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# Metal ion complexation by products of the Maillard reaction

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Potentiometric proton-liberation experiments were used to study the interaction between metal ions and a crude Maillard reaction product (MRP) of glucose–glutamate or a pure product of the Maillard reaction, the Amadori compound fructosyl glycine. The present study is the first to report values for stability constants for complex formation involving products of the Maillard reaction and metal ions. Proton displacement suggested binding of $Zn^{2+}$, $Cu^{2+}$, $Mg^{2+}$ and $Ca^{2+}$ by the glucose–glutamate MRP. The strength of binding occurred in the order $Mg^{2+} > Cu^{2+} = Ca^{2+} > Zn^{2+}$. The order of binding affinity differs from that expected for a mononuclear binary system as predicted by the Irving–Williams series which might suggest the presence of two or more distinct ligands in the MRP.

Proton displacement suggested the formation of complexes of fructosyl glycine with zinc but not with calcium. The $pK_a$ value of fructosyl glycine was notably higher than that of glycine (3.25 vs 2.23, respectively), whereas the $pK_b$ value was reduced (8.93 vs 9.73, respectively). Such changes in protonation may help to explain the lower affinities for zinc exhibited by fructosyl glycine compared with glycine (glycine: log $K_1$, 5.40; log $K_2$, 4.47; log $K_3$, 2.73. Fructosyl glycine: log $K_1$, 4.27, log $K_2$, 3.83; log $K_3$, 1.92). The results of the present study may explain some of the effects of Maillard reaction products on mineral homoeostasis *in vivo*. However, it is likely that the biokinetics and metabolism of such compounds also play a major role in mediating observed effects. Copyright © 1996 Elsevier Science Ltd

## INTRODUCTION

There is accumulating evidence for the ability of Maillard reaction products to complex metal ions. Most of the evidence is circumstantial and derives from studies on mineral homoeostasis *in vivo* both in animals and in humans.

Intravenous infusion of heat-sterilized parenteral solutions containing Maillard reaction products resulted in increases in the urinary excretion of copper, zinc and iron in human subjects (Stegink *et al.*, 1981). Oral administration of such solutions appeared not to influence urinary mineral excretion. Johnson *et al.* (1983) also concluded that urinary trace metal excretion was not affected by the oral administration of a diet containing products of the Maillard reaction. However, the experimental diet containing cornflakes resulted in a decrease in long-term $^{65}Zn$ retention compared with the control diet containing corn grits. Significantly, the urine of subjects fed the cornflake diet contained high molecular weight compounds that appeared to bind zinc.

Urinary zinc excretion increased dramatically (up to six-fold) in rats fed heat-treated (3 days at 60 °C) spray-dried casein–glucose (Furniss *et al.*, 1989). Urinary excretion was increased and retention of $^{67}Cu$ was decreased (~30%) in rats fed a sterilized product containing 8514 mg fructosyl lysine per kg protein compared with a pasteurized product (2314 mg fructosyl lysine per kg protein) (Rehner & Walter, 1991). O'Brien *et al.* (1994) reported a reduction in zinc status of rats fed 0.5% of a soluble glucose–glutamate MRP, which they attributed to increases in both faecal and urinary zinc excretion. Copper and iron status were not affected.

Based on proton liberation, Rendleman (1987) reported binding of copper to insoluble Maillard products of fructose–glycine or glucose–glycine at pH 5.0. Calcium appeared to bind weakly to insoluble Maillard products of glucose–glycine, fructose–glycine and glucose–glutamic acid at pH 7.0. The binding of zinc by MRPs of glycine–glucose, lysine–glucose, leucine–glucose, proline–glucose, glutamate–glucose, glycine–lysine–glucose and

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glycine–lysine–glutamate–glucose appeared to be largely reversible under simulated digestion conditions (Whitelaw & Weaver, 1988). However, the binding of $Zn^{2+}$ by a browned corn meal appeared to be only partially reversible under similar conditions. Rendleman (1987) demonstrated an increase in the calcium and copper binding capacity of toasted bread containing lactose. Affinity for copper was greater than that for calcium.

