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Maillard Browning Kinetics in a Liquid Model System

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Liquid model systems containing varying concentrations of glucose and glycine in a 0.1 M phosphate buffer at pH 7.0 were stored at 37 °C. Formation of brown pigments and fluorescent compounds as well as the loss of glucose and glycine was monitored over time. Glucose and glycine loss individually followed first-order kinetics and second order overall, as predicted mechanistically. Amino acid loss went into a no-loss period, as expected mechanistically from Bodenstein steady-state kinetics. The rate of browning and fluorescence formation followed pseudo-zero-order kinetics after an induction period and increased when either reactant concentration was increased with the other remaining constant up to a certain level.

INTRODUCTION

Despite the large amount of interest generated by the reaction, the kinetics of the Maillard browning reaction (NEB) are not well understood. Of considerable interest today is the influence of the Maillard reaction on in vivo aging reactions (Finot et al., 1990). A significant indication that glycation of human tissue proteins has occurred is the formation of fluorescence (Monnier and Baynes, 1989). Although fluorescence has been used to measure reaction intermediates that have biological significance (Bunn et al., 1981), there are no reports of the kinetics of fluorescence formation and its use in studying the Maillard reaction in foods is minimal. One objective of this study was to investigate the kinetics of fluorescence formation in liquid model systems.

The ratio of reducing sugar to amino acid has been suggested as being an important factor in determining the rate of Maillard browning and would have important implications both from a food formulation standpoint and in the case of in vivo glycation of proteins. O'Brian and Morrissey (1989) stated that "it is generally agreed that an excess of reducing sugar over amino compound promotes the rate of Maillard browning", the explanation being that there are "mechanistic differences" in the destruction of the sugar compared to the amino acid. They, however, did not clarify what they meant by this. Lerici et al. (1990) stated in their introduction that the "velocity" of the Maillard reaction depends on the different characteristics of the sugars and amino acids and on the different ratios between them.

Two studies that reported results concerning the effect of the sugar:amino acid ratio are that of Wolfrom et al. (1974) and Warmbier et al. (1976). The former found that the browning rate increased with increasing glycine to glucose ratios in the range 0.1:1 to 5:1 in a model orange juice system at 65 °C. The Warmbier et al. (1976) study of an intermediate moisture model system at a water activity of 0.52 at 45 °C showed that the rate of browning increased as the molar ratio of glucose:lysine (as present in casein) increased from 0.5:1 to 3.0:1 and then leveled out.

Lea and Hannan (1950) also observed a maximum in browning rate at a 3:1 ratio with a glucose/casein system held at 37 °C and 70% relative humidity for 30 days. As in the Warmbier et al. (1976) study, only the glucose was varied, with an 8:1 glucose to casein ratio being the maximum studied. It should be noted that in all three studies no statistical evaluation was made to determine if indeed the rates were significantly different at the different ratios.

Dworschak and Orsi (1977) studied a 0.2 M phosphate buffer model system that contained varying concentrations of glucose and methionine. The concentration ranges used were 0.01–1.0 M glucose and 0.02–0.2 M methionine. The samples were heated in closed ampules at 130 °C for 2 h. They concluded that the amount of brown pigment produced was highest when the glucose was in excess of methionine in the ratio 3:2. Three of the four studies discussed here determined that the rate of the Maillard reaction was fastest when the glucose was in excess, but they disagree at which ratio the maximum rate was achieved.

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The other objective of this research was to study the Maillard browning reaction at various glucose to glycine ratios and determine what effect, if any, the ratio of the initial reactants has on the reaction and the kinetic basis for this effect.

MATERIALS AND METHODS

A model system consisting of varying concentrations of D-glucose and L-glycine (Sigma Chemical Co., St. Louis, MO) was prepared in a 0.1 M, pH 7.0, phosphate buffer and stored at 37 °C. Sodium azide (Sigma) was added at 1% to prevent microbial growth. The concentrations of both glucose and glycine were varied from 0.05 to 0.4 M to give glucose:glycine ratios from 1:0.125 to 1:8.

Glucose loss was followed enzymatically using a glucose diagnostic kit (No. 315, Sigma). A quinonimine dye with an absorbance maximum at 505 nm is produced. The intensity of the color is proportional to the glucose concentration and is correlated by the use of a calibration curve.

