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Effect of buffer phosphate in the reaction of hemoglobin with fructose

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Abstract
The reaction of hemoglobin with fructose is catalyzed by buffer phosphate. The kinetics exhibited both, a buffer independent term ($k_0$) and a second-order term in buffer ($k_B$). The catalytic constants ($k_B$) for phosphate is $(11.40 \pm 0.60) \times 10^{-10} \text{ s}^{-1} \text{ M}^{-1}$. The spontaneous rate reaction ($k_0$) for phosphate is $(5.30 \pm 1.60) \times 10^{-7} \text{ s}^{-1}$. The glucation of hemoglobin with fructosa exhibits identical results in buffer phosphate prepared in H$_2$O and D$_2$O. The constant rate for phosphate in H$_2$O and D$_2$O are $(5.31 \pm 0.30) \times 10^{-6} \text{ s}^{-1}$ and $(5.30 \pm 0.09) \times 10^{-6} \text{ s}^{-1}$. The absence of solvent isotope effect is indicative that a group basic in the molecule of hemoglobin may be involved in the abstraction of proton 2 in the reorganization of Amadori.

Keywords: Hemoglobin; Glycation; Fructose; Phosphate

Introduction
Non-enzymatic glycation, is a natural phenomenon, which is the attachment of free sugar molecules to certain amino acid residues of proteins. The reaction between glucose and free amino structures in proteins forms complex cyclic structures that are related to diabetes complications and ageing.

Studies with a number of proteins indicate that glycation is not a random chemical modification of amino groups in proteins. There is preferential glycation of β-chain terminal valine residues in hemoglobin$^1$ and a selective modification of a limited set of lysine residues in hemoglobin$^1$, albumin$^2$ and lipoprotein$^3$. As shown in Scheme 1, the initial reversible condensation step of glucose and hemoglobin forms an aldimine or Schiff base which is able to undergo a nearly irreversible intermolecular Amadori rearrangement$^4$.

Several factors have been reported that can influence the rate of glycation of hemoglobin: Ph$^{4,6}$, sugar concentration$^7$, catalysis by buffer phosphate$^{6,8}$, arsenate and catalysis by

Scheme 1: Glycation of Hemoglobin.
buffer phosphate, arsenate and carbonate, catalysis by 2,3-diphosphoglycerate, 3-phosphoglycerate, 2-phosphoglycerate and 2-glycerolphosphate. Clearly inorganic and organic phosphates play an important role in determining the kinetics and specificity of glycation of hemoglobin. The role of inorganic phosphate and carbonate is not classical, proteolytic general acid-base catalysis, showing that a functional group on the hemoglobin is the abstracting base in the Amadori rearrangement. The catalytic constants for carbonate and phosphate buffer are the same with glucose.

Glucose is the most abundant reducing sugar in the body. This implies that the reducing sugar is generally considered in the non-enzymatic glycation reactions of biological interest. No clutch, any sugar that has a free carbonyl group can react with primary amino groups of proteins to form Schiff base.

The reactivity of the different sugars is given by the availability of its carbonyl group. Sugar molecules get stabilized by a balance between the open form and at least two closed forms (cyclic anomers) in which the carbonyl group disappeared. In 1953 Kastchalsky group showed a correlation between the reaction rate and the glycation ratio of the open form of the sugar. Have demonstrated the relative reactivity of hemoglobin with certain monosaccharides, where the correlation is observed between the rate of interaction with the protein and the open form of the sugar.

Fructose, as is the case of other reducing sugars, undergoes Maillard reaction with proteins and amino acids. The initial stages of the reaction occur faster than with glucose fructose, approximately the rate of formation of Schiff base from amino groups of the hemoglobin with fructose is 7.5 times faster than that formed with glucose.

Fructose is also formed in the reaction of fructose with hemoglobin. Because of all these factors that involve high consumption of fructose, we propose to perform a mechanistic study of the behavior of fructose directly with hemoglobin in the presence of phosphate buffer, if they react faster than glucose with hemoglobin formation HbA1c glycated hemoglobin should not be a substitute for glucose as side reactions accelerate diabetes and normal aging.

**Experimental**

Human hemoglobin was obtained from Sigma Chemical Co., fructose Sodium dihydrogen phosphate (NaH2PO4), disodium hydrogen phosphate (Na2HPO4·7H2O) and sodium chloride were purchased from Merck. Acrodisc filters 0.2 µm was from Gelman Sciences.

[Scheme 2: Fructose Maillard reaction causes two (a and b) products Heyns.]

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**References**

1. Kastchalsky group (1953)
2. Glucose is the most abundant reducing sugar in the body.
3. Fructose, as is the case of other reducing sugars, undergoes Maillard reaction with proteins and amino acids.
4. Fructose is also formed in the reaction of fructose with hemoglobin.
5. Because of all these factors that involve high consumption of fructose, we propose to perform a mechanistic study of the behavior of fructose directly with hemoglobin in the presence of phosphate buffer, if they react faster than glucose with hemoglobin formation HbA1c glycated hemoglobin should not be a substitute for glucose as side reactions accelerate diabetes and normal aging.
Phosphate buffer solutions at pH = 7.3 were prepared by mixing appropriate volumes of sodium dihydrogen phosphate (10 mM), disodium hydrogen phosphate (10 mM) and NaCl (0.15 M).