Homma *et al.* (1986), using equilibrium gel filtration and potentiometric titration, demonstrated the ability of brown pigments in coffee to bind to copper, iron or zinc. Hashiba (1986), using a simple spectrophotometric assay, showed that melanoidins of a glucose–glycine system may bind iron. Rendleman (1987) reported considerable soluble complex formation (43% loss of free $Ca^{2+}$) upon adding $Ca^{2+}$ to coffee. In addition, there was evidence of small amounts of insoluble complex formation when $Ca^{2+}$ or $Cu^{2+}$ was added. However, it is unclear to what extent MRPs were responsible for this effect. The most active Zn-binding fraction in coffee as separated by Cu-chelating Sepharose 6B, contained a compound of molecular weight less than 3 kDa (Homma *et al.*, 1990). More recently, Homma & Murata (1994) reported the presence of ligands for $Zn^{2+}$ and $Fe^{2+}$ in coffee with molecular weights of ~36 and ~50 kDa. The complexes with iron were more stable than those with zinc.

However, previous studies of the mineral interactions of MRPs have failed to characterize the binding sufficiently to express the strength of the interaction. Furthermore, no information is presently available on the ability of low molecular weight Maillard products to bind metal ions. In the absence of such information, it is difficult to determine whether individual MRPs are likely to influence mineral metabolism *in vivo*. Considering the avidity of most free amino acids for metal ions (Hay & Williams, 1978), it is reasonable to assume that many free Amadori compounds would possess similar activity. In addition, since ingestion of low amounts of free amino acids does not normally result in increased urinary losses of metal ions, it may be hypothesized that MRPs might bind metal ions more strongly than the amino acid from which they were derived.

Thus, the present studies were conducted to: investigate the ability of a crude MRP of glucose–glutamate to bind zinc, copper, calcium or magnesium *in vitro*; investigate the ability of a pure Amadori product, fructosyl glycine, to bind calcium or zinc *in vitro* (fructosyl glycine was studied because of the ease of preparation compared with other Amadori products derived from amino acids); and compare the strength of metal ion binding, where applicable, by glycine and fructosyl glycine.

## MATERIALS AND METHODS

### Synthesis, isolation and purification of fructosyl glycine

Fructosyl glycine was synthesized according to the method of Hagan *et al.* (1970). Glucose (50 g) and glycine (5 g) were suspended in 1 litre of absolute methanol and refluxed for 3 h. This solution was concentrated by rotary evaporation *in vacuo* (Rotavapor, Buchi, Switzerland) and the resulting brown syrup dissolved in 200 ml of water for ion-exchange chromatography.

Fructosyl glycine was separated by stepwise trichloroacetic acid (TCA) elution on a bed of cation exchange resin (Dowex 50W X8, $H^+$ form, 100–200 mesh, Sigma Chemical Co., Poole, Dorset, UK). Aliquots of fractions were tested for the presence of glucose, Amadori compound and amino acid. Glucose was determined using a glucose oxidase–*o*-dianisidine assay (Sigma kit No. 510, Sigma Chemical Co., Poole, Dorset, UK). Fructosyl glycine concentration was determined using the cold alkaline ferricyanide test (Borsook *et al.*, 1955). The presence of glycine was determined by reaction with ninhydrin on filter papers upon heating.

The column was first washed with distilled water until fractions were negative for glucose. The Amadori compound was eluted with 0.5 M TCA. Resin was regenerated by the method of Moore & Stein (1951) with 4 M HCl and subsequently washed with distilled water until the eluate was negative for chloride using silver nitrate solution.

Fractions containing the Amadori compound were pooled and extracted ~10 times with small volumes of ether to remove TCA. These solutions were then freeze-dried using a New Brunswick Scientific freeze drier (New Brunswick Scientific, New Brunswick, USA) to yield a light brown syrup. The syrup was dissolved in absolute methanol; crystallization was initiated by scratching the base of the beaker with a glass rod and adding a few drops of ether to the solution. A second crystallization was conducted in a similar way and the white product obtained was washed several times in absolute ethanol before drying *in vacuo* over phosphorus pentoxide. The material was subsequently stored over phosphorus pentoxide at room temperature prior to use.

The purity of the Amadori compound was examined by TLC on silica gel $60F_{254}$ plates (Merck, Darmstadt, Germany) using a butanol–acetic acid–water (12:5:3) solvent system. Chromatograms were developed using either ninhydrin, alkaline ferricyanide or anthrone reagent (Borsook *et al.*, 1955).

