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KINETICS OF THE GLYCATION OF BOVINE SERUM ALBUMIN BY MANNOSE AND FUCOSE IN VITRO

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Glycation of bovine serum albumin was measured for mannose and fucose at 37°C. Mannose as well as fucose demonstrated an initial rapid increase in rate of formation of total adducts followed by a slower secondary reaction. The equilibrium constant for Schiff base formation was almost two times larger for mannose than fucose, although the Schiff base formed by fucose rearranged 1.5 times faster than that for mannose. Both sugars showed parallel lines for the formation of total and acid stable products after three hours. Discussion integrates new mechanistic data with previously suggested mechanisms.

Nonenzymatic glycosylation (glycation) commonly occurs in body proteins(1). This posttranslational modification has been shown to be an early step in crosslinking of proteins (2), formation of fluorescent pigments (3,4) and also may well be a source of free radicals.(5,6) However, the mechanism of the initial condensation of glucose with a protein has been partially but not entirely explained by the theory presented by Higgins and Bunn (7). In a study regarding the reaction of various reducing sugars with hemoglobin, they proposed that the rate of a sugar reactivity with the protein is dependent on the quantity of open chain species of the sugar. They rationalized that since glucose had the lowest percentage of open chain species, it shows the slowest rate of condensation with protein among reducing sugars. McPherson et al (8) have suggested that the reactivity of a sugar with various proteins depends also upon the particular protein utilized. Specifically, individual proteins have distinct sites for catalysis of the Schiff base formation and this appears to be influenced by nucleophilicity of amino groups in the protein not involved in the condensation. In this report we have examined

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the kinetic behavior of the formation of the Schiff base and the rearrangement products of albumin with the sugars mannose and fucose to further elucidate the reaction mechanism of glycation.

MATERIALS AND METHODS

All reagents were purchased from Sigma (USA) unless otherwise noted. A Sephadex G-25 fine (27 x 1 cm) column was prepared according to instructions from the manufacturer (Pharmacia,Sweden). When not in use the column was stored in physiological saline(9), pH 7.4, with 0.002 % NaN_3. The column was prepared, stored and utilized in a cold room (temp = 4° C.) Measurement of sugar ([^{14}C]L-fucose, [^{14}C]D-mannose, Amersham, UK) incorporation was determined by the method of Baynes et al.(10) with the following modifications. The incubation mixtures of 5.5 mM sugar, 1.1 mM bovine serum albumin, 0.004% NaN_3 with [^{14}C] labeled sugar added to make 8 x 10^7 cpm, were dissolved in physiological saline and incubated at 37° C. At each time point two 35 µl aliquots were withdrawn, the first diluted in 0.465 ml of cold physiological saline in ice and immediately layered upon the column, the second diluted in 0.465 ml of 0.2 M sodium acetate buffer pH 4.5 and incubated for an additional hour at 37° C before gel filtration following the same protocol as described above. The column flow rate was 0.5 ml/min and 0.75 ml fractions were collected directly into microvials (Packard, Switzerland). Instagel (Packard) (2.25 ml) was added to the microvials, mixed to homogeneity and counted for 10 minutes with a Packard liquid scintillation spectrometer model C2425. The ratio between the radioactivity bound to the protein and total radioactivity was determined. The quantity of Schiff base was calculated by subtracting the relative cpm of the acid stabile products from the cpm of the total adducts.

RESULTS

The top line in figure 1 shows the percent of total adducts formed in the reaction of albumin with [^{14}C]mannose. The formation of total adducts has a fast initial phase followed by a more gradual rise in percent of total adducts. Acid stable adducts also present a biphasic curve, with an initial rapid reaction followed by a slower one. After three hours the curves were parallel. Subtraction of acid stable products from total adducts furnished a curve of the formation of acid labile adducts. This curve showed rapid attainment of equilibrium concentrations of the acid labile Schiff base.

Figure 2 depicts the percent generation of [^{14}C]fucose adducts with albumin. The formation of total adducts shows a biphasic curve where an initial rapid phase is followed by a slower one. The acid stable adducts also exhibit a rapid formation stage followed by a slower one. After three hours the two curves were parallel. Subtraction of the percent of acid stable products from

