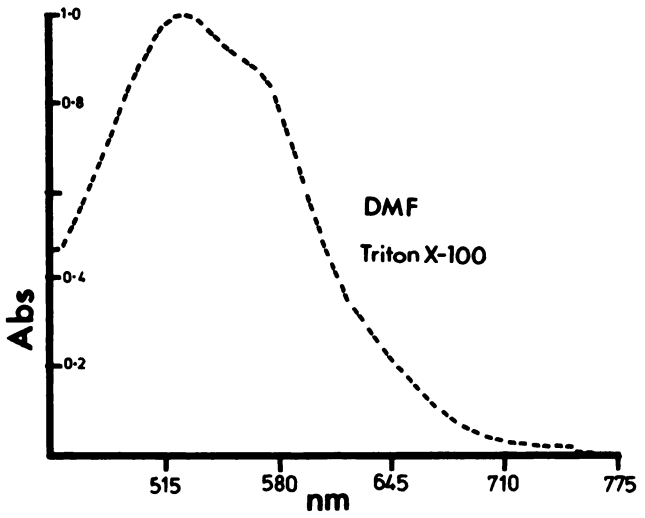

Fig. 2. Change in spectral characteristics for DMF reduction of NBT in presence of dispersing agent, Triton X-100 (20 g/L)
Other conditions exactly as in Fig. 1

Table 1. Regression Equations for DMF, DHA, and Glyceraldehyde in the Fructosamine Reaction[a]

Standard[b]	Intercept	Slope	Triton X-100[c]
DMF	1.86	19.1	0
DMF	−0.90	47.3	20
DHA	3.30	120.2	20
Glyceraldehyde	5.50	84.4	20

[a] *y* (absorbance × 1000 at 525 nm) vs *x* (concn, mmol/L). Conditions: pH 10.0, NBT 0.5 mmol/L, temp 37 °C.
[b] Concentrations (mmol/L): DMF 1.0, 2.0, 4.0, and 6.0 (no Triton X-100); 0.5 1.0, 2.0, and 3.0; DHA 0.2, 0.5, 1.0, 1.5, and 2.0; glyceraldehyde 0.5, 1.0, 1.5 and 2.0.
[c] Concn, g/L, in assay buffer.

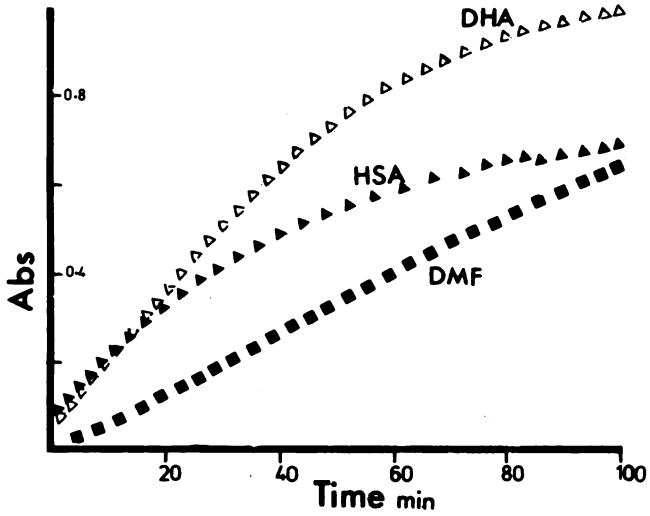

Fig. 3. Kinetic plot of absorbance at 525 nm versus time for DMF, HSA, and DHA

Conditions: NBT 0.5 mmol/L, pH 10.0, Triton X-100 20 g/L, temp 37 °C

the Amadori-rearrangement product on protein is secondary, the explanation is inconsistent with the finding reported by Lever et al. (*25*) that fructose-valine is not exceptionally reactive in the fructosamine assay. The latter findings establish that the reaction profile of DMF is quite dissimilar from that observed for protein Amadori-rearrangement products.

The suitability of both DMF and DHA as calibration standards for the fructosamine reaction was evaluated by investigating the response for numerous combinations of pH and NBT. At each pH/NBT combination a calibration curve was constructed and the concentration of a standard HSA solution (40 g/L) estimated.

From the preceding values we computed contour plots for HSA concentration (mmol/L) with respect to pH and NBT concentration (mmol/L) for both DMF and DHA (Figures 4 and 5). Figure 4 highlights the sensitivity of the DMF/NBT reaction to the experimental conditions, in comparison with that noted for DHA. A similar finding—that is, the sensitivity of the DMF standard to reaction conditions—was reported by Baker et al. (*5*) and formed the basis of their decision to use glycosylated bovine serum albumin as a secondary standard. The pronounced increase in DMF response with respect to that for HSA as the pH is increased again underlines the obvious structural differences between DMF

