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The influence of Cu^{2+} in the Maillard reaction

Jacob A. Rendleman, Jr., and George E. Inglett
Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604 (U.S.A.)*

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ABSTRACT

Cupric ions augmented the rate of nonenzymic browning in D-glucose–glycine, glyoxal–glycine, and 5-(hydroxymethyl)-2-furaldehyde–glycine systems. Rates of formation of both melanoidin and precursors of melanoidin were influenced in the D-glucose–glycine system. In the pH range of 4–6, study of the D-glucose–glycine reaction was complicated by the tendency for cupric ion to undergo reduction to metallic copper in systems depleted of molecular oxygen. An investigation was made of the ability of cupric ion to form strong complexes with melanoidins. In general, two H^+ ions were released for each Cu^{2+} that became bound. The binding data suggested that at least half of the glycine moieties in the melanoidins contain a free carboxyl group.

INTRODUCTION

Nonenzymic browning reactions, in particular those caused by the Maillard reaction[1–3], have been of great interest to the food industry. In foods the reaction of reducing sugars with substances containing free amino groups produces not only compounds that contribute to aroma and flavor but also brown polymeric pigments (melanoidins). Considerable attention has been given to a variety of variables that can influence the production of pigments. Among those variables were: type of sugar, type of amino acid, type of buffer, concentration of reactants, pH, temperature, presence or absence of oxygen, and the amount of moisture in systems of low water-content. Comparatively few studies have been made of the effect of polyvalent metal cations on browning[4–17]. In studies of cationic influence, the results of one investigator would often conflict with those of another. Deschreider[4], working with dilute solutions of glucose and amino acids at pH 3 and 5.2, reported an inhibiting effect of Cu^{2+}. However, Markuze[5] later reported that Cu^{2+} accelerated browning in solutions of glucose and lysine buffered with phosphates at pH 6.5 and 7.0. A subsequent kinetic study at 50° by Wang, Bobbio, and Bobbio[9] indicated that Cu^{2+} accelerated browning in glucose–glycine systems at both pH 3 and pH 6.2 and that reaction rate increased with increase in copper concentration. Comparisons of extents of melanoidin formation (by absorbance measurements at 450 nm) at the end of 240-h reaction periods, indicated that enhance-

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ment of browning at pH 3 was much greater than that at the higher pH. At pH 3 in a buffer-free solution containing 100 p.p.m. of copper, the rate was 5 times faster than that in a similar system without copper. However, at an initial pH of 6.2, the rate was only 21% greater. No explanation was offered for the pronounced effect that pH had on the catalytic influence of copper.

Recent quantitative studies by Rendleman[18,19] of the complexation of calcium ion by both soluble and insoluble melanoidins indicated that the properties of pigments formed by the interaction of reducing sugar and amino acid are similar to those of acidic forms of anionic polymer. Interaction of Ca^{2+} with many melanoidins occurs with concurrent release of hydrogen ions to give moderately strong complexes.

The object of the present research was to reinvestigate the influence of Cu^{2+} on the browning reaction between D-glucose and glycine and to study *in vitro* complexation between Cu^{2+} and various model melanoidins prepared by reacting amino acids with reducing sugars, 5-(hydroxymethyl)-2-furaldehyde (HMF), and glyoxal. Complexations were conducted at or below pH 5 in order to preclude formation of highly insoluble copper hydroxide that might occur at a higher pH. Browning reactions, which were conducted without buffering and without any special pH control, were followed by u.v. and visible spectrophotometry and by fluorimetry. For comparative purposes, reaction conditions used by Wang, Bobbio, and Bobbio[9] for buffer-free systems were followed as closely as possible. The reason for our exclusion of buffers was to eliminate complications that might arise from copper–buffer interaction or catalysis[20] by buffer anions.

EXPERIMENTAL

General methods. — All chemicals were reagent grade. Water was distilled and deionized. Model melanoidins were prepared in aqueous solution under a nitrogen atmosphere in the dark by the interaction of carbohydrate with amino acid under conditions of temperature and pH that led either to soluble forms or to insoluble forms at pH 5. Because melanoidin composition varies significantly with initial molar ratio of reactants in the reaction mixture, this ratio has been applied as a prefix to names of melanoidins mentioned in this study. Except where specifically mentioned to be otherwise, all melanoidins were isolated as finely-divided, dark-brown powders. All products contained water of hydration, which varied with relative humidity. Preparation details of some of the melanoidins used in this study can be found in a previous publication[19]. Details for other melanoidins are in Table III.

Nitrogen analyses were by either the Kjeldahl method or a conventional microanalytical method. Both methods gave comparable results. Absorbance and fluorescence measurements were conducted at 25° in 1-cm cells. Quinine sulfate solution (0.1 μg per mL of 0.05M H_2SO_4) was the fluorescence reference standard; its intensity level was arbitrarily set at 6.10. Excitation and emission wavelengths were 350 and 430 nm, respectively. A Metrohm pH-stat was used for pH control and for monitoring the course of complexation, which often required several days to reach equilibrium. As H^+ ions were released by the melanoidin, the automatic titrator released an amount of

standard NaOH solution needed to maintain a constant pH of 5.00.

