Kinetic Significance of the Schiff Base Reversion in the Early-Stage Maillard Reaction of a Phenylalanine−Glucose Aqueous Model System

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The kinetic constants (k) and activation energy (E_a) for the formation (k_1, E_{a1}) and reversion (k_{-1}, E_{a-1}) of the Schiff base complex and for the Amadori rearrangement (k_2, E_{a2}) of the Maillard reaction were determined in a phenylalanine−glucose system to elucidate its early-stage mechanism. The k_{-1} and k_2 were 10^3 times greater than the k_1, indicating the Schiff base formation, but not the Amadori rearrangement, was the rate-limiting step of the reaction. The E_{a2} (8.01×10^4 kcal/mol) was slightly greater than the E_{a1} (6.52×10^4 kcal/mol) and the E_{a-1} (7.49×10^4 kcal/mol), revealing that the Amadori rearrangement was more sensitive to temperature changes and was more favorable at higher temperatures. These suggested that the chemical nature of the reactants, determined by their structures and pH condition, is more important to control the reaction. These results were also supported by the pH and temperature profiles of the Amadori compound formation.

Keywords: *Maillard reaction; kinetics; Amadori compound; Schiff base; phenylalanine; fructosylphenylalanine*

INTRODUCTION

Effective control of the Maillard reaction relies on understanding its mechanism, especially for the formation and conversion of the Amadori compounds at the very early stages. Yaylayan and Huyghues-Despointes (1994) and Labuza (1994) reviewed the synthesis, analysis, chemical reactions, and kinetics of the Amadori compounds. It is believed generally that the kinetics of the Maillard reaction is very complex, even for some well-studied initial steps. Because the Schiff base is difficult to determine quantitatively, the exact kinetic order of the Amadori compound formation and the rate-determining step in the early stages of the reaction are still not clear. Most of the previous kinetic studies were based on the determination of the loss of either amino acid or sugar during the reaction, assuming that the reversion of the Schiff base complex was negligible. The Amadori compound was treated as the initial product due to ignorance of the Schiff base complex. However, reported results on the kinetic order of the Amadori compound formation were confusing in the amino acid−sugar Maillard reaction system (Lee et al., 1984; Huang, 1988; Baisier and Labuza, 1992).

Higgins and Bunn (1981) demonstrated that the reversion rate constant of the Schiff base complex was greater than those of its formation and rearrangement in a protein−sugar Maillard reaction system. The reversion of the Schiff base complex cannot be ignored at least in the protein−sugar Maillard reaction system. Therefore, it is very important to clarify if the reversion of the Schiff base complex is a kinetically significant step in the amino acid−sugar model Maillard reaction system.

Lee et al. (1979) found that the Amadori compound could reach its maximum concentration before an appreciable amount of brown color developed in a tryptophan−glucose model system. According to their results, it is possible to terminate the Maillard reaction at the very early stages by freezing the reaction mixture before the Amadori compound converts to the advanced products. At this stage, the concentration of the Amadori compound may be high enough for an accurate determination but still too low to initiate a conceivable amount of conversion to the advanced products. With the concentration data of the Amadori compound, the kinetic constants can be calculated, and thus the rate-limiting step in the early stages of the Maillard reactions can be determined.

Phenylalanine−glucose is an ideal model system for the kinetic study of the Maillard reaction, because both phenylalanine and its Amadori compound can be detected by UV absorption at 260 nm. In addition, a high-resolution HPLC method has been developed in our laboratory for the separation and determination of fructosylphenylalanine and phenylalanine simultaneously in the reaction mixture of phenylalanine and glucose (Ge and Lee, 1996).

The objective of the present study was to elucidate the kinetic significance of the Schiff base reversion in the early stages of the Maillard reaction in a phenylalanine−glucose aqueous model system.

THEORETICAL MODEL

Development of a Kinetic Model. For the formation of the Amadori compound, a simplified kinetically based early-stage scheme of the Maillard reaction is represented as

$$A + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [AS]_{sb}^* \xrightarrow{k_2} AR \qquad (1)$$

where A is amino acid, S is sugar, $[AS]_{sb}^*$ is the Schiff base complex intermediate, and AR is the Amadori compound.