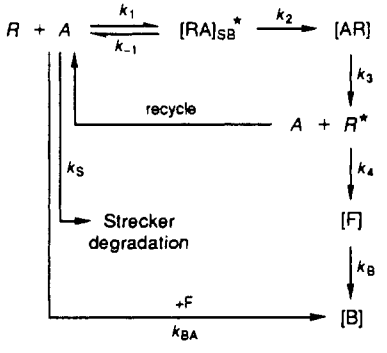
Glycine concentration was determined by using a modified version of the reversed-phase HPLC procedure developed by Peterson and Warthesen (1979), which was derived from the fluorodinitrobenzene (FDNB) method of Sanger (1945). The basic assumption is that glycine, rendered unavailable by the Maillard browning reaction, will not react with FDNB, while available amine groups will react and form the characteristic yellow color of dinitrophenylglycine (DNP-glycine). The HPLC of DNP-glycine was performed with a Spectra-Physics SP8810 precision isocratic pump at a flow rate of 1.0 mL/min equipped with an Applied Biosystems 757 absorbance detector set at 436 nm. The absorption unit was set at 0.02, while the filter rise time was set at 0.5 s. The data were recorded and electronically integrated by a Hewlett-Packard HP3396A recorder integrator. Separation of DNP-glycine from interfering compounds was accomplished by the use of a 15 cm × 3.9 mm Water Novapak C_{18} column (Millipore Corp., Milford, MA) with a mobile phase of 85% 0.01 M acetate buffer at pH 4.0 and 15% acetonitrile (Fisher Scientific HPLC grade). A calibration curve was used for quantitation.

The formation of intermediate stage products was monitored by the formation of fluorescent compounds. The samples were diluted 1:100 in 8% sodium bicarbonate before being analyzed in a Perkin-Elmer 650-10S fluorescence spectrophotometer utilizing emission and excitation wavelengths of 415 and 347 nm, respectively. A quinine sulfate solution (0.00055 g/L) was used daily as a standard for calibration of the instrument.

The formation of advanced Maillard products was monitored by measuring the absorbance of the solutions at 420 nm using a 1 cm cell.

Statistical analysis of the data was done by linear regression of the appropriate rate function to get the rate constants using the methods described by Labuza (1984). In this method, two systems show a statistically significant difference at >95% significance if the 95% confidence limits of the rate constants do not overlap. On this basis, they are different at >95% confidence.

Kinetics of Sugar:Amine Loss. A simplified kinetic-based browning reaction mechanism in foods can be represented as



where R is the reducing sugar concentration, A is the reactive amine concentration, RA is the Schiff base, AR is the Amadori rearrangement product, R^* is the reactive reducing compound intermediate which may react to form fluorescent pigment, F_i, and B_i is the brown pigment concentration. The loss of sugar or amine in the early stages can be represented by

$$\frac{-dR}{dt} = \frac{-dA}{dt} = k_1[A][R] \quad (1)$$

which is an overall second-order reaction and assumes that the back reaction (k_{-1}) is negligible and the amount of amine going into recycling, going into brown pigment, or reacting via the Strecker degradation reaction is small. This is a valid assumption since Higgins and Bunn (1981) found k_{-1} to be 3 times smaller than k_1. If indeed the rate depends on both reactants and we assume k_2 to be very fast, then the first step controls the overall reaction rate. Thus, the rate of formation of AR is also overall second order and has the same solution

$$\frac{dAR}{dt} = k_1[A][R] = k_1(A_0 - AR)(R_0 - AR) \quad (2)$$

where A_0 and R_0 represent the initial concentrations of the reactants. If the initial concentrations of the reactants are equal (C_0) and k_{-1} is small, then

$$\frac{-dR}{dt} = \frac{-dA}{dt} = \frac{d[AR]}{dt} = k_1[C_0 - AR]^2 = k_1A^2 = k_1R^2 \quad (3)$$

Integrating for the sugar or the amine concentration with time gives

$$\frac{1}{R} - \frac{1}{R_0} = k_1t = \frac{1}{A} - \frac{1}{A_0} \quad (4)$$

Thus, a plot of $1/A$ or $1/R$ vs time is a straight line of slope k_1 [mol/(L^{-1} h^{-1})].

If neither concentration is the same, then the solution is

$$\frac{1}{[R_0 - A_0]} \ln \frac{A_0R}{AR_0} = k_1t \quad (5)$$

A plot of ln (A_0R/AR_0) vs time gives a straight line with a slope $k_1[R_0 - A_0]$. As shown, k_1 can be determined by using this second-order equation, since this accounts for the loss of both reactants simultaneously. Integrating eq 2 with [AR] as a function of time gives

$$\frac{1}{A_0R_0} \ln \frac{AR - A_0}{AR - R_0} - \frac{1}{A_0R_0} \ln \frac{A_0}{R_0} = k_1t \quad (6)$$

This solution, if the task of measuring [AR] can be accomplished, also gives k_1, when ln $(AR - A_0)/AR - R_0)$ is plotted vs time.