The carbon, hydrogen and nitrogen content of the purified material was determined in duplicate on ~1-mg samples using a Perkin-Elmer 240 elemental analyser (Perkin-Elmer Ltd, Beaconsfield, Buckinghamshire, UK). The content of oxygen was calculated by difference. Melting points were obtained using capillary tubes in a melting point apparatus (Electrothermal). FT-$^{13}$C-NMR spectra were recorded on a JOEL FX60 Fourier transform $^{13}$C-NMR spectrometer (JOEL Ltd, Colindale, London, UK) using 10-mm sample tubes. Trimethylsilane was used as internal standard. The $^{13}$C-NMR spectrum of fructosyl glycine in DMSO resulted from 48 000 scans, a pulse width of 1.2 s and a pulse angle of 4 μs.

The mass spectrum of the isolated Amadori compound was recorded using a VG Analytical 70E mass spectrometer with a fast atom bombardment probe (VG Analytical Ltd, Manchester, UK) attached to a Super Incos 2400 data system (Finnigan Mat, Hemmel Hempstead, UK). The fast atom bombardment (FAB) mass spectrum was read in the positive ion mode.

## Materials

Maillard reaction product (MRP) was prepared as described previously (O'Brien *et al.*, 1994) from monosodium glutamate and glucose and the freeze-dried material was used to prepare solutions for potentiometric titration. All materials, unless otherwise specified, were obtained from BDH Ltd, Poole, Dorset, UK and were of analytical reagent grade. The $ZnCl_2$, $CuCl_2$, $MgCl_2$ and $CaCl_2$ used to prepare metal solutions were dried overnight at 100 °C prior to dissolution. Glycine was obtained from Reidel de Haen AG, Seelze, Germany.

## Methods

*Potentiometric titrations*

Proton liberation experiments exploit the competition between protons and metal ions for the ligand to derive stability constants from pH measurements. The approach involves the measurement of pH as a function of the concentration of acid or base added at constant metal ion and ligand concentration. Titrations were performed using a PHM-85 digital pH meter (Radiometer, Copenhagen, Denmark) interfaced via an RS-232C interface to an IBM personal computer, model XT. Data storage was facilitated using a program which stored paired values of pH and base concentration for each titration until further data analyses could be conducted. Base (5 M KOH) was added in 50-μl increments and pH was recorded to 0.001 units. Base was standardized daily against 1 M HCl (Convol Volumetric Standard) and a two-point calibration, using pH 4.0 and 7.0 buffers, was performed before each titration.

The electrode pair consisted of a Radiometer G202C glass electrode and a K401 calomel reference electrode. The volume of solution taken for each titration was 50 ml and all titrations were performed in duplicate at 25 ± 1 °C. All glassware used for titrations was acid-washed overnight in 5% $HNO_3$, followed by two rinses in distilled water to remove interfering metal ions.

All solutions contained known concentrations of HCl to lower the starting pH to ~pH 1.0. In order to relate this system to physiological conditions, 0.15 M NaCl was chosen as a background electrolyte. In addition, using NaCl concentrations in excess of that of the reacting species permitted the use of concentration terms instead of activities of the reactants by holding the ionic strength and hence the activity coefficients constant. The metal concentration used in all cases was 20 mM and solutions were standardized by comparison with Spectrosol (BDH Ltd) standards using atomic absorption spectroscopy (Pye-Unicam SP-9, Pye Unicam Ltd, Cambridge, UK). The concentrations of MRP used for these titrations were 0.8, 1.6 or 3.2 g/100 ml. The concentrations of glycine and fructosyl glycine employed were 40 or 80 mM. Equilibration periods of up to 20 min were employed in some regions of these titration curves to produce steady pH readings. Proton liberation curves for the crude MRP were prepared by plotting the means of least-squares slopes of $[H^+]$-ligand concentration plots vs pH. Determination of protonation and stability constants of glycine and fructosyl glycine were conducted as described below.

*Determination of protonation constants*

The determination of both the protonation constants and the stability constants for metal complex formation were based on Bjerrum's formation function, $\bar{n}$, as described by Beck (1970). The $\bar{n}$ function is a secondary concentration variable expressing the average number of ligand molecules, $L$, attached to the metal ion, $M$, or, in the case of protonation, the average number of protons per ligand molecule.

