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Artículo científico

Effect of buffer phosphate in the reaction of hemoglobin with fructose

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Resumen

La reacción de la hemoglobina con fructosa es catalizada por el buffer de fosfato. La cinética exhibe tanto, un término independiente del buffer (ko) como un término de segundo orden dependiente del buffer (k_B). La constante catalítica (k_B) para el fosfato es (11,40 ± 0,60) x 10^{-10} s^{-1} M^{-1}. La velocidad de reacción espontánea (k_0) para el fosfato es (5,30 ± 1,60) x 10^{-7} s^{-1}. La glucación de la hemoglobina con fructosa exhibe idénticos resultados en buffer fosfato preparado en H_2O y D_2O. Las constantes de velocidad para el fosfato en H_2O y D_2O son (5,31 ± 0,30) x 10^{-6} s^{-1} y (5,30 ± 0,09) x 10^{-6} s^{-1}. La ausencia de efecto isotópico de solvente es indicativo que un grupo básico en la molécula de hemoglobina pueda estar involucrado en la abstracción del protón 2 en el reordenamiento de Amadori.

Palabras claves: hemoglobina; glucación; fructosa; fosfato

Abstract

The reaction of hemoglobin with fructose is catalyzed by buffer phosphate. The kinetics exhibited both, a buffer independent term (k_o) and a second-order term in buffer (k_B). The catalytic constants (k_B) for phosphate is (11.40 ± 0.60) x 10^{-10} s^{-1} M^{-1}. The spontaneous rate reaction (k_o) for phosphate is (5.30 ± 1.60) x 10^{-7} s^{-1}. The glycation of hemoglobin with fructose exhibits identical rates in protium and deuterium oxides in phosphate buffer. The first rate constant for phosphate in H_2O and D_2O are (5.31 ± 0.30) x 10^{-6} s^{-1} and (5.30 ± 0.09) x 10^{-6} s^{-1}. The absence of solvent isotope effect is indicative that a catalyst base group on the hemoglobin molecule may be involved in the abstraction of proton 2 in the Amadori rearrangement.

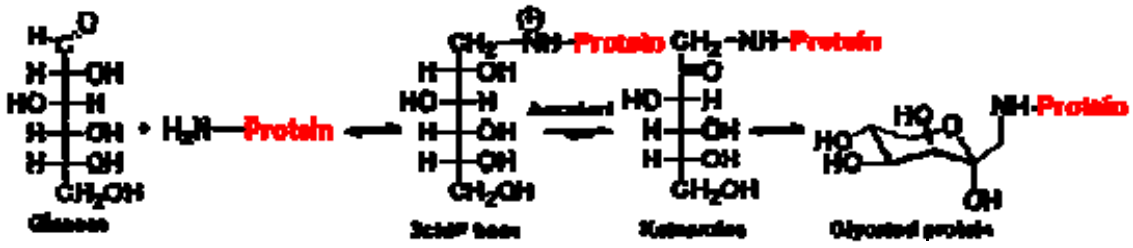
Keywords: Hemoglobin; Glycation; Fructose; Phosphate

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[6,8], arsenate and carbonate[9], catalysis by 2,3-diphosphoglycerate[5,10-12], 3-phospho-glycerate, 2-phospho-glycerate and 2-glycerolphosphate[13]. Clearly inorganic[6,8] and organic phosphates[10,13,14] 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[8]. The catalytic constants (k_B) for carbonate[9] and phosphate[8] 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 monosac-charides[18], 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[18].

The Maillard reaction with fructose is very similar to that with glucose (Scheme 2), but presents another grouping is called "Heyns rearrangement" and is shown generally as the replacement of 2-amino-2-deoxyaldosa[19,20] as shown in Scheme 2. Others found to the 1-amino-1-deoxy-D-fructose is also formed in the reaction of fructose with amino acids[19,20] and protein[21].

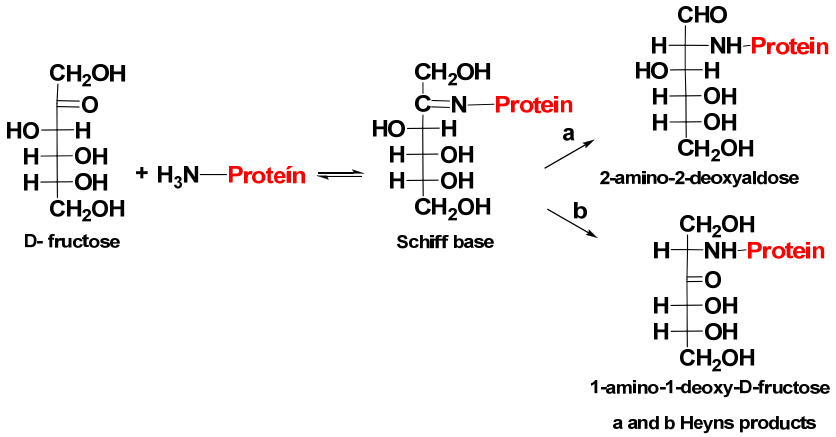
Studies indicate that high levels of fructose postprandial plasma are associated with retinopathy and possibly nephropathy in patients with type 2 diabetes[22]. Diets high in fructose (72 % by weight) in rats caused the development of diabetes mellitus and diffuse glomerulosclerosis[23].