If either amine or reducing sugar is in excess to the other, then for sugar or amine loss, the rate constant can be combined with the initial concentration of the compound in excess, assuming the loss is small over the time of the experiment. Thus

$$k_R = k_1[A]_0 \quad \text{or} \quad k_A = k_1[R]_0 \quad (7)$$

From this, the initial rate of loss of sugar or amine is

$$\frac{-dR}{dt} = k_R[R] \quad \text{or} \quad \frac{-dA}{dt} = k_A[A] \quad (8)$$

which when integrated gives

$$\ln \frac{R}{R_0} = -k_Rt \quad \text{or} \quad \ln \frac{A}{A_0} = -k_At \quad (9)$$

Thus, a plot of the natural log of the fraction of sugar or amine remaining vs time during the initial period of the reaction gives a straight line with a slope of k_R or k_A, respectively. From this, k_1 can be found by simply dividing the observed slope by the initial concentration of the other reactant, again assuming that data at a loss of less than 50% are used, i.e., initial reaction rate kinetics.

RESULTS AND DISCUSSION

Amino acid loss in the NEB reaction, although more complicated than sugar loss, has been found to follow

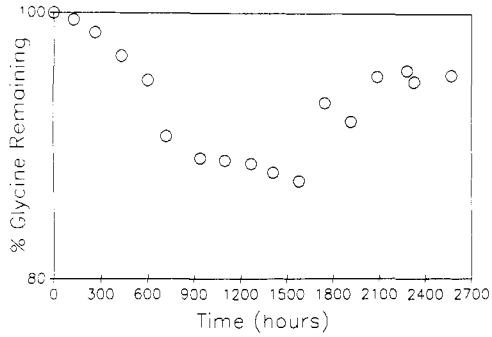


Figure 1. Semilog plot of percent amino acid vs time showing initial first-order loss of glycine followed by the regeneration of glycine and a no-loss period (pH 7, 37 °C, 0.05 M glucose with 0.4 M glycine).

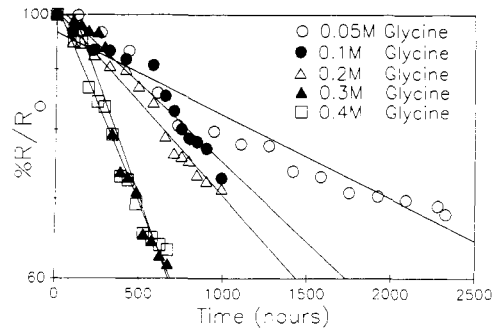


Figure 2. First-order loss of glucose with an initial concentration of 0.4 M glucose and varying concentrations of glycine at pH 7 and 37 °C. Slope is k_R (h^{-1}).

pseudo-first-order kinetics initially, as predicted by eq 9 (Warmbier et al., 1976; Massaro and Labuza, 1990). After the initial first-order period, however, the loss rate tapers off into a no-loss period, which can be explained by application of steady-state kinetics (Massaro and Labuza, 1990). Wolf et al. (1977) were the first to speculate on a no-loss period, while Massaro and Labuza (1990) were the first to show a first-order period followed by a no-loss period and work out the mathematics. Most other published studies have not collected enough data to quantitatively establish that the no-loss period occurred. An example of the amino acid concentration vs time for the 0.05 M Glu/0.4 M Gly sample of the present study is shown in Figure 1. To determine the reaction rate constant, k_1, only the initial first-order part of the data is used, neglecting the points after the concentration begins to increase.

In most cases in the present study, the no-loss period occurred when about 20–28% of the glycine was lost. This compares to 23% for lysine, 26% for cysteine, and 26% for tryptophan reported by Massaro and Labuza (1990) in model TPN solutions. Of critical importance here is that if the amino acid loss is followed in NEB studies, one needs to collect enough data points to show both the initial rate period and the no-loss period. In addition, the initial data cannot be used to project final losses during storage.

Figure 2 shows a semilog plot of the glucose loss data in the present study for samples with 0.4 M glucose and varying concentrations of glycine from 1:1 to 8:1. As can be seen, the slope, which is proportional to k_R, increases as the glycine concentration increases, as would be expected from eq 7. Table I lists the calculated reaction rate constants (k_R) for glucose loss for the samples shown in Figure 2. As seen, there is a statistically significant increase in the observed reaction rate constant as the amount of amino acid was increased at constant reducing sugar concentration, except that there was no difference between

Table I. First-Order Rate Constant (k_R) for Glucose Loss in a System at 37 °C and pH 7 Containing 0.4 M Glucose and Varying Glycine Concentrations[a]

sample	ratio (Glu:Gly)	k_R, $h^{-1} \times 10^4$	R^2	upper 95% limit, $\times 10^4$	lower 95% limit, $\times 10^4$
0.4/0.05	8	1.62[a]	0.93	1.87	1.36
0.4/0.1	4	3.64[b]	0.93	4.25	3.03
0.4/0.2	2	3.62[b]	0.97	4.03	3.21
0.4/0.3	1.3	8.97[c]	0.92	10.63	7.32
0.4/0.4	1	7.68[c]	0.98	8.44	6.91

[a] Superscripts of the same letter denote no statistical difference at >95%.