General procedure for kinetic studies. — Measured volumes of freshly prepared stock amino acid solution and freshly prepared carbohydrate solution were mixed to produce a mixture with the desired reactant concentrations. After the pH had been adjusted to the desired level by adding appropriate amounts of either HCl or KOH, standard 0.0787M $CuCl_2$ solution was then added. Aliquots (10 mL each) of the final solution were immediately placed in 20-mL Pyrex tubes equipped with Teflon-lined screw caps. The lower ends of the capped tubes were then immersed in a constant-temperature bath kept in a dimly illuminated room. At intervals of time, tubes were removed for absorption, fluorescence, and pH measurements. For fluorescence analysis, appropriate dilutions were made in order to minimize quenching. All fluorescence measurements were conducted at pH 3; relative intensity (r.i.) values were obtained at emission/excitation settings of either 430/350 nm or 480/390 nm.

General procedure for studying complexation of Cu^{2+} with melanoidins. — Into a thermostated (25°) Metrohm pH-stat titration vessel, equipped with stirrer, pH electrode, and burette (for automatic introduction of increments of 0.02M NaOH), was placed 100 mL of water whose pH was then adjusted to exactly 5.00 by means of dilute HCl. A weighed amount of melanoidin (60–100 mg) was added and the mixture was allowed to stir until the pH stabilized (usually ~15 min). Stock 0.0787M $CuCl_2$ (2 mL) was added to impart a Cu^{2+} concentration of 98 p.p.m. (1.54mM), causing a further drop in pH. When no further decrease in pH was noted, standard 0.02M NaOH was added to bring the pH back up to 5.0 and all vessel openings were then closed. The pH-stat mode of the instrument was put into operation; and additions of standard NaOH were automatically recorded as a function of time on a chart strip. No attempt was made to maintain a constant ionic strength. Establishment of reaction equilibrium was evidenced by no further uptake of NaOH by the system. An appropriate amount (usually 10 mL) of reaction mixture was then removed and centrifuged. The resulting clear supernatant was analyzed for total Cu concentration either by atomic absorption spectroscopy or by the convenient spectrophotometric method outlined next.

Spectrophotometric analysis of Cu^{2+} as a glycine complex. — The copper–glycine complex at pH 3 absorbs strongly in the u.v., the magnitude increasing with decreasing wavelength (see Fig. 1). Absorption in the u.v. range of 240–285 nm was found to obey Beer's Law over a wide range of Cu^{2+} concentration (at least as high as 220 p.p.m.) and the choice of wavelength was determined by a combination of such factors as the concentration of Cu^{2+} and the contribution of small amounts of dissolved melanoidin to the observed absorption at a chosen wavelength. A reasonably close estimate of the contribution of dissolved melanoidin at a chosen wavelength (λ_a) was obtained by the commonly known technique of making an absorption measurement at a wavelength (λ_b) where no significant absorption by the Cu–glycine complex occurs but where absorption by melanoidin occurs. The ratio of A_a/A_b found by separate measurements on a saturated, copper-free solution of melanoidin at pH 3, permitted a value to be obtained for the absorbance contribution of melanoidin at λ_a in the presence of Cu^{2+}. This contribution, and the contribution of glycine itself at pH 3, were subtracted from

the overall observed absorbance at the chosen wavelength λ_a. The general analytical procedure used in the present Cu-binding studies was to mix equal volumes of reaction mixture supernatant and 1.32M glycine (pH 3) and measure the absorbance of the resulting mixture at 240 nm. The absorbance at 240 nm for 0.66M glycine at pH 3 (usually 0.225) and the absorbance at 240 nm for trace amounts of dissolved melanoidin [= absorbance at 285 nm times the predetermined value for A_{240}/A_{285} for a glycine-free sample of supernatant mixed with an equal volume of dil. HCl (pH 3)] were subtracted from the absorbance at 240 nm for the 1:1 mixture of supernatant and 1.32M glycine (pH 3). From the net absorbance, the Cu^{2+} concentration in the 1:1 mixture was determined by referring to the chart in Fig. 1. Multiplying this concentration by 2 gave the concentration of Cu^{2+} in the original reaction mixture, whose volume was known from a knowledge of the quantities of water, stock $CuCl_2$ solution, and standard NaOH that were used.

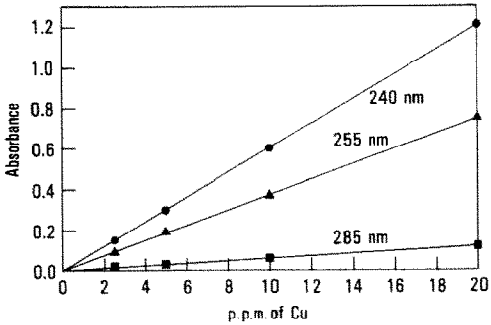

Fig. 1. Absorption of copper–glycine complex at pH 3.0. [Glycine] = 0.66M. Plotted value equals observed value minus value of 0.66M glycine blank.

RESULTS AND DISCUSSION

Reaction of D-*glucose with glycine at 50° and pH 3.00.* — Selected absorption and fluorescence data for the reaction between D-glucose (1.25M) and glycine (0.66M) at 0, 20 (0.315mM) and 100 p.p.m. (1.574mM) of Cu^{2+} are presented in graph form in Figs. 2–4. At pH 3 the presence of Cu^{2+} had a tremendous rate-augmenting effect that was evidenced by both visible and u.v. spectrophotometry and by fluorimetry. Even after 900 h, augmentation was apparent (not shown in Figs.). The results from absorption measurements at 450 nm (Fig. 1) agreed favorably with those of Wang, Bobbio, and Bobbio[9]; absorbance at the end of 240 h for the solution of 100 p.p.m. Cu^{2+} was 7 times greater than that for the solution containing no Cu^{2+}. Even at 20 p.p.m., Cu^{2+} augmentation was large (4-fold). Acidity of the reaction mixtures remained remarkably constant, never changing (decreasing) by more than 0.08 pH unit over a 2000-h period.