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Kinetic constants for this type of scheme cannot be calculated directly by conventional methods because the concentration of the intermediate complex such as $[AS]_{sb}$* is not easy to determine (Beringer and Gindler, 1955). One possible way to simplify this problem is to confine the reaction in its early stages. We assume that at the early stages of the Maillard reaction (1) the decomposition of amino acid through the Strecker degradation and of sugar through caramelization will be negligible and (2) the conversion of the Amadori compound to the advanced Maillard reaction products and its reversion to its parent compound or compounds will also be negligible at a low concentration.

Therefore, the Maillard reaction shown in the eq 1 is the only major reaction taking place in the reaction system. Because both phenylalanine and fructosylphenylalanine can be determined using our HPLC method, it is possible to determine the kinetic constants using the samples of different initial concentrations.

The rate equations of the early-stage Maillard reaction can be represented as follows:

$$-\mathrm{d[A]}/\mathrm{d}t = k_1[\mathrm{A}][\mathrm{S}] - k_{-1}[\mathrm{AS}] \quad (2)$$

$$\mathrm{d[AS]}/\mathrm{d}t = k_1[\mathrm{A}][\mathrm{S}] - (k_{-1} + k_2)[\mathrm{AS}] \quad (3)$$

$$\mathrm{d[AR]}/\mathrm{d}t = k_2[\mathrm{AS}] \quad (4)$$

The equilibrium constant K among the Schiff base complex intermediate $[AS]_{sb}$*, amino acid, and glucose can be represented by eq 5

$$K = [\mathrm{AS}]/([\mathrm{A}][\mathrm{S}]) \quad (5)$$

$$[\mathrm{AS}] = [\mathrm{A}]_0 - [\mathrm{A}] - [\mathrm{AR}] \quad (6)$$

where $[A]_0$ is the initial concentration of the amino acid.

If the initial concentrations of amino acid and sugar are the same, then eq 5 can be changed into eq 7.

$$K = \frac{[\mathrm{A}]_0 - [\mathrm{A}] - [\mathrm{AR}]}{[\mathrm{A}][\mathrm{A}]} \quad (7)$$

From the eqs 4 and 5, the following equation can be obtained.

$$\mathrm{d[AR]}/\mathrm{d}t = k_2[\mathrm{AS}] = k_2K[\mathrm{A}][\mathrm{S}]$$

Since [A] = [S], the above equation can be converted into

$$\mathrm{d[AR]}/\mathrm{d}t = k_2K[\mathrm{A}]^2 \quad (8)$$

From the eqs 2 and 6, we can get eq 9.

$$-\mathrm{d[A]}/\mathrm{d}t = k_1[\mathrm{A}][\mathrm{S}] - k_{-1}([\mathrm{A}]_0 - [\mathrm{A}] - [\mathrm{AR}])$$

$$-\mathrm{d[A]}/\mathrm{d}t = Kk_{-1}[\mathrm{A}]^2 - k_{-1}([\mathrm{A}]_0 - [\mathrm{A}] - [\mathrm{AR}]) \quad (9)$$

Determination of the Kinetic Constants. For a given temperature and pH, the equilibrium constant K value should not vary with the change of the concentrations of reactants. The K value can be calculated from eq 7, and it should be equal to the average K value of different initial reactant concentrations.

The k_2 value can be calculated from eq 8 with the plot of $\mathrm{d[AR]}/\mathrm{d}t$ against $[\mathrm{A}]^2$. The slope of the line equals the value of k_2K.

The k_{-1} value can be calculated from eq 9 with the plot of $\mathrm{d[A]}/\mathrm{d}t$ against $K[\mathrm{A}]^2 - ([\mathrm{A}]_0 - [\mathrm{A}] - [\mathrm{AR}])$ of the different initial phenylalanine and glucose concentrations at the early stage of the reaction.

The k_1 value can be calculated from the K and k_{-1} values.