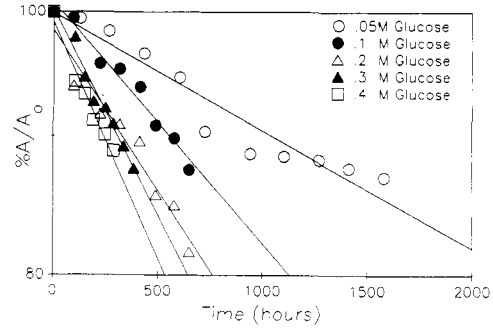


Figure 3. First-order loss of glycine with an initial concentration of 0.4 M glycine and varying concentrations of glucose at pH 7 and 37 °C. Slope is k_A (h^{-1}).

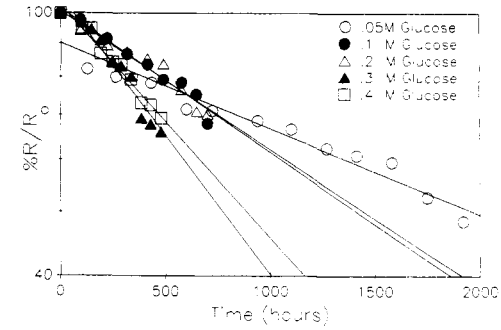


Figure 4. First-order loss of glucose with an initial concentration of 0.4 M glycine and varying concentrations of glucose at pH 7 and 37 °C. Slope is k_R (h^{-1}).

the rate constants at 0.1 and 0.2 M glycine (4:1 vs 2:1) and no difference between 0.3 and 0.4 M glycine. Apparently, because of other reactions occurring, the error introduced in assuming a first-order reaction is large enough that one cannot determine whether the theoretical equation holds over concentration differences of about 2-fold.

Equation 7 demonstrates that the rate constant for amino acid loss, k_A, is dependent on the initial reducing sugar concentration, R_0. Therefore, it would be expected that as the initial glucose concentration increases, the reaction rate constant for glycine loss, as represented by the slope, would do likewise. This effect is seen in Figure 3 with the corresponding rate constants, 95% confidence limits, and R^2 values listed in Table II. As with the results in Table I for constant glucose, three levels of significance are found in Table II. The sample with 0.05 M glucose is significantly different from that with 0.1 M glucose, while the sample with 0.1 M glucose is not different from that with 0.2 M glucose but is significantly different from the samples with 0.05, 0.3, and 0.4 M glucose. The rate constants should theoretically all be different, but due to the error introduced by competing reactions, such as Strecker degradation, the results deviate from strict mathematical kinetics.

Equation 7 shows not only that k_R depends on the initial glycine concentration but also that it should be indepen-

Table II. First-Order Rate Constant (k_A) for Glycine Loss in a System at 37 °C and pH 7 Containing 0.4 M Glycine and Varying Concentrations of Glucose[a]

sample	ratio (Glu:Gly)	k_A, $h^{-1} \times 10^4$	R^2	upper 95% limit, $\times 10^4$	lower 95% limit, $\times 10^4$
0.05/0.4	0.125	1.00[a]	0.93	1.21	0.78
0.1/0.4	0.25	2.01[b]	0.97	2.34	1.68
0.2/0.4	0.5	3.23[bc]	0.88	4.29	2.18
0.3/0.4	0.75	4.71[c]	0.91	5.90	3.52
0.4/0.4	1	8.26[c]	0.80	12.35	4.17

[a] Superscripts of the same letter denote no statistical difference at >95%.

Table III. First-Order Rate Constant (k_R) for Glucose Loss in a System at 37 °C and pH 7 Containing 0.4 M Glycine and Varying Glucose Concentrations[a]

sample	ratio (Glu:Gly)	k_R, $h^{-1} \times 10^4$	R^2	upper 95% limit, $\times 10^4$	lower 95% limit, $\times 10^4$
0.05/0.4	0.125	3.97[a]	0.97	4.75	3.18
0.1/0.4	0.25	5.50[b]	0.98	6.02	4.99
0.2/0.4	0.5	5.44[b]	0.97	6.09	4.79
0.3/0.4	0.75	9.55[c]	0.97	10.60	8.50
0.4/0.4	1	7.68[c]	0.98	8.44	6.91

[a] Superscripts of the same letter denote no statistical difference at >95%.