Cataract formation in diabetes occurs through non - enzymatic glycation of proteins in the lens due to higher concentrations of sugars in the lens of the diabetic patients. Research conducted with [^{14}C]fructose and gamma-II-crystalline bovine radiolabeled indicated that fructose had reacted in one site of the protein. The results of the amino acid analysis to the amino acid sequence of gamma-II-crystalline, it was concluded that the labeled peptide corresponded to the N-terminal dipeptide[24]. The site of glycation of bovine gamma-II-crystalline by fructose was thereby identified as the alpha-NH_2 group of the N-terminal glycine[24]. Molecular modeling study revealed that the most likely to join between fructose and hemoglobin sites are lysines: Lys 7 (α), Lys 127 (α) and Lys 66 (β)[25].

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 (NaH_2PO_4), disodium hydrogen phosphate ($Na_2HPO_4{\cdot}7H_2O$) 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[19].

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[15]. Glycohemoglobin HbA_{1C} was eluted from a cation-exchange column and measured spectrophotometrically at 415 nm (Teco Diagnostcs Kit[15,17]**.** 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[15,17].

In the experiments to determinate 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 HbA_{1C} 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 mM. The increase of HbA_{1C} 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 as a function of phosphate buffer concentrations at pH = 7.30, NaCl (0.15 M) at 37°C.The concentration of hemoglobin and fructose were held constant at 9.2×10^{-2} mM and 40 mM, respectively, and phosphate was varied from 1.6 mM to 8.0 mM. The first-order rate constants were calculated as the best-fit parameters which were obtained from a non-linear least squares fitting procedure to the first order equation:

$$[HbA_{1C}]_t = [HbA_{1C}]_\infty + ([HbA_{1C}]_0 - [HbA_{1C}]_\infty)e^{-k_{obs}.t}$$

Figure 2 shows an increase in the first order rate constants for glycation of hemoglobin as a function of phosphate buffer concentrations, pH 7.30, NaCl 0.15 M at 37°C. The concentration of hemoglobin and fructose were held

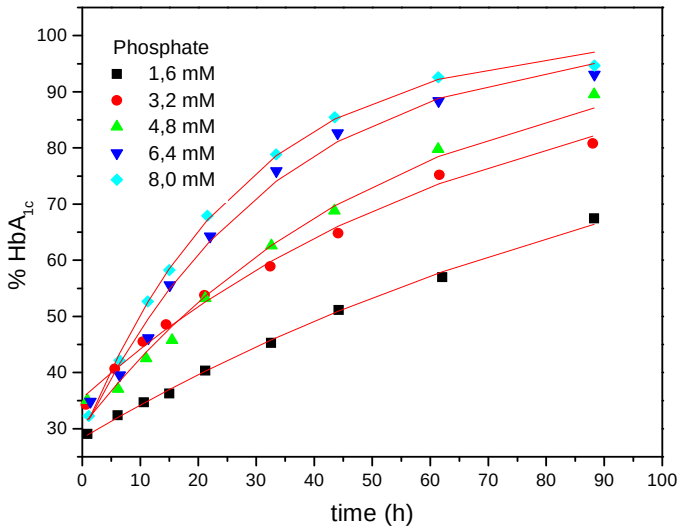


Fig. 1: Plot of formation of HbA_{1C} as a function of time in diferents concentration of phosphate buffer, pH 7.30 for glycation of hemoglobin at 37°C.

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.

[Fosfato] mM	k_{obs}x 10^6 s^{-1}
1.6	2.38 ± 0,05
3.2	4.04 ± 0,10
4.8	5.31 ± 0,30
6.4	8.33 ± 0,40
8.0	10.07 ± 0,30

constant at 9.2×10^{-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 increase of rate constants (k_{obs}) 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 ($k_{obs} = k_0 + k_B$ [Buffer]). The second order rate constants (k_B) calculated from the slopes (Figure 2) and the first order rate constant (k_0) for the spontaneous reaction from the intercepts (Figure 2). The catalytic constants (k_B) (slope) for phosphate is, $(11.40 \pm 0.60) \times 10^{-10}$ s^{-1} M^{-1}, the spontaneous rate reaction (k_o) (intercept) is $(5.30 \pm 1.60) \times 10^{-7}$ s^{-1}. A similar effect with hemoglobin and glucose was reported. $k_B = (1{,}67 \pm 0{,}11) \times 10^{-10}$ s^{-1} M^{-1} y $k_o = (0{,}57 \pm 0{,}19) \times 10^{-7}$ 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 %[16].