Protonation constants ($K_a$ and $K_b$) for glycine and fructosyl glycine were determined by effectively treating the proton as a ligand and the glycine or fructosyl glycine as a metal ion in a titration of a metal-free system. The formation function $\bar{n}_L$ for protonated species was defined as follows:

$$\bar{n}_L = \frac{[H]_L}{[L]_U} \tag{1}$$

where $[H]_L$ = concentration of bound protons corrected for dilution, $[L]_U$ = total concentration of ligand molecules not complexed with metal ion ($= [L] + [LH] + [LH_2]$) corrected for dilution. Since $[H]_T$ (total concentration of protons) $>> [H]$, $[H]_T \sim [H]_L$ and therefore equation (1) approximates to:

$$\bar{n}_L = \frac{[H]_T}{[L]_U}. \tag{2}$$

Assuming that at $\bar{n}_L < 0.8$, only the protonation reaction

$$L + H^+ \overset{K_b}{\rightleftharpoons} LH \tag{3}$$

need to be considered as taking place, $\bar{n}_L$ approximates to:

$$\bar{n}_L = \frac{[LH]}{[L] + [LH]} \tag{4}$$

Substituting for equation (3),

$$\bar{n}_L = \frac{K_b[L][H]}{[L] + K_b[L][H]} \tag{5}$$

Rearranging and taking logs:

$$\frac{\bar{n}_L}{1 - \bar{n}_L} = K_b[H] \tag{6}$$

$$\log K_b = \log\left(\frac{\bar{n}_L}{1-\bar{n}_L}\right) + \text{pH} \quad (7)$$

Similarly, at $\bar{n}_L > 1.2$, only the protonation reaction:

$$L + H^+ \overset{K_a}{\rightleftharpoons} LH_2 \quad (8)$$

need be considered as taking place, and the function

$$\log K_a = \log\left(\frac{\bar{n}_L - 1}{2-\bar{n}_L}\right) + \text{pH} \quad (9)$$

can be derived from equations (2) and (8). Equations (7) and (9) were used to calculate values for $K_a$ and $K_b$ for each suitable data pair for each titration. Protonation constants derived as such were subsequently averaged to minimize experimental error.

*Determination of stability constants*

The stepwise stoichiometric stability constants for glycine or fructosyl glycine with zinc were determined in a similar manner (Beck, 1970). The value of $\bar{n}$ for each point on a titration curve was calculated as follows:

$$\bar{n} = \frac{\text{Total bound ligand}}{\text{Total metal}} = \frac{[L]_T - [L]_U}{[M]_T} \quad (10)$$

Substituting equation (1),

$$\bar{n} = \frac{[L]_T - [H]_L/\bar{n}_L}{[M]_T} \quad (11)$$

where: $[L]_T$ = total ligand concentration corrected for dilution, $[M]_T$ = total metal ion concentration corrected for dilution and

$$[H]_L = [L]_T - [\text{KOH}] + [\text{HCl}] - [\text{H}] + [\text{OH}] \quad (12)$$

Once $K_a$ and $K_b$ are known, equation (13) holds for all points on the titration curve

$$\bar{n}_L = \frac{K_b[H] + 2K_aK_b[H]^2}{1 + K_b[H] + K_aK_b[H]^2} \quad (13)$$

The right-hand side of equations (12) and (13) contain variables that are either known or measurable. Thus, paired values of $\bar{n}_i$ and $[L]_i$ were calculated for each titration point. The determination of values of $\bar{n}_i$ and $[L]_i$, as such, made no assumptions concerning the complexity of the system. The complexation reactions were assumed to occur in order of increasing complexity as follows:

$$M + L \overset{K_1}{\rightleftharpoons} ML \quad (14)$$

$$ML + L \overset{K_2}{\rightleftharpoons} ML_2 \quad (15)$$

$$ML_2 + L \overset{K_3}{\rightleftharpoons} ML_3 \quad (16)$$

The calculation of the stepwise stability constants ($K_1$, $K_2$ and $K_3$, for the formation of a maximum of three complexes $ML_1$, $ML_2$ and $ML_3$, respectively) from these data was performed by the numerical method of Block & McIntyre (1953, 1955) as described by Beck (1970) which, in contrast to other techniques, enabled such calculation directly from the raw data without the need for interpolation. Stability constants derived from each titration curve were averages of three numerical solutions to the $\bar{n}_i$ and $[L]_i$ data. All titrations were performed in duplicate.