Finely divided, highly insoluble melanoidin began to form in all three reaction mixtures at the end of about 600 h. Up to this time, the solutions had gradually darkened

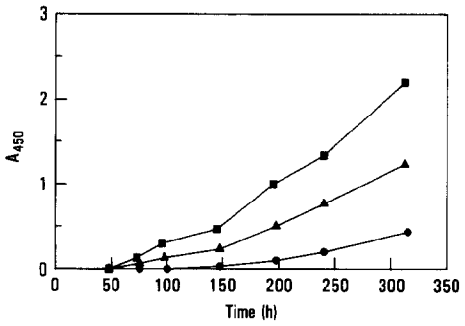

Fig. 2. Visible absorbance study of 2:1 D-glucose–glycine reaction at pH 3.0 and 50°. [D-Glucose] = 1.25M; [glycine] = 0.66M (100 p.p.m. Cu^{2+}, ■; 200 p.p.m. Cu^{2+}, ▲; no Cu^{2+}, ●).

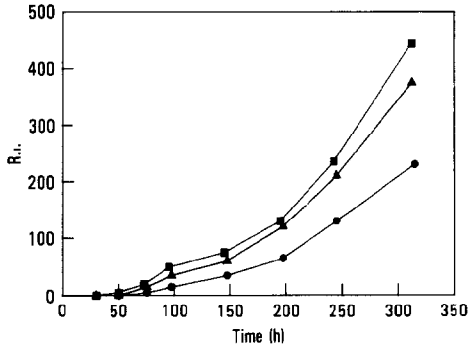

Fig. 3. Fluorescence study of 2:1 D-glucose–glycine reaction at pH 3.0 and 50°. Excitation and emission wavelengths are 350 and 430 nm, respectively. [D-Glucose] = 1.25M; [glycine] = 0.66M (100 p.p.m. Cu^{2+}, ■; 20 p.p.m. Cu^{2+}, ▲; no Cu^{2+}, ●).

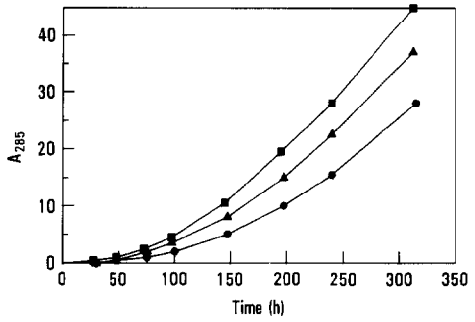

Fig. 4. U.v. absorbance study of 2:1 D-glucose–glycine reaction at pH 3.0 and 50°. [D-Glucose] = 1.25M; [glycine] = 0.66M (100 p.p.m. Cu^{2+}, ■; 20 p.p.m. Cu^{2+}, ▲; no Cu^{2+}, ●).

and remained clear and homogeneous. Yields of insoluble melanoidin increased with time and were greatest at highest Cu^{2+} concentration. After about 2000 h of reaction time, the dark-brown solids were removed, washed, and dried. The melanoidin isolated from the system containing 100 p.p.m. of copper was found to contain a considerable amount of bound Cu^{2+}, a fact that led to our initial discovery that this and other

melanoidins formed by the interaction of reducing sugars with amino acids have the properties of acidic forms of anionic polymer. Treatment of copper-free melanoidins with dilute $CuCl_2$ solution resulted in strong copper binding with concurrent release of H^+ ions by the melanoidin. Results of copper-binding studies will be presented and discussed later in this paper.

Increases in relative intensity of fluorescence (r.i.) and in absorbance at 450 nm are possibly associated mostly, if not entirely, with increases in concentration of soluble melanoidin. However, as is evident from comparing Figs. 2 and 3, visible spectrophotometry reveals a far greater rate difference between a 20 p.p.m. Cu^{2+} system and a 100 p.p.m. Cu^{2+} system than does fluorimetry. For this reason, fluorimetry is probably not the preferred means for quantitatively following the course of browning in systems containing Cu^{2+} and, perhaps, other polyvalent metal catalysts. Adhikari and Tappell[15] have reported that polyvalent cations have the potential for complexing with melanoidins in solution and that, by doing so, they can diminish r.i. Nevertheless, in systems *not* containing polyvalent cations, fluorimetry might be of assistance where visible spectrophotometry is of little or no value. For example in the 2:1 D-glucose–glycine system (pH 3, 50°) now under discussion, no observable absorbance at 450 nm occurred until after 90 h; at 100 h A_{450} was only 0.012. On the other hand, fluorimetric measurements showed a significant increase in r.i. (1.52) as early as 28 h after reaction had been initiated. After a total of 100 h, the r.i. increase was 15.0 [Note: r.i. at zero time (time of mixing) was 2.09, which was subtracted from all subsequent intensity readings].