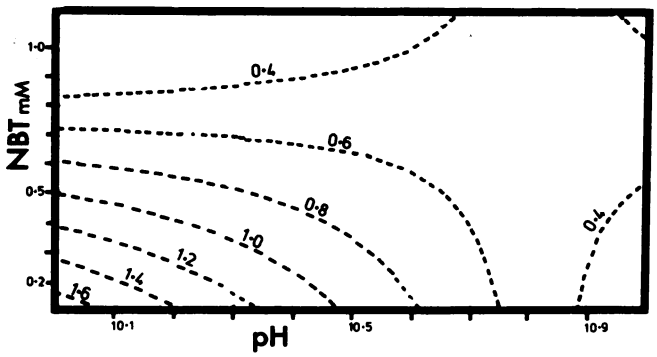

Fig. 4. Contour plot for value of HSA standard (mmol/L) based on DMF calibration curve at individual pH and NBT concentration

Temp 37 °C and Triton X-100 20 g/L added

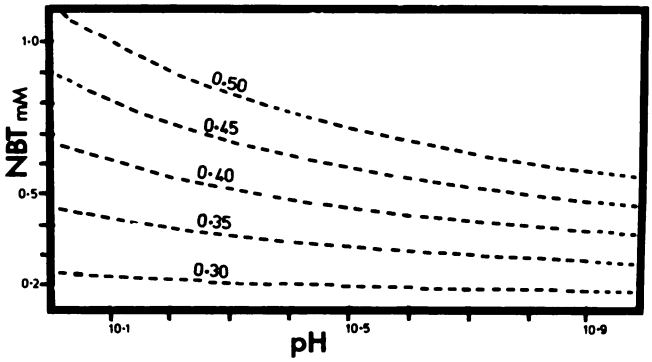

Fig. 5. Contour plot as in Fig. 4, but for DHA as calibration standard

and the respective protein Amadori-rearrangement product. Whereas DHA is directly oxidized as its respective enetriol by NBT, it is quite apparent that a secondary product of DMF reduces NBT. The rate of formation for the latter compound is clearly base-dependent. Given all the preceding facts, DHA is undoubtedly a superior standard to DMF for the fructosamine reaction.

In all our experiments the absorbance difference for the 10- to 15-min interval was used. To examine whether any other time interval would yield comparable results, preferably over a shorter period, the rate of absorbance change for DHA, HSA (40 g/L), and plasma was evaluated every 10 s for 20 min. In contrast to the recently reported results of Howey et al. (*12*), we found that a 5-min interval was an absolute necessity, with the earliest possible valid initiation of reaction at 7 min. Lim and Staley (*26*) have previously cited similar findings with respect to pre-incubation time requirements.

Using standard conditions (pH 10.3; NBT, 0.25 mmol/L; Triton X-100, 20 g/L; and 37 °C), we repeatedly measured the response of an HSA solution (40 g/L, 615 μmol/L), with DHA as the calibration standard. The calculated value was 0.33 mmol/L and the inter- and intra-assay coefficients of variation were 3.9% and 1.1%, respectively (n = 5). The number of NBT reducing sites therefore is calculated to be 0.54 per mole of HSA. This may be compared with a value of 0.80/mol obtained by Olufemi et al. (*27*) for the same type of commercial sample by use of the periodate oxidation procedure. These authors also reported a value of 0.37/mol for specifically purified human albumin, which agrees closely with Schleicher and Wieland's (*28*) value of 0.28/mol based on the specific measurement of furosine. The number of glycated residues on HSA estimated by our procedure is therefore similar to results obtained by alternative and more specific methods.

The use of DHA as a standard leads to markedly lower values for plasma fructosamine. For example, the assay of plasma samples from 38 non-diabetic pregnant women (24–28 weeks of gestation) gave an average value of 0.38 mmol/L (SD 0.04, range 0.29–0.50 mmol/L). This may be compared with the value of 2.41 (SD 0.14) mmol/L recently cited by Roberts and Baker (*29*), who used DMF as the primary calibration standard.

The overall findings of this study have several important implications for the fructosamine reaction. The use of a HSA/DMF calibration matrix with its attendant "albumin blank correction" (*5, 10, 12, 14, 16–19*) is no longer relevant. It should be noted, however, that the relationship between concentrations of fructosamine and albumin in individual patients with respect to expression of results has not been

considered in our study. However, there is uncertainty with respect to the exact nature of the moiety in plasma that actually reduces NBT, because analogs of the Amadori-rearrangement product are shown to have quite dissimilar kinetic profiles when compared with glycated protein. Supporting this latter argument, Walker et al. (*30*) reported that only half of the total value for fructosamine in plasma is attributable to HSA. More recently, Wincour et al. (*31*) have shown major relational differences between fructosamine and glycated albumin. The present paper presents evidence that trioses, represented by DHA and glyceraldehyde, are readily measured in the fructosamine reaction.

Because the response for protein is also enhanced by added Triton X-100, values measured without a dispersing agent may partly reflect the ability of the plasma matrix or specific proteins to solubilize the final nitro blue diformazan product.

Although we advocate here the use of DHA, and possibly also glyceraldehyde, as calibrators for the fructosamine reaction, they are not structurally equivalent to glycated proteins. Previously, Lever et al. (*25*) have presented limited results on the use of synthetic α-glycated valine and ϵ-glycated lysine derivatives. Further investigation of the latter compounds and other synthetic ketoamine analogs under the new protocol would seem most justified.

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