MATERIALS AND METHODS

D-(+)-Glucose, sodium metabisulfite, ammonium hydroxide (29.1%), and sodium acetate were ACS analytical grade reagents from Sigma Chemical Co., St. Louis, MO. L-Phenylalanine was a SigmaUltra reagent. Amberlite CG120 (200−400 mesh), Dowex-50W (200−400 mesh), and Amberlite I-6766 (100−200 mesh), were also purchased from Sigma. Other chemicals used in this study were all ACS analytical grade reagents.

The model system was prepared by solubilizing equimolar ratios of glucose and phenylalanine, from 10 to 100 mmol, in a 0.05 M phosphate buffer, pH 7.0. A 15 mL scintillation vial was used as the reactor. Each vial was filled with 10 mL of reaction mixture and stored at −20 °C before use. The reaction was started when the temperature was raised to that of the water bath. The reaction was terminated by putting the vial into an ice−water bath and then transferring it to a −20 °C freezer for storage before compositional analysis.

The phenylalanine Amadori compound, fructosylphenylalanine, was synthesized according to a procedure modified from that of Hashiba (1976) previously developed in our laboratory (Ge and Lee, 1996).

The Maillard reaction mixture was analyzed using the HPLC method previously developed in our laboratory on a CarboPack PA-1 column, 4.6 × 250 mm (Dionex Corp., Sunnyvale, CA) (Ge and Lee, 1996). The HPLC system used for this study was a Dionex BioLC system (Dionex), which consisted of an advanced gradient pump, a UV detector, and a reagent storage module. The effective gradient program for the Maillard reaction mixture analysis was as follows: Elutant A (0.1 M ammonium hydroxide) was linearly decreased from 100% to 40%, whereas elutant B (0.1 M ammonium hydroxide−0.5 M sodium acetate) was linearly increased from 0% to 40% in 16.7 min at the same time. A good separation of the reaction mixture using the HPLC method was obtained, and the chromatography is shown in Figure 1. The retention times for phenylalanine and fructosylphenylalanine were 11.73 and 13.5 min, respectively. The flow rate of the elutant was kept constant at 1 mL/min. Both phenylalanine and fructosylphenylalanine were detected using a UV detector at 260 nm. The data were acquired and recorded by a Waters chromatography workstation Maxima 820 (Waters Dynamic Solutions, Ventura, CA). A calibration curve was used for determination.

Statistical analysis of the data was done using linear regression of the appropriate rate function listed in the above section to determine their rate constants.

RESULTS AND DISCUSSION

Test for the Validation of the Assumption. As stated above, we assumed the reaction shown in eq 1 was the only major reaction taking place in the early stages of the Maillard reaction and that all other reactions were negligible. To determine the kinetic constants with the above theoretical model, two essential problems must be resolved. One is that the resolution of the HPLC method should be high enough for an accurate determination of phenylalanine and its Amadori compound fructosylphenylalanine simultaneously. As shown in Figure 1a, the HPLC method developed in our laboratory can separate phenylalanine and its Amadori compound fructosylphenylalanine from other compounds with a high resolution. The other is that the samples taken before the yellow-brown color development in the reaction system should match the requirement of the above theoretical model, which is at