Because aqueous solutions of 2:1 D-glucose–glycine melanoidin absorb strongly in the u.v. region (λ_{max} = 285 nm), the reaction between D-glucose and glycine was also monitored by absorption measurements at 285 nm. From the data presented in Fig. 4, it is obvious that Cu^{2+} had an augmenting effect on absorption. Part of this absorption was due to melanoidin itself. However, it must also be recognized that 5-(hydroxymethyl)-2-furaldehyde (HMF), a product of the Maillard reaction and a known precursor in the formation of melanoidin, also has an absorption maximum at 285 nm. Furthermore, there are possibly other precursors formed in the Maillard reaction that absorb at or near this same wavelength. Absorption at 285 nm is probably a composite of contributions from a variety of reaction mixture components — the melanoidin plus its precursors. These precursors, often referred to as premelanoidins, would be expected to accumulate to a measurable extent long before any measurable amount of melanoidin appears. Thus, it was highly probable that u.v. measurements at 285 nm in the very early stages of reaction (that is, during the first 2–3 days in our studies) might provide reasonably accurate reflections of premelanoidin concentration. During this early period, a rapid, easily measured rise in absorption at 285 nm did occur. At the same time, in the visible region at 450 nm, where only melanoidin would be expected to contribute significantly to absorption, there was no absorbance greater than 0.001, except in those systems where catalytic amounts of Cu^{2+} were present.

Table I contains selected absorbance data at various reaction times for the 2:1 D-glucose–glycine systems with Cu^{2+} (100 p.p.m.) and without Cu^{2+}. With this information it was possible to show that Cu^{2+} accelerates not only the formation of

melanoidin but also the formation of premelanoidin. In Expt. 3 where no Cu^{2+} was used, the absence of measurable absorbance at 450 nm (the value of 0.001 was within the expected experimental error of ± 0.001) was indicative of very little or no melanoidin. However, in this same experiment, absorption in the u.v. at 285 nm was large (A_{285} = 0.85). In Expt. 4, at the end of 148 h, the presence of melanoidin, although small, was evident (A_{450} = 0.043); the premelanoidin concentration appeared to have increased to a level that was 6 times greater than that at 65 h (Expt. 3). At the end of 296 h (Expt. 6), both the concentration of melanoidin and the concentration of premelanoidin had increased considerably. However, up to this time, the contribution of melanoidin to u.v. absorbance at 285 nm had been very small. Percent contributions by melanoidin to absorbance at this wavelength were calculated from knowledge of the ratio A_{285}/A_{450} for a saturated solution of "insoluble" 2:1 D-glucose–glycine melanoidin in water (pH 3) and are shown in the last column of Table I. Expts. 1 and 2 illustrate the enhancing effect that Cu^{2+} had upon the production of both melanoidin and premelanoidin. By the end of 40 h (Expt. 1), Cu^{2+} had accelerated the formation of melanoidin and led to an A_{450} of 0.043; the calculated contribution of melanoidin to absorbance at 285 nm was 13%. In the absence of Cu^{2+}, the contribution of melanoidin to absorbance at 285 nm during the first 65 h was no greater than 0.3% (see Expt. 3). Expts. 1 and 3 provided evidence that premelanoidin formation was likewise accelerated by Cu^{2+}. At the end of 40 h in the copper-containing system (Expt. 1), u.v. absorbance at 285 nm, caused primarily by premelanoidins, was 0.85. In the absence of Cu^{2+}, the time required to reach the same level of absorbance (0.85) was much greater, 65 h (Expt. 3). An additional illustration of the accelerating effect of Cu^{2+} upon premelanoidin formation is provided by comparing u.v. data for Expt. 2 with that for Expt. 4. A_{285} for each of these reaction mixtures was 5.00. However, in that system where copper was present (Expt. 2), the time required to reach this absorbance level was 68% less than that required by the copper-free system (Expt. 4).

Reaction of D-*glucose with glycine at 50° and initial pH 6.18–6.86.* — Selected absorption and fluorescence data for the reaction between D-glucose (1.25M) and glycine

TABLE I

Effect of Cu^{2+} on formation of D-glucose–glycine melanoidin and premelanoidin at pH 3 and 50°

Expt. no.	*Conditions*	*Reaction time (h)*	*Absorbance increase*		*Contribution of melanoidin to* A_{285} *(%)*[a]
			A_{450}	A_{285}	
1	100 p.p.m. Cu^{2+}	40	0.043	0.85	13
2	100 p.p.m. Cu^{2+}	100	0.30	5.00	15
3	No Cu^{2+}	65	0.001	0.85	0.3
4	No Cu^{2+}	148	0.043	5.00	2
5	No Cu^{2+}	280	0.30	22.00	3
6	No Cu^{2+}	296	0.35	24.9	4

[a] Based upon A_{285}/A_{450} = 2.53 for a saturated solution of 2:1 D-glucose–glycine melanoidin in water at pH 3 and 25°. The % contribution to A_{285} = (2.53 × A_{450}/A_{285}) × 100.