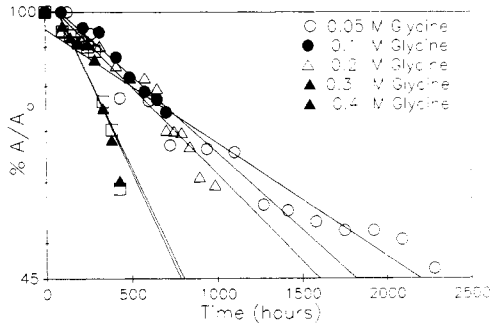


Figure 5. First-order loss of glycine with an initial concentration of 0.4 M glucose and varying concentrations of glycine. Slope is k_A (h^{-1}).

dent of the initial glucose concentration. In other words, increasing the initial glucose concentration should have no effect on the glucose loss rate constant. Figure 4 is a semilog plot of glucose loss in samples with an initial glycine concentration of 0.4 M and varying glucose concentrations. Theory would dictate that the slope of all of the systems tested would be equal. This is the case for the systems with concentrations of up to 0.2 M, but the calculated results in Table III for 0.4 M glycine show considerable deviation. The high R^2 values would indicate that the data are good and that the deviation must be related to some side reactions as a result of the higher concentrations of reactants or a possible water activity effect as the glucose concentration increases, which would increase the rate.

The same theory would apply to the rate of loss of amino acid being unaffected by the initial amino acid concentration. Figure 5 is a semilog plot of glycine loss for samples with 0.4 M glucose and varying initial concentrations of glycine. Equation 7 shows that, in theory, the rate constants should all be the same and therefore the slopes should all be equal. Table IV lists the rate constants, confidence limits, and R^2 values for the samples shown in Figure 5. With the exception of the sample containing 0.4 M glucose and 0.05 M glycine, all of the samples have equivalent slopes at the 95% level, since at or above 0.1 M glycine there is no statistically significant difference in the rate constants.

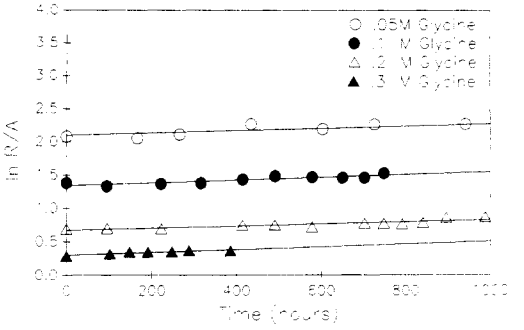


Figure 6. Second-order loss plot of glucose and glycine with an initial glucose concentration of 0.4 M and pH 7 and 37 °C. Slope is k_1 (h^{-1} M^{-1}).

Table IV. First-Order Rate Constant (k_A) for Glycine Loss in a System at 37 °C and pH 7 Containing 0.4 M Glucose and Varying Glycine Concentrations[a]

sample	ratio (Glu:Gly)	k_A, $h^{-1} \times 10^4$	R^2	upper 95% limit, $\times 10^4$	lower 95% limit, $\times 10^4$
0.4/0.05	8	3.40[a]	0.94	3.93	2.87
0.4/0.1	4	4.61[ab]	0.96	5.48	3.74
0.4/0.2	2	5.22[b]	0.94	6.05	4.38
0.4/0.3	1.3	10.47[b]	0.76	15.72	5.22
0.4/0.4	1	8.26[b]	0.80	12.35	4.17

[a] Superscripts of the same letter denote no statistical difference at >95%.

Table V. Comparison of Values for k_1 Calculated from the First-Order Loss of Glucose and Glycine and the Second-Order Loss of Both ($h^{-1} \times 10^4$)

sample	k_1, glucose loss	k_1, glycine loss	k_1, second order
0.05/0.1	25.9	4.6	25.2
0.05/0.2	15.2	3.5	22.8
0.05/0.4	9.9	2.5	3.7
0.1/0.05	27.6	3.6	65.5
0.1/0.1	22.0	18.7	29.2
0.1/0.2	14.1	5.9	16.6
0.1/0.4	13.8	5.0	8.0
0.2/0.05	29.8	25.0	9.1
0.2/0.1	16.3	11.5	4.9
0.2/0.2	19.1	11.2	19.6
0.2/0.4	13.6	8.1	4.6
0.3/0.3	25.0	17.6	42.8
0.3/0.4	23.9	11.8	44.4
0.4/0.05	32.4	68.0	4.8
0.4/0.1	36.4	46.1	7.0
0.4/0.2	18.1	26.1	5.8
0.4/0.3	29.9	34.9	21.2
0.4/0.4	19.2	20.7	24.6
av	21.8 ± 3.7	18.0 ± 8.6	20.0 ± 8.5