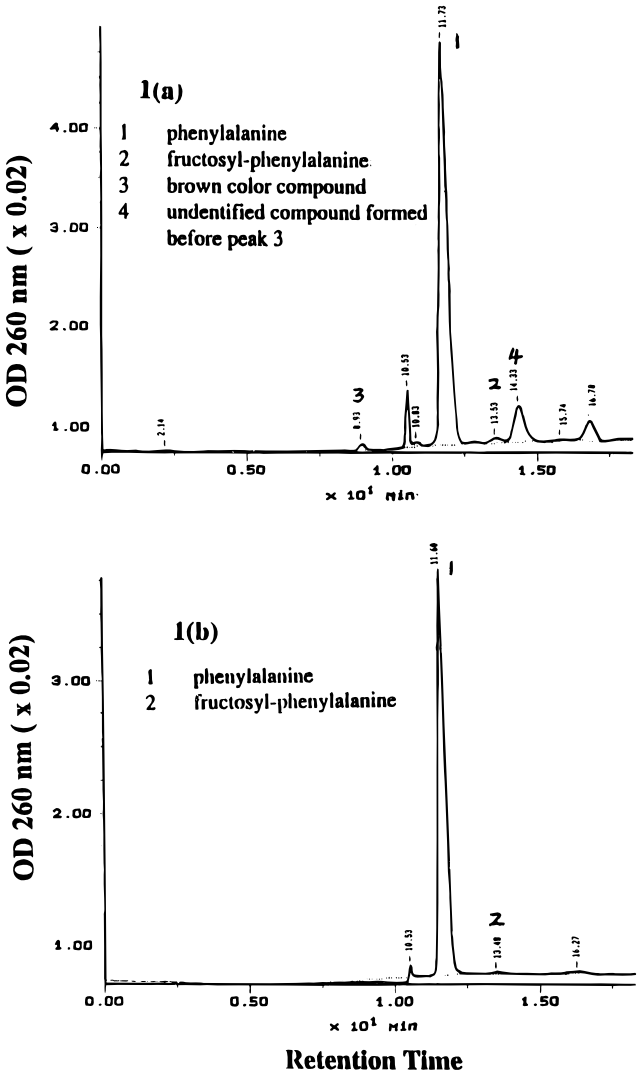

Figure 1. Typical HPLC chromatography of the Maillard reaction mixture of phenylalanine−glucose: (a) after development of the yellow-brown color; (b) before development of the yellow-brown color. Concentrations of phenylalanine and glucose were 0.05 M in 0.05 M phosphate buffer, pH 7.0. The samples were taken from the reaction mixture at the temperature of 76 °C for 24 h (a) and 8 h (b).

the point that the Amadori compound is high enough in concentration but still not significantly converted to the advanced Maillard reaction products. Figure 1b was a typical chromatography of the model reaction mixture before the yellow-brown color development. As shown in this figure, the Amadori compound can reach the concentration at which an accurate determination is feasible before converting to the advanced Maillard reaction products. This confirms that the assumption was reasonable and that the requirement of the theoretical model can be satisfied experimentally.

Kinetic Constants of the Early-Stage Reactions. In Figure 2, we have plotted d[AR]/dt against K[A]^2, on the basis of the experimental data and eq 8. The k_2 values and the regression correlation coefficients for each plot are listed in Table 1. It is indicated in Table 1 that the experimental data fit eq 8 very well because the correlation coefficients were much greater than the critical value 0.929 ($\alpha = 99\%$). Figure 3 shows the plots of −d[A]/dt against (K[A]^2 − [A]^0 + [A] + [AR]) based on the experimental data of the different initial phenylalanine and glucose concentrations and eq 9. As shown

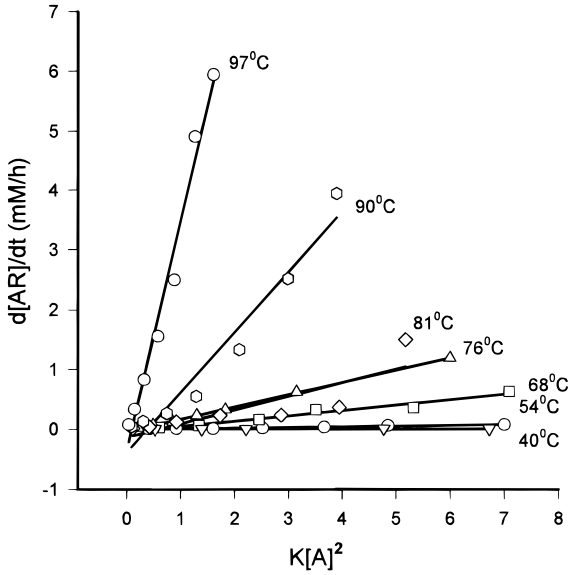

Figure 2. Linear regression plots of d[AR]/dt with K[A]^2. Concentrations of the phenylalanine and glucose were 0.05 M in 0.05 M phosphate buffer, pH 7.0. The reaction temperatures ranged from 40 to 97 °C.