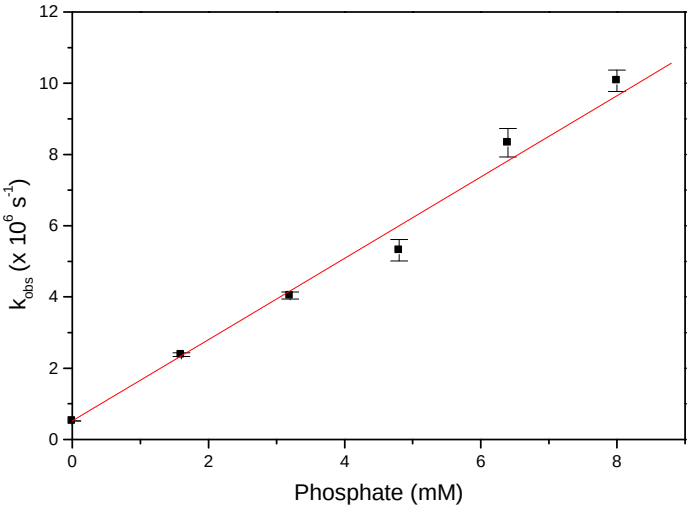


Fig. 2: Plot of firt order rate constants for the glycation of hemoglobin with fructuose as a function of phosphate concentration 37 °C y pH 7.3

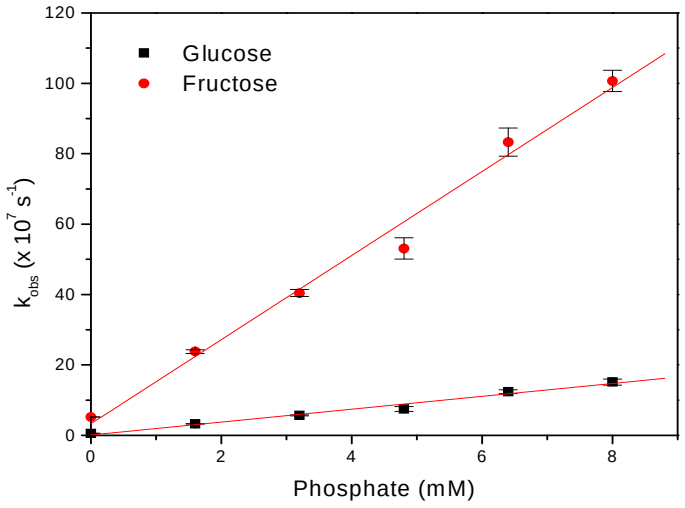


Fig. 3: Plot of firt order rate constants for the glycation of hemoglobin with fructuose and glucose as a function of phosphate concentration 37 °C y pH 7.3

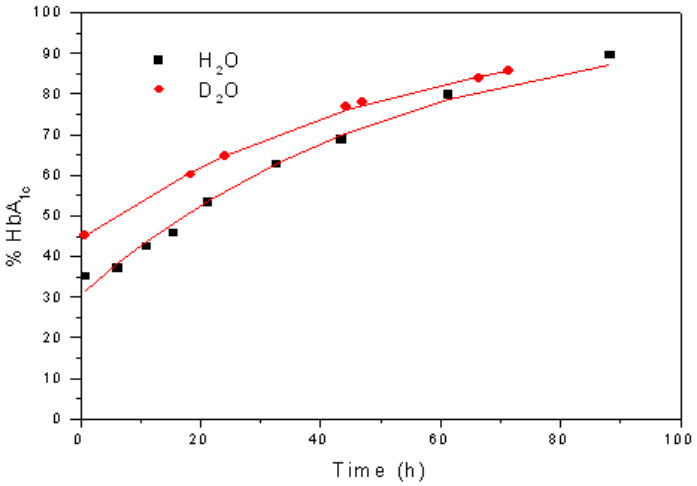


Fig. 4: Plot of formation of HbA_{1C} as a function of time in H_2O nd D_2O phosphate 4.8 mM, pH 7.30, pD 7.84 at 37°C.

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_2O and D_2O are (5.31 ± 0.30) x 10^{-6} s^{-1} and (5.30 ± 0.09) x 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 (kB) for fructose is seven times higher that glucose.
3. The spontaneous reaction (k_0) for fructose is nine times bigger that 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.

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