(0.66M) at 0, 20, and 100 p.p.m. Cu^{2+} are presented graphically in Figs. 5–8. The results of following both pH and absorbance at 450 nm for over 600 h are shown in Fig. 5. No precipitation of melanoidin occurred over the entire course of reaction. Unquestionably, reaction at pH 6.2 is faster than at pH 3.0, but because the first absorption measurement was not made until 25 h after commencement of reaction, we were initially misled by these data into thinking that Cu^{2+} had little or no catalytic influence on the Maillard reaction at pH 6.2. In fact, the small differences in absorbance between the three systems at the end of 240 h were nearly the same as the small differences found by Wang, Bobbio, and Bobbio[9] for their similar systems. The tendency for pH to decrease most rapidly in systems of greatest copper content (see Fig. 5) suggested, early in our investigations, that differences in pH between the three systems were probably the primary reason for the observed absence of pronounced catalytic effect. However plausible this explanation seemed at first, subsequent experiments at, or near, pH 6.2 soon revealed another factor that probably contributed much to the observed similarity in reaction rate; and proof was obtained that moderately strong catalysis by Cu^{2+} can indeed occur at this pH.

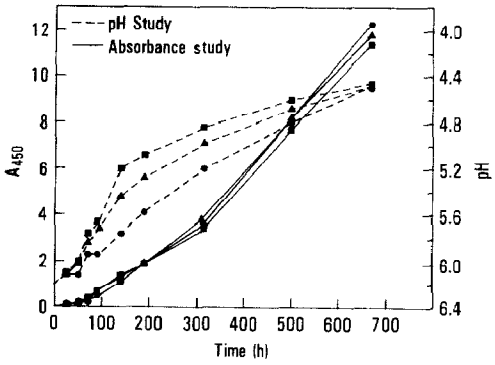

Fig. 5. pH and visible absorbance study of D-glucose–glycine reaction at $pH_{initial}$ 6.2 at 50°. [D-Glucose] = 1.25M; [glycine] = 0.66M (100 p.p.m. Cu^{2+}, ■; 20 p.p.m. Cu^{2+}, ▲; no Cu^{2+}, ●).

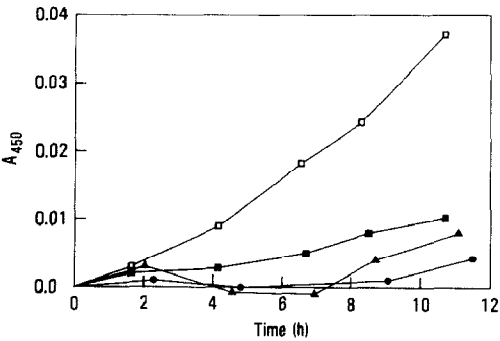

Fig. 6. Visible absorbance study of 2:1 D-glucose–glycine reaction at $pH_{initial}$ 6.18 and 6.86 at 50° during early stages of reaction. [D-Glucose] = 1.25M; [glycine] = 0.66M. (At pH 6.18: 100 p.p.m. Cu^{2+}, ■; 20 p.p.m. Cu^{2+}, ▲; no Cu^{2+}, ●. At pH 6.86: 100 p.p.m. Cu^{2+}, □).

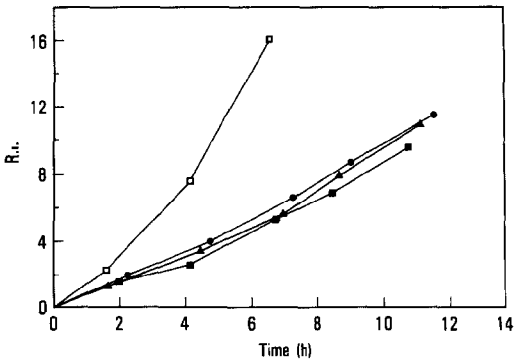

Fig. 7. Fluorescence study of 2:1 D-glucose–glycine reaction at $pH_{initial}$ 6.18 and 6.86 at 50° during early stages of reaction. [D-Glucose] = 1.25M; [glycine] = 0.66M. (At pH 6.18: 100 p.p.m. Cu^{2+}, ■; 20 p.p.m. Cu^{2+}, ▲; no Cu^{2+}, ●. At pH 6.86: 100 p.p.m. Cu^{2+}, □).

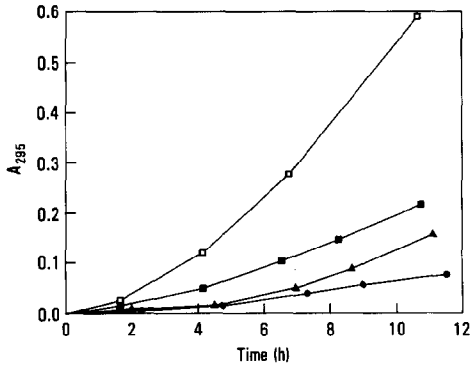

Fig. 8. U.v. absorbance study of 2:1 D-glucose–glycine reaction at $pH_{initial}$ 6.18 and 6.86 during early stages of reaction. [D-Glucose] = 1.25M; [glycine] = 0.66M. (At pH 6.18: 100 p.p.m. Cu^{2+}, ■; 20 p.p.m. Cu^{2+}, ▲; no Cu^{2+}, ●. At pH 6.86: 100 p.p.m. Cu^{2+}, □).

Within 24 h, after D-glucose–glycine reaction mixtures of pH 6.2 were placed in capped tubes and heated to 50°, a finely divided, powdery precipitate of reddish color was observed to form in those systems containing Cu^{2+}. Formation of this substance commenced after only 15 h in the mixture containing 100 p.p.m. Cu^{2+}. A sample of the red material was isolated, washed with water, dried under vacuum at 25°, and dissolved in dilute HNO_3 (in which it was easily soluble). Atomic absorption analysis on the nitric acid solution revealed that the unknown precipitate was 100% metallic copper (Cu^0). Further experimentation showed that precipitation of elemental copper in these systems can occur over the pH range of 4–6 and that exhaustion of O_2 inside the reaction tube is a prerequisite for its formation. Introduction of air or O_2 into a reaction mixture from which metallic copper had been precipitated led to a slow dissolution of the metal. We observed this same effect of O_2 on a few mg of a commercial sample of copper powder. However, it is important to note that the presence of glycine was essential for solubilization, and complete solution to give a clear blue solution of copper–glycine complex required at least one day of vigorous stirring.