Table 1. Linear Regression of the Experimental Data with Equation 8[a]

temp (°C)	k_2 (1/h)	R^{2b}	K
40	1.27×10^{-3}	0.9320	1.47
54	1.19×10^{-3}	0.9605	1.09
68	6.74×10^{-2}	0.9772	0.71
76	2.04×10^{-1}	0.9979	0.53
81	2.29×10^{-1}	0.9415	0.96
90	1.007	0.9755	0.85
97	3.97	0.9887	0.37

[a] Conditions: concentrations of phenylalanine and glucose were equal and in the range of 10−100 mM in 0.05 M phosphate buffer solution, pH 7.0. Triplicate samples were taken for each concentration, and at least seven different concentrations were tested for each temperature. [b] The critical value for Spearman rank correlation coefficient is $r_s = 0.929$ for $\alpha = 99\%$.

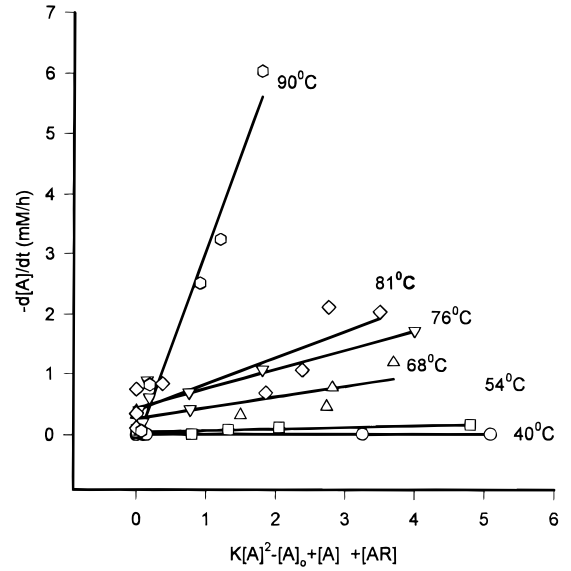

Figure 3. Linear regression plots of −d[A]/dt with K[A]^2 − [A]$_0$ − [A] − [AR]. The reaction conditions were the same as those of Figure 2.

in Table 2, the data also fit well to eq 9 because all of the correlation coefficient values were >0.714 ($\alpha = 90\%$).

It can be seen from the above results that the formation of the Schiff base complex intermediates

Table 2. Linear Regression of the Experimental Data with Equation 9[a]

temp (°C)	k_{-1} (1/h)	R^{2b}	k_1 (mM^{-1} h^{-1})
40	1.33×10^{-3}	0.8942	1.97×10^{-6}
54	2.40×10^{-2}	0.6046	2.63×10^{-5}
68	1.29×10^{-1}	0.7398	1.54×10^{-4}
76	3.26×10^{-1}	0.7172	1.73×10^{-4}
81	4.35×10^{-1}	0.7417	4.90×10^{-4}
90	1.26	0.7150	1.07×10^{-3}
97	9.42	0.9484	3.54×10^{-3}

[a] The experiment conditions were the same as those of Table 1. [b] The critical value for Spearman rank correlation coefficient is $r_s = 0.714$ for $\alpha = 90\%$.

follows second-order kinetics. However, formation of the Amadori compound follows first-order kinetics, as can be predicted from eq 1. Comparing the above results to those reported by Higgins and Bunn (1981), Lee et al. (1984), Huang (1988), and Baisier and Labuza (1992), we show that the Schiff base complex intermediate in the phenylalanine−glucose Maillard reaction system is very important, because its formation is the rate-limiting step in the early stages of the reaction. The reversion of the Schiff base was significant and thus cannot be ignored. This was demonstrated by the k values listed in Tables 1 and 2. The k_1 values were 10^3 times smaller than both the values of k_{-1} and k_2. The results were similar to those of the protein−sugar reaction system reported by Higgins and Bunn (1981) in the hemoglobin and glucose reaction system, where k_1 was 0.3×10^{-3} 1/mM·h and k_{-1} was 0.33 1/h for the glycosylation of the hemoglobin. Furthermore, the formation of the Schiff base complex intermediate followed second-order kinetics, as can be predicted from eq 1. This is logical because the loss of both amino acid and glucose in the Maillard reaction follows first-order kinetics as reported by Baisier and Labuza (1992). In addition, the Amadori rearrangement followed first-order kinetics if the reaction was considered to start from the Schiff base complex intermediate. The Amadori rearrangement reaction rate depends on both the concentrations of the parent compounds and the equilibrium constant as predicted in eq 8.