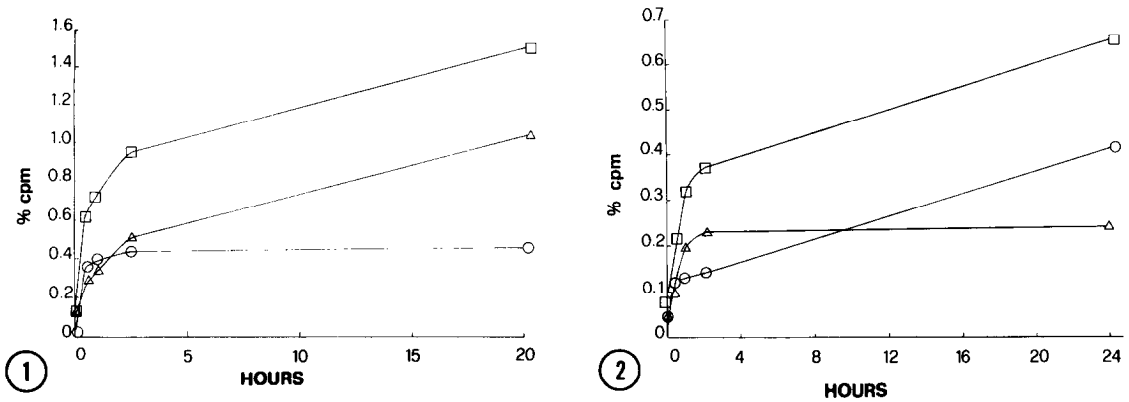

Figure 1 Kinetics of mannose induced glycation of bovine serum albumin. Incorporation of [^{14}C]-mannose in total adducts (□—□), acid stabile adducts (○—○), acid labile adducts (△—△). The percent sugar incorporated was calculated by comparing radioactivity at the column void volume with total radioactivity recovered in column fractions.

Figure 2 Kinetics of fucose induced glycation of bovine serum albumin. Incorporation of [^{14}C]-fucose in total adducts (□—□), acid stabile adducts (○—○), acid labile adducts (△—△) as described in Figure 1.

total adducts produced a curve of the percent formation of the acid labile Schiff base. It can be seen that equilibrium concentrations are reached by three hours and that mannose generated almost twice the equilibrium concentration of acid labile adducts than fucose.

Table I compares the kinetic parameters found in this study for the reactions of mannose and fucose with bovine albumin. The

TABLE I
KINETIC CONSTANTS FOR THE REACTION OF SUGARS WITH BOVINE SERUM ALBUMIN[a]

	Mannose	Fucose
$K_{eq}(M^{-1})$	4.18	2.18
$k_2'(hr^{-1}\ M^{-1})$	0.62	0.48
$k_2(hr^{-1})$	0.15	0.22

[a] Reaction scheme

$$Glc + Prot \underset{k-1}{\overset{k_1}{\rightleftharpoons}} Glc{=}Prot \xrightarrow{k_2} GlcProt$$

Kinetic constants were calculated as in reference 10, from Figures 1 and 2.

equilibrium constant of the formation of Schiff base for mannose (C2 epimer of glucose) is almost twice that for fucose. The k_2', apparent velocity of formation of all species of adducts, is slightly higher for mannose than for fucose. On the other hand k_2, velocity of formation of Amadori products is almost 1.5 times higher for fucose than for mannose.

DISCUSSION

The Schiff base formed by the condensation of glucose and albumin has been shown to rapidly attain an equilibrium concentration (10) followed by a slower rate of rearrangement to form Amadori products. In order to explain the lower reactivity of glucose in comparison to other reducing sugars, Higgins and Bunn (8) hypothesized that this phenomenon may be due to lower concentrations of the open chain, reactive form, of glucose. This hypothesis was supported by results showing a general, but not an absolute correlation between the concentration of open chain form of a sugar and its reactivity with hemoglobin. In this study we have observed that mannose and fucose rapidly attain equilibrium concentrations of the Schiff base but that mannose has a larger K_{eq} for this reaction than fucose. On the contrary the K_{eq} for glucose is very similar to that of mannose (10). We also observed that the overall condensation rate of mannose with albumin was faster than that of fucose, in spite of the fact that there are more open chain molecules available for fucose (7). Our data together with that reported by Baynes et. al.(10) extend the previous hypothesis of Higgins and Bunn such that the overall rate of glycation may be directly related to the reactivity of the Schiff base. Recently, the Schiff base form has been shown to exist not only in the open chain configuration but also in the pyranosyl form (11). Therefore it may also be hypothesized that the Schiff base reactivity depends on the extent of its cyclization. Mechanisms of rearrangements between the sugars and proteins which we have studied may have some physiological significance. In fact, the monosaccharides L-fucose and D-mannose as well as their BSA conjugates have been found to greatly stimulate migration of macrophages at the same concentrations used in this study (12). Furthermore, the Amadori products formed from glucose can undergo further rearrangements and dehydrations to form AGE proteins (advanced glycosylated endproducts)(13), and macrophages have been reported to have specific receptors for

these AGE proteins (14). In our study with albumin, we have confirmed that the tendency of a sugar to react with a protein is in part but not entirely predicted on the basis of the quota of sugar present in the open chain form, and may be in part determined by the formation, stability and reactivity of the Schiff base.

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