The effect of oxygen on the solubility of copper metal in a glycine solution explained why 15 h or more had to elapse before precipitation of metal commenced in the sealed tubes in our studies of the D-glucose–glycine reaction. In the reaction mixture containing 100 p.p.m. of Cu^{2+}, the combined amounts of dissolved O_2 and gaseous O_2 present in the air space above the liquid surface was just sufficient to prevent metallic copper from forming during the first 15 h of reaction. During those 15 h, Cu^{2+} ions probably oxidized compounds that are known to have substantial reducing power (such as D-glucose and certain Maillard reaction products), and, in the process, would normally be reduced to the metallic Cu^0 state were it not for the presence of molecular oxygen which tended to restore reduced copper to the divalent form. The presence of glycine was a requirement for conversion of reduced copper to Cu^{2+}. After depletion of oxygen through the oxidation–reduction processes just mentioned, this conversion to the divalent state was no longer possible and metallic copper commenced to precipitate from solution. Precipitation was complete or nearly complete after 5 days of reaction time. Thus, the similarity in the appearance of the curves in Fig. 5 can be explained plausibly by two factors: (*1*) the rapid disappearance of Cu^{2+} from the two copper-containing systems, which left all three systems very similar in Cu^{2+} content, and (*2*) the greater acidity of those systems that initially contained the greater amounts of cupric ion.

With this knowledge of the behavior of Cu^{2+} in the presence and absence of air, the series of kinetic experiments with a 2:1 -glucose–glycine mixture at 0, 20, and 100 p.p.m. Cu^{2+} was repeated. This time all measurements were conducted over a period no longer than 12 h, in order to preclude errors caused by elimination of Cu^{2+} through conversion into metallic copper. The reaction conducted in the presence of 100 p.p.m. Cu^{2+} was carried out at both $pH_{initial}$ 6.18 and $pH_{initial}$ 6.86 in order to determine the effect of small pH changes on rate. At the end of the study, pH in those systems of $pH_{initial}$ 6.18 had fallen only to 6.13; pH in those of $pH_{initial}$ 6.86 fell to 6.76.

The visible spectrophotometric measurements at pH 6.18 (see Fig. 6), although difficult to make because of the extremely low levels of absorbance during the first 12 h of reaction, revealed an accelerating effect of Cu^{2+}. Not unexpectedly, fluorimetric data (Fig. 7) on the pH 6.18 systems gave curves that were somewhat erratic, yet very similar to each other. Furthermore, the copper-free solutions appeared to fluoresce more intensely than the corresponding solutions containing copper, probably because of possible complexation of Cu^{2+} with Maillard reaction intermediates (premelanoidins) and melanoidins, which might result in a reduction of the ability of these substrates to fluoresce. The u.v. absorbance curves (Fig. 8), constructed from data at 295 nm seem quite normal, and they adequately illustrate the augmenting effect of Cu^{2+}. All three instrumental methods of analysis gave results (Figs. 6–8) that illustrate the greatly enhancing effect that a small increase in pH can have on Maillard reaction kinetics.

Binding of Cu^{2+} by melanoidins. — All melanoidins prepared in this laboratory from the interaction of amino acids with carbohydrates (including glyoxal and HMF) had acidic properties. This acidity was found to be augmented by the presence of such divalent cations as Ca^{2+}, Zn^{2+}, and Cu^{2+}, which, presumably, effect the release of

protons through complexation of the metal with ionizable groups on the melanoidin. The subject of Ca^{2+}–melanoidin interaction has been treated in recent papers[18,19] where calcium was reported to effect the release of H^+ from a variety of soluble and insoluble melanoidins at neutral pH. The ease with which Cu^{2+} effects a similar release is illustrated by the interaction of Cu^{2+} with insoluble 2:1 D-glucose–glycine melanoidin at 25° and pH 5.00, as shown in Fig. 9. The reaction was initially very rapid, reaching 80% completion within 1 h. However, total reaction was not achieved until after 2 days. The important thing to note is that two H^+ ions were released for each Cu^{2+} ion bound by melanoidin. With possibly one exception, all of the binding studies revealed this 2:1 relationship (see Table II). The complexing ability of Cu^{2+} is so great that almost all binding sites of a melanoidin substrate are filled when the amount of Cu^{2+} in the system is present in only slight excess (compare Expts. 3 & 4 and Expts. 6 & 7, where it is shown that increasing the Cu^{2+} concentration results in little additional complexation).