As discussed previously, if second-order plots of the results are made, as in Figure 6, k_1 can be calculated from the slope. Although the slopes look similar, when the values for k_1 are calculated (listed in Table V), there are significant differences. Table V shows the values of k_1 calculated by the three methods described for all of the systems. The range of values found is due to routine errors in the analytical procedure and the assumptions that were made to perform the calculations; e.g., to calculate k_1 from the first-order loss, the assumption had to be made that the other reactant was in such excess that its concentration does not change. The second-order calculation takes this loss into account and should theoretically be more correct; however, since the amino acid is reacting in other pathways, k_1 varies from 3.7×10^{-4} to 65×10^{-4} M^{-1} h^{-1}, almost a 16-fold difference, which is quite large. The first-order loss rate constant for glucose shows the least amount of deviation between systems, as would be expected since glucose only participates in the first step of the reaction

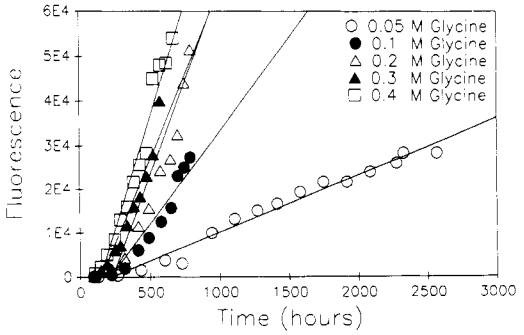


Figure 7. Pseudo-zero-order formation of fluorescence with an initial glucose concentration of 0.4 M with varying glycine concentrations at pH 7 and 37 °C.

Table VI. Zero-Order Rate Constant for Fluorescence Formation in a System Containing 0.4 M Glucose with Varying Concentrations of Glycine

sample	ratio (Glu:Gly)	k, fluor/h	R^2	upper 95% limit, $\times 10^4$	lower 95% limit, $\times 10^4$
0.4/0.05	8	13.43[a]	0.98	14.61	12.25
0.4/0.1	4	54.57[b]	0.97	64.93	44.21
0.4/0.2	2	102.24[c]	0.95	119.42	85.06
0.4/0.3	1.3	81.71[c]	0.95	93.82	69.60
0.4/0.4	1	100.79[c]	0.96	113.74	87.84

and does not recycle. In any case, the average values of k_1 for all systems, calculated by the three different methods, are not statistically different from each other at the 95% confidence level, which indicates the validity of the method of analysis but shows the problem in data interpretation if only a few concentrations are tested. The average value of k_1 for this system is about 20×10^{-4} M^{-1} h^{-1}, which is roughly 450 times less than the value calculated by Higgins and Bunn (1981) for the glycation of hemoglobin at 37 °C and pH 7.4. It would be expected that a reaction between glucose and an amino acid would be faster than with a protein, but it must be kept in mind that hemoglobin has multiple sites of glycation, whereas glycine can only react with glucose at one site. Also, since blood was used, there may be other compounds involved that are native to blood that contribute to the increased reaction rate.

Fluorescence and Sugar:Amine Ratio. Patton and Chism (1951) and Overby and Frost (1952) demonstrated that fluorescence formation showed an induction period followed by a period of increasing concentration until a maximum is reached, after which the concentration decreased. Figure 7 shows a pseudo-zero-order plot for the formation of fluorescence in systems with 0.4 M glucose and varying initial concentrations of glycine. As can be seen, the reaction rate constant increases as the glycine concentration increases, although the rate constant appears to level off above a glycine concentration of 0.2 M. Table VI lists the rate constants, confidence limits, and R^2 values for the systems shown in Figure 7. Increasing the glucose concentration at constant glycine levels has the same effect and is shown in Figure 8.

Figures 7 and 8 are almost identical and show that a maximum rate of fluorescence production is achieved after one reactant level is 0.4 M and the other is 0.2 M. This would imply that the ratio of the initial reactant concentrations plays a role in the reaction rate constants, as suggested by Wolfrom et al. (1974) and Warmbier et al. (1976), with a maximum rate of fluorescence formation when the ratio is 2:1 or more for either reactant. However, this is more complicated when one looks at the different absolute concentrations with the same ratio as shown in Figure 9. This graph shows the rate constants

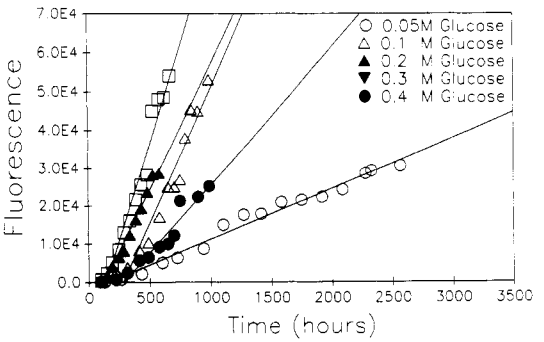


Figure 8. Pseudo-zero-order formation of fluorescence with an initial glycine concentration of 0.4 M with varying glucose concentrations at pH 7 and 37 °C.