The conclusion derived from these results is different from the general notion that the Amadori rearrangement was the rate-limiting step at the early stages of the Maillard reaction. As stated above, the Schiff base was thought to have no kinetic significance in the Maillard reaction. However, no evidence has been reported to support the insignificant reversion of the Schiff base. We conclude that the control of the Maillard reaction should rely on controlling the formation of the Schiff base. This includes selecting the low reactive reactants and controlling the reaction condition that can inhibit the formation of the Schiff base.

Effects of Temperature on the Kinetic Constants. Temperature changes had a significant effect on the rate constants of the Maillard reaction. As shown in Figure 4, all kinetic constants increased with the rise in temperature. The activation energy (E_a) for each step or the direction of the reaction at the early stages is listed in Table 3. The E_a value was calculated on the basis of the linear regression of k values at different temperatures as shown in Figure 5. The E_a values in Table 3 explain that because the activation energies of the reactions are very high, as predicted by Labuza (1994), the Maillard reaction is slow at room temperature. Of all the steps in eq 1, the Amadori rearrangement reaction has the highest value of activation energy.

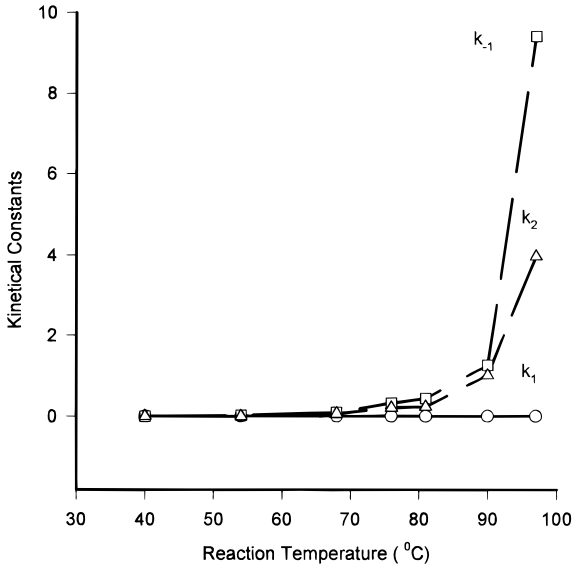

Figure 4. Effect of the reaction temperature on the kinetic constants k_1, k_{-1}, and k_2. The reaction conditions were the same as those of Figure 2.

Table 3. Activation Energy (E_a) of the Maillard Reactions

	reaction step		
	k_1	k_{-1}	k_2
E_a (kcal/mol)	6.52×10^4	7.49×10^4	8.01×10^4
R^{2a}	0.9908	0.9881	0.9685

[a] The critical value for Spearman rank correlation coefficient is $r_s = 0.929$ for $\alpha = 99\%$.

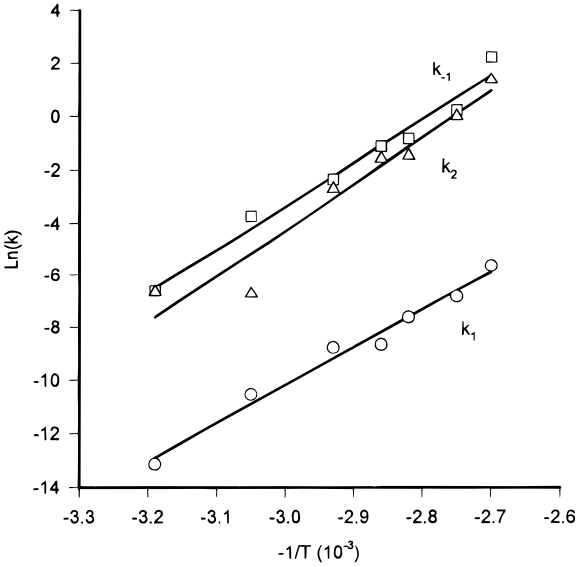

Figure 5. Linear regression plots of Ln(k) with $-1/T$. The reaction conditions were the same as those of Figure 2.