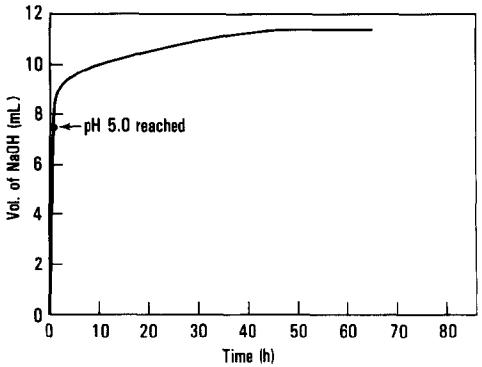

Fig. 9. Titration of insoluble 2:1 D-glucose–glycine melanoidin at pH 5.0 and 25° with 0.0213M NaOH in the presence of Cu^{2+}. Weight of melanoidin (6.14% N), 99.4 mg; initial solution volume, 102 mL.

In the last column of Table II are values for degree of metal binding calculated on the basis of melanoidin nitrogen content. The expression $Cu^{2+}/4N$, which signifies the number of Cu^{2+} ions bound per 4 nitrogen atoms in the complex, was used to draw attention to the possibility that binding is related to specific structural features of melanoidin. With the exception of the 1:2 D-glucose–glycine complexes in Expts. 3 and 4, the 1:2 D-glucose–glycine complexes contained 1.0 ($\pm$0.1) Cu^{2+} ion per group of 4 nitrogens. In this respect the 3:5 glyoxal–glycine complex and the 1:5 D-fructose–glycine complex belonged to the same category. The relatively high $Cu^{2+}/4N$ value of 1.6–1.7 for the HMF–glycine complex was unexpected. Perhaps a relationship of Cu^{2+} to a group of 2 or 3 nitrogens, where $Cu^{2+}/2N$ and $Cu^{2+}/3N$ would have values of 0.81 ($\pm$0.02) and 1.24 ($\pm$0.06), respectively, would be more reasonable.

The nature of the binding sites on melanoidins is not known. Most likely they are carboxyl groups, because of the strongly acidic properties possessed by these melanoidins. The acidity is much too strong to be due simply to enolic structures. If the binding sites can be shown to be free carboxyl groups, there is a possibility that these

TABLE II

Copper binding by melanoidins at pH 5.00 and 25°

Expt. no.	*Melanoidin*[a]	*% N in melanoidin*	*Weight of melanoidin (mg)*	*Analytical data*					
				Initial volume of reaction mixture (mL)	*Initial Cu (mmol)*	*Bound Cu (mmol)*	*(%)*	*H^+ released*[b] *(mmol)*	*$Cu^{2+}/4N$*[c]
		Insoluble melanoidins							
1	4:1 Glc–Gly	5.16	97.0	102	0.157	0.097	62	0.191	1.09
2	2:1 Glc–Gly[d]	6.14	99.4	102	0.161	0.108	67	0.243	1.02
3	1:2 Glc–Gly	6.50	62.3	102	0.157	0.094	60	0.188	1.30
4	1:2 Glc–Gly	6.50	70.6	102	0.472	0.110	23	0.204	1.37
5	1:5 Glc–Gly	6.93	74.3	102	0.157	0.091	58	0.174	0.99
6	1:11 HMF–Gly	6.97	61.8	102	0.157	0.121	77	0.247	1.57
7	1:11 HMF–Gly	6.97	69.8	100	0.472	0.150	32	0.300	1.72
8	1:5 Fru–Gly	7.10	73.5	102	0.157	0.100	64	0.186	1.07
		Soluble melanoidins							
9	2:1 Glc–Gly[e]	6.27	60.6	102	0.157	0.075	48	0.113	1.11
10	3:5 Glyoxal–Gly	11.22	60.6	102	0.157	0.138	88	0.268	1.14

[a] The symbols Glc, Fru, HMF, and Gly refer respectively to D-glucose, D-fructose, 5-(hydroxymethyl)-2-furaldehyde, and glycine. [b] Equivalent to mmol of standard 0.0213M NaOH consumed during complexation. [c] This ratio represents the number of Cu^{2+} ions bound per unit of 4 nitrogen atoms in the melanoidin. [d] From a large-scale preparation; 6.14% N. [e] Hydrated at 50% RH; 6.27% N; 16.3% H_2O.

TABLE III

Preparation of melanoidins[a] at pH 3 and 60°

	Initial concentration of reactants (M)		*Reaction*			*Product analysis*				
Type	*Carbohydrate*	*Amino acid*	*Volume (mL)*	*time (days)*	*Yield (g)*	*% H_2O*	*% C*	*% H*	*% N*	*Product solubility*
4:1 Glc–Gly	2.4	0.6	200	21	1.45	9.4	54.42	5.35	5.16	Insoluble pH 3–7
4:1 Glc–Gly	1.2	0.3	200	45	0.22	9.9	52.59	4.95	5.35	Insoluble pH 3–7
2:1 Glc–Gly	1.2	0.6	200	27	0.43	10.1	53.97	5.22	5.79	Insoluble pH 3–7
1:1 Glc–Gly	0.6	0.6	200	33	0.28	10.2	52.14	4.86	6.18	Insoluble pH 3–7
1:1 Glc–Gly	1.2	1.2	200	19	1.08	9.7	54.04	5.34	6.08	Insoluble pH 3–7
1:2 Glc–Gly	0.6	1.2	200	24	0.68	9.7	53.01	5.11	6.50	Insoluble pH 3–7
1:5 Glc–Gly	0.5	2.5	200	17	1.58	9.3			6.93	Insoluble pH 3–7
1:11 HMF–Gly	0.06	0.66	130	20	0.28	10.9			6.97	Insoluble pH 3–5

[a] All reaction mixtures were acidified with HCl and heated in the dark under N_2 for the desired length of time. Products were washed with water, dried under vacuum at 25°, and equilibrated at 31% r.h.