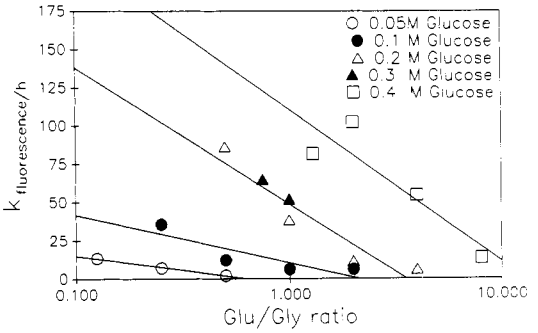


Figure 9. Pseudo-zero-order fluorescence formation at pH 7 and 37 °C as a function of the initial Glu:Gly ratio.

for fluorescence formation for all of the systems in the present study. There are four systems that have initial reactant ratios of 1, all of which have significantly different rate constants at the 95% level that range from 6.2 to 110.8 fluorescence units/h. Therefore, the formation of fluorescence is much more complex. Labuza and Baisier (1992) have derived the analytical function for fluorescence which takes the form

$$F = k_3 k_A [R_0] \left\{ \frac{e^{-k_A t}}{(k_3 - k_A)(k_B - k_A)} + \frac{e^{-k_3 t}}{(k_A - k_3)(k_B - k_3)} + \frac{e^{-k_B t}}{(k_A - k_B)(k_3 - k_B)} \right\} \quad (10)$$

Unfortunately, since there are probably many different fluorescent compounds formed, and we have no idea what their individual concentrations or molecular weights are, eq 10 cannot be tested analytically.

Browning and Sugar:Amine Ratio. Most of the literature has considered browning a pseudo-zero-order reaction where the amount of browning is plotted vs time and linear regression is used to get the rate constant (Warmbier et al., 1976; Labuza and Saltmarch, 1981).

Labuza and Baisier (1992) have also derived the analytical expression for brown pigment formation, which is

$$B = [R_0] + [B_0] + [F_0](1 - e^{-k_B t}) - k_3 k_A k_B [R_0] \left\{ \frac{e^{-k_3 t}}{(k_A - k_3)(k_B - k_3)} + \frac{e^{-k_A t}}{k_A(k_3 - k_A)(k_B - k_A)} + \frac{e^{-k_B t}}{k_B(k_3 - k_B)(k_A - k_B)} \right\} \quad (11)$$

As with fluorescence, since we do not have molecular weights or distributions of the various pigments, this equation cannot be tested analytically. Thus, the simple zero-order approach is applicable until this information can be obtained.

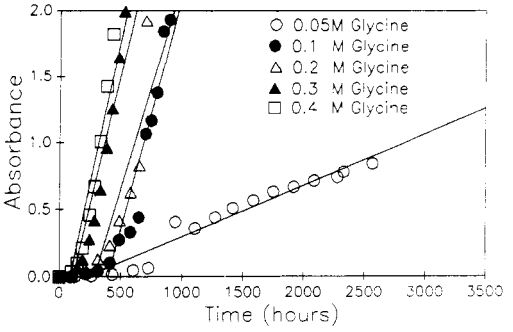


Figure 10. Pseudo-zero-order formation of brown pigment in a system with an initial glucose concentration of 0.4 M at pH 7 and 37 °C.

Table VII. Zero-Order Rate Constant (OD/h) for Pigment Formation in a System Containing 0.4 M Glucose with Varying Concentrations of Glycine

sample	ratio (Glu:Gly)	k, (OD/h) × 10^4	R^2	upper 95% limit, ×10^4	lower 95% limit, ×10^4
0.4/0.05	8	2.97[a]	0.97	3.33	2.61
0.4/0.1	4	46.34[b]	0.93	59.42	33.26
0.4/0.2	2	48.91[b]	0.75	101.08	-3.26
0.4/0.3	1.3	62.30[b]	0.99	70.56	52.04
0.4/0.4	1	67.72[b]	0.98	80.89	54.55

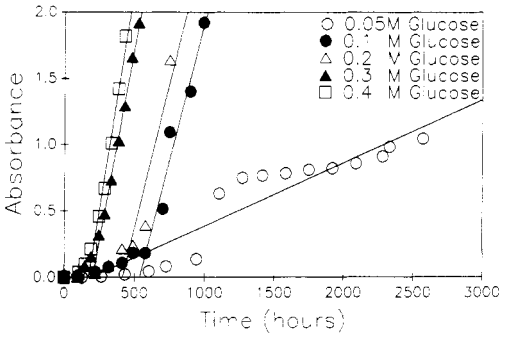


Figure 11. Pseudo-zero-order formation of brown pigment in a system with an initial glycine concentration of 0.4 M at pH 7 and 37 °C.