This means the Amadori arrangement is more sensitive to temperature changes than any other steps in the early stages of the reaction. Thus, high temperature is favorable to the formation of the Amadori compound, which leads to the formation of the brown coloring materials.

Effect of the pH Value on the Rate of Amadori Compound Formation. The rate of Amadori compound formation, as shown in Figure 6, increased significantly with the increase of pH. This is due to the nucleophilic nature of the Schiff base formation reaction. The active group of amino acid is amino group but not

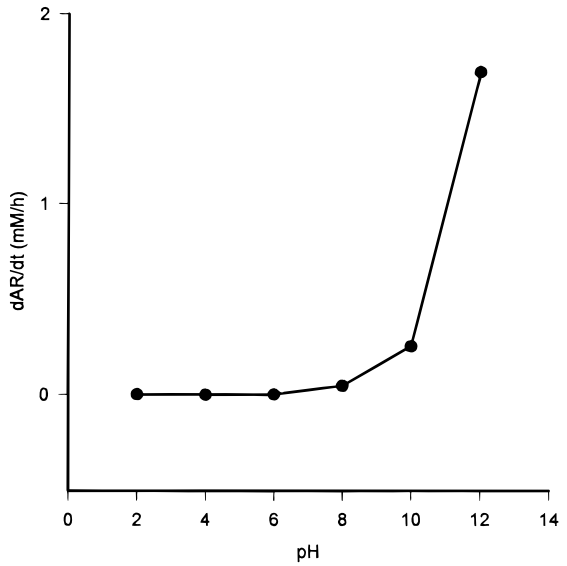

Figure 6. Effect of pH on the formation rate of the phenylalanine Amadori compound. Concentrations for phenylalanine and glucose were the same at 0.05 M. The reaction temperature was 80 °C. The pH buffer used was 0.01 N hydrochloric acid for pH 2.0, 0.05 M acetic buffer for pH 4.0, 0.05 M phosphate buffer for pH 6.0 and 8.0, 0.05 M boric buffer for pH 10, and 0.01 N sodium hydroxide for pH 12.

amine ion. Under the alkaline pH conditions, the proton is released from the amine ion, increasing the effective concentration of amino acid, which takes part in the reaction of the Schiff base formation. Increasing the pH value of the reaction system creates a favorable condition for the formation of the Schiff base complex, thus accelerating the formation of the Amadori compound. Because the formation of the Schiff base complex is the rate-determining step, any favorable conditions accelerating this step, such as the alkaline pH condition, could greatly increase the rate of the Maillard reaction overall. The Amadori rearrangement contains both an acid and an alkali accelerating step (Nursten, 1996). If the Amadori rearrangement were the rate-determining step, the alkaline pH condition could not have had such a great impact on the rate of the Maillard reaction. The results shown in Figure 6 provide further support of our conclusion that the Schiff base formation is the rate-determining step of the early-stage reaction.

These results reveal the significant kinetic importance of the Schiff base complex intermediate and its relationship with the Amadori compound formation. The formation of the Schiff base complex intermediate is a kinetic second-order reaction. The formation of the Amadori compound is a kinetic first-order reaction in the chain reactions of the Maillard reaction based on the significant existence of the Schiff base complex intermediates. Because the kinetic constant for the formation of the Schiff base complex (k_1) is much smaller than that of the Amadori rearrangement (k_2) and the reversion (k_{-1}) of the Schiff base complex, the rate-determining step for the Maillard reaction is the formation (k_1) of the Schiff base. The control of the Maillard reaction should be based on controlling the formation of the Schiff base, which is mainly determined by the molecular structures of reactants and some environmental conditions that affect the structures of the reactants such as pH.

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