The reaction mixtures were prepared by mixing known volumes of hemoglobin with different concentrations of buffer phosphate, NaCl (0.15 M), pH = 7.3, fructose (40 mM) at 0°C. The final pH for phosphate buffer was 7.3 for all solutions. The reaction was started and maintained at 37 °C. All solutions were sterilized by ultrafiltration through a Gelman 0.2 µm filter (Acrodisc) in sterile plastic tubes.

The rates of glycation of hemoglobin under an atmosphere of air were measured by the appearance of the glycated hemoglobin. Glycohemoglobin HbA1C was eluted from a cation-exchange column and measured spectrophotometrically at 415 nm (Teco Diagnostics Kit). Hemoglobin concentration of 9.2 × 10−2 mM was employed with fructose (40 mM). The reaction media were 0.15 M of NaCl, buffered at 37 °C with mixtures of sodium dihydrogen phosphate and disodium hydrogen phosphate.

In the experiments to determine solvent isotope effects for phosphate buffer was held at 4.8 mM, pH 7.30, pD 7.84, at 37°C under similar condition of hemoglobin and fructose concentration. All solutions were sterilized by ultra-filtration thorough a Gelman 0.2 µm filter (Acrodisc) in sterile plastic tubes.

**Results and discussion**

Figure 1 shows an increase in the percent of HbA1C formation with time at fixed concentrations of phosphate buffer at pH = 7.3, NaCl (0.15 M) at 37°C. The concentrations of hemoglobin and fructose were kept at 9.2 × 10−2 mM and 40 mM, respectively. Phosphate buffer was varied from 1.6 mM to 8.0 mM. The increase of HbA1C formation with increase in phosphate concentration is indicative of catalysis by phosphate buffer.

Table 1 list the first order rate constants for Glycation of hemoglobin with fructose as a function of phosphate buffer pH 7.30, NaCl 0.15 M at 37°C.

<table>
<thead>
<tr>
<th>[Fosfato] mM</th>
<th>kobs x 10^6 s^(-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6</td>
<td>2.38 ± 0.05</td>
</tr>
<tr>
<td>3.2</td>
<td>4.04 ± 0.10</td>
</tr>
<tr>
<td>4.8</td>
<td>5.31 ± 0.30</td>
</tr>
<tr>
<td>6.4</td>
<td>8.33 ± 0.40</td>
</tr>
<tr>
<td>8.0</td>
<td>10.07 ± 0.30</td>
</tr>
</tbody>
</table>

The increase of rate constants (kobs) with phosphate buffer is indicative of catalysis by phosphate. The kinetics exhibited both, a buffer independent term reflecting that the reaction assisted by water, ions (hydroxide or hydronium ions), or protein functional groups and a second-order term in buffer (kobs = k0 + kB [Buffer]). The second order rate constants (kB) calculated from the slopes (Figure 2) and the first order rate constant (k0) for the spontaneous reaction from the intercepts (Figure 2). The catalytic constants (k0) (slope) for phosphate is, (11.40 ± 0.60) x 10^-10 s^-1 M^-1, the spontaneous rate reaction (k0) (intercept) is (5.30 ± 1.60) x 10^-7 s^-1. A similar effect with hemoglobin and glucose was reported. kB = (1.67 ± 0.11) x 10^-10 s^-1 M^-1 y k0 = (0.57 ± 0.19) x 10^-7 s^-1. The glycation of hemoglobin is greater with fructose that glucose, figure 3.

This observation is consistent with the extent to which the sugar exists in the open form. The equilibrium between the open and ring structures for glucose is 0.002 % and from fructose is 0.7 %.

![Fig. 1](image-url): Plot of formation of HbA1C as a function of time in different concentration of phosphate buffer, pH 7.30 for glycation of hemoglobin at 37°C.

**Table 1:** First order rate constants for glycation of hemoglobin with fructose as a function of phosphate buffer pH 7.30, NaCl 0.15 M at 37°C.

- constant at 9.2x10^-2 mM and 40 mM respectively, phosphate was varied from 1.6 mM to 8.0 mM. The first-order rate constants were calculated as the best-fit parameters obtained from a non-linear least squares fitting procedure to the first order equation.
The glycation of hemoglobin with fructose exhibits identical rates in protium and deuterium oxides in phosphate buffer, figure 4. The first rate constant for phosphate in H$_2$O and D$_2$O are $(5.31 \pm 0.30) \times 10^{-6}$ s$^{-1}$ and $(5.30 \pm 0.09) \times 10^{-6}$ s$^{-1}$. Phosphate buffer was held at 4.8 mM, pH 7.30, pD 7.84, at 37°C under similar condition of hemoglobin and fructose concentration. The absence of solvent isotope effect is indicative that the a catalyst base group on the hemoglobin is the proton-abstracting base in the Amadori rearrangement.

Conclusions

1. The reaction of hemoglobin with fructose is catalyzed by buffer phosphate.
2. The catalytic constant ($k_B$) for fructose is seven times higher than glucose.
3. The spontaneous reaction ($k_0$) for fructose is nine times bigger than glucose.
4. The glycation of hemoglobin with fructose exhibits identical rates in protium and deuterium oxides in phosphate buffer.
5. Non solvent isotope effect for phosphate is indicative that a functional group in the hemoglobin is the abstracting base in the Amadori rearrangement.

References