Figure 10 shows the formation of brown pigment at 420 nm for systems with 0.4 M glucose and varying initial concentrations of glycine. As seen, brown pigment formation has an induction period which varies in length depending on the experimental conditions. Figure 10 looks very similar to the plot of fluorescence formation in that after an induction period, a pseudo-zero-order period begins. The rate of reaction, as measured by the slope of this pseudo-zero-order plot, increases as the concentration of glycine increases and appears to level out above a 0.4 M glucose to 0.1 M glycine ratio. There is no significant difference in the rate constant between systems with 0.1, 0.2, 0.3, and 0.4 M glycine, as can be seen in Table VII. Figure 11 shows the formation of brown pigment for systems with a constant 0.4 M glycine concentration and varying concentrations of glucose. This figure is almost identical to Figure 10 for the samples with 0.4 M glucose, in that the 0.05 M glycine system is the only one that is significantly different from the others. Figure 12 shows the rate constants for brown pigment formation for all of the systems in this study. As in Figure 9 for fluorescence, one can see that at any constant glucose concentration, as the glucose:glycine ratio decreases, the reaction rate increases, as would be expected from the kinetics of the first step since more AR is produced. At a constant molar ratio, increasing the amount of both reactants increases the rate, as would be expected. In addition, one can use this figure to show that if the glycine level is kept constant

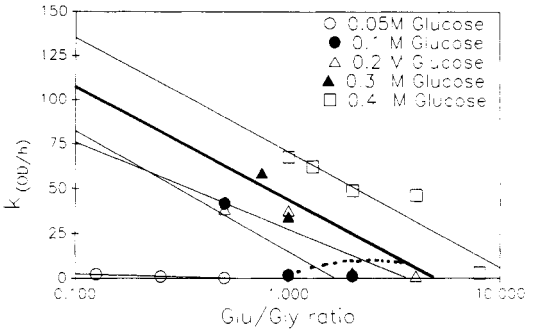


Figure 12. Pseudo-zero-order formation of brown pigment (OD/h) as a function of initial Glu:Gly ratio at pH 7 and 37 °C. Dashed line represents the browning rate for constant glycine at 0.05 M with increasing glucose levels.

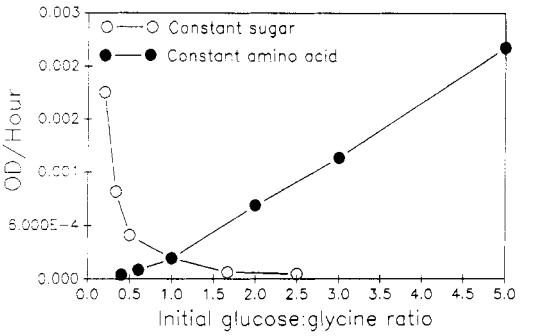


Figure 13. Pseudo-zero-order formation of brown pigment (OD/h) as a function of initial Glu:Gly ratio at pH 7 and 37 °C from Binder (1989).

and glucose increased (the curved dashed line), the browning rate increases slowly to a maximum. This is consistent with the findings of Warmbier et al. (1976) since they kept the casein constant and increased the glucose concentration and found a maximum above a 3:1 ratio.

Figure 13 is from the work of Binder (1989), who followed the procedures of the present study to determine the kinetics of glucose loss in two types of systems: one that held glucose at 0.1 M and varied glycine from 0.04 to 0.5 M and the other that held glycine at 0.1 M and varied glucose from 0.04 to 0.5 M. This study might explain the Wolfrom et al. (1974) results which were interpreted as a decrease in rate as the glucose:glycine ratio increased. Wolfrom et al. (1974) apparently varied the glycine concentration while keeping glucose constant and therefore made some incorrect conclusions regarding the kinetics of the NEB reaction. The mistake could have been identified earlier had it been more clearly stated in the publication whether one or either reactant was kept constant. This indicates the need for better reporting of reactant conditions in the literature.

Conclusions. Glucose and glycine loss individually follow first-order kinetics and second order overall, as predicted mechanistically. In the case of amino acid loss, however, the loss rate tapers off after the initial first-order period as described by Bodenstein steady-state kinetics. Increasing the amino acid concentration while keeping the reducing sugar concentration constant causes an increase in the rate constant for sugar loss. On the other hand, increasing the reducing sugar concentration while keeping the amino acid concentration constant increases the rate constant for amino acid loss.

Fluorescence and brown pigment formation exhibit an induction period and then follow zero-order kinetics. It was shown in this study that an increase in either initial glucose or glycine concentration caused either an increase or a decrease in the rate constant for brown pigment

formation depending on how the ratio is calculated on the basis of which component is kept constant. This also held true for the rate of formation of fluorescent compounds when either reactant concentration varied.

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