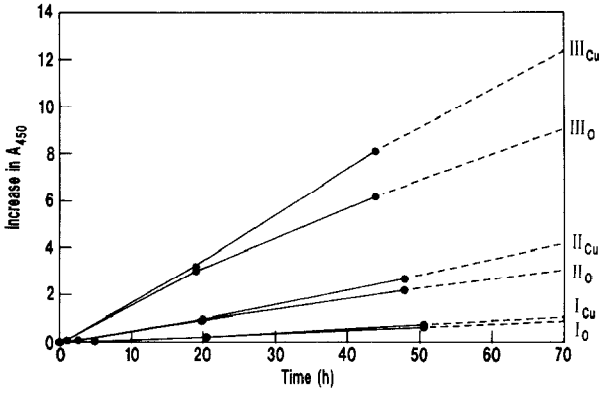

Fig. 10. Visible absorbance study of the influence of Cu^{2+} on rate of melanoidin formation in glyoxal–glycine reactions at pH 3.0 and 60°. The initial concentrations of glyoxal in systems I, II, and III are, respectively, 0.66, 0.132, and 0.264M. The initial concentration of glycine is 0.66M in each system. The subscripts *Cu* and *O* refer, respectively, to systems with Cu^{2+} (100 p.p.m.) and without Cu^{2+}.

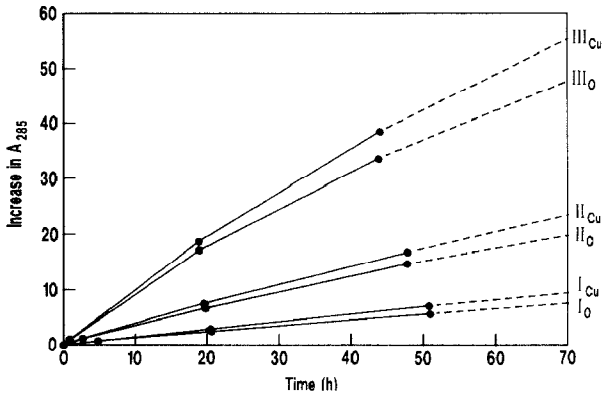

Fig. 11. U.v. absorbance study of the influence of Cu^{2+} on glyoxal–glycine reactions at pH 3.0 and 60°. These systems are the same as those described in Fig. 10.

carboxyls are associated with amino acid moieties in the polymer. Radioactive-labeling and ^{13}C-n.m.r. studies by Feather and Nelson[21] have shown that, in a D-glucose–glycine reaction, both carbon atoms of glycine are incorporated into the melanoidin polymer. It is conceivable that, under the reaction conditions that we used for preparing melanoidins for copper-binding studies, one of the two carbon atoms of a large number of glycine moieties belongs to a free carboxyl group. If this is assumed to be true, then the Cu^{2+}/4N values in Table III would indicate that every glycine moiety in the HMF–glycine melanoidin contains close to one free carboxyl and that, in general, half of the glycine moieties in the other melanoidins in Table III contain a free carboxyl.

Reaction of glycine with glyoxal and HMF. — Because glyoxal, a product of sugar degradation during caramelization[22], and HMF, a product of the Maillard reaction[1], can both be looked upon as direct precursors in melanoidin formation, a set of

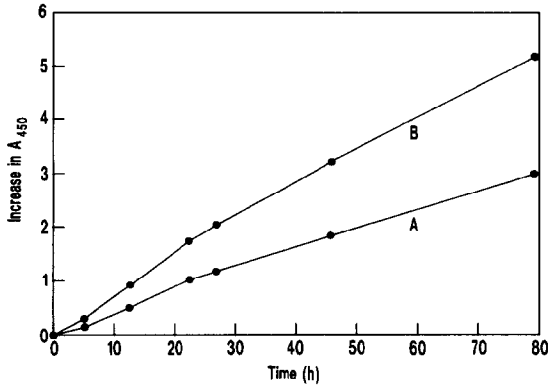

Fig. 12. Visible absorbance study of the influence of Cu^{2+} on rate of melanoidin formation in HMF–glycine reactions at pH 3.0 and 60°. System A: 0.066M HMF, 0.66M glycine, and no Cu^{2+}. System B: 0.066M HMF, 0.66M glycine, and 100 p.p.m. Cu^{2+}.

experiments was designed to determine whether Cu^{2+} has any catalytic effect upon reaction of these two compounds with glycine at pH 3.0. The rates of both reactions were increased by the presence of Cu^{2+} as is shown in Figs. 10–12. From these experiments it may be concluded that augmentation of melanoidin formation in a glycine–hexose system is not derived exclusively from metal catalysis of an early step in the Maillard reaction that produces HMF or some other direct precursor.

In the glycine–glyoxal reaction (Fig. 10), although no precipitate formed in either system I_o or system I_{Cu} for more than 100 h, a trace of brown, insoluble melanoidin began to appear in II_o and III_o at 100 and 70 h, respectively. In systems II_{Cu} and III_{Cu}, formation of insoluble melanoidin was more rapid; precipitation began there at about 95 and 50 h, respectively. In the HMF–glycine reaction (Fig. 12), insoluble melanoidin first appeared in the copper-free system at 22 h and in the Cu^{2+} solution at about 18 h.

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