The overall (cumulative) stability constants $\beta_2$ and $\beta_3$ for the formation of complexes $ML_2$ and $ML_3$, respectively, were calculated as follows:

$$b_n = K_1.K_2.K_3...K_n. \quad (17)$$

Examination of the species distribution vs pH for glycine and fructosyl glycine was facilitated by plotting the partial mole fraction of total metal bound, $\alpha_c$, for complex component $ML_C$ vs pH, where:

$$\alpha_C = \frac{[ML_C]}{[M]_T} = \frac{\beta_C[L]^C}{1 + \sum_{n=1}^{n=N} \beta_n[L]^n} \quad (18)$$

thus, for a system with three complexes ($N = 3$):

$$\alpha_0 = \text{Free metal ion} = \frac{1}{1 + \beta_1[L] + \beta_2[L]^2 + \beta_3[L]^3} \quad (19)$$

$$\alpha_1 = \frac{\beta_1[L]}{1 + \beta_1[L] + \beta_2[L]^2 + \beta_3[L]^3} \quad (20)$$

$$\alpha_2 = \frac{\beta_2[L]^2}{1 + \beta_1[L] + \beta_2[L]^2 + \beta_3[L]^3} \quad (21)$$

and

$$\alpha_3 = \frac{\beta_3[L]^3}{1 + \beta_1[L] + \beta_2[L]^2 + \beta_3[L]^3} \quad (22)$$

The present approach neglected any possible contribution from the sugar hydroxyl groups which is likely to be negligible under the pH conditions used compared

with the activity of the carboxyl and amino groups (Burger & Nagy, 1990).

*Statistical analysis*

Statistical analysis of data was performed using the Minitab Statistical Package (Ryan *et al.*, 1976) on a DEC Vax 11/780 computer.

## RESULTS

### Chemical characterization

The isolated Amadori compound appeared to be pure as assessed by thin-layer chromatography. Fructosyl glycine appeared as a single spot ($R_f = 0.14$) whether visualized using ninhydrin or alkaline ferricyanide reagent. In the same system, glycine appeared at $R_f = 0.25$, and glucose, which was detected using anthrone reagent, had an $R_f$ value of 0.30.

Similarly, the results of elemental analysis suggested a high degree of purity for the Amadori preparation [found (%): C, 40.45; H, 6.24; N, 5.85; calculated (%): C, 40.51; H, 6.33; N, 5.91]. The crystalline fructosyl glycine had an average melting point of 151 °C compared with 155 °C for glucose and 245 °C for glycine. However, the melting point obtained in the present study for fructosyl glycine is slightly higher than the value of 146 °C obtained by Anet (1957) and the values 146.5–147.5 °C obtained by Mossine *et al.* (1994).

The $^{13}$C-NMR chemical shift values obtained for the carbons of the sugar moiety of fructosyl glycine (Table 1) were close to those of pure fructose (Levy *et al.*, 1980) and even closer to those of the fructosyl moieties of other Amadori products (Moll *et al.*, 1982). An up-field shift was evident for the carboxyl group of the Amadori compound compared to free glycine (180.56 compared to 168 ppm) (presumably associated with a reduction in electron density). The present $^{13}$C-NMR data confirm the tautomeric equilibrium of fructosyl glycine. The conditions used in the NMR experiment did not permit calculation of the abundance of individual tautomers due to saturation effects. However, the C-2 signals suggested an abundance of the α-furanose and β-pyranose configurations.

Mass spectral analysis of fructosyl glycine by the conventional chemical ionization technique yielded no parent peak, probably due to the instability of the compound. However, using a fast atom bombardment probe, the mass spectrum (Fig. 1) yielded a parent peak of 238 ($MH^+$) and one of the fragments probably corresponds to glycine [75($M$)]. This pattern is consistent with previously published mass spectra of other Amadori compounds (Moll *et al.*, 1982) with the exception that a fragment which clearly corresponded to fructose (180) was not observed in the present study. Peaks at 257, 297 and 330 are attributable to the glycerol matrix (Dell, 1987).

The results confirm the structure of the Amadori compound prepared and suggest a high degree of purity.

**Table 1. $^{13}$C-NMR peak assignments for fructosyl glycine in DMSO solution**

| δ (ppm)/TMS | Fructosyl carbon | Configuration | Glycine carbon |
|---|---|---|---|
| 168.67 | | | COOH |
| 168.48 | | | |
| 101.36 | 2 | α-f, β-f | |
| 99.41 | | | |
| 95.44 | 2 | α-p, β-p | |
| 95.18 | | | |
| 83.17 | 3 | α-f | |
| 82.19 | 5 | α-f, β-f | |
| 81.48 | | | |
| 78.29 | 4 | α-f | |
| 75.56 | 3 | β-f | |
| 74.79 | 4 | β-f | |
| 70.37 | 3 | α-p | |
| 68.94 | 4 | α-p, β-p | |
| | 5 | α-p, β-p | |
| 63.54 | | | |
| 63.28 | | | |
| 62.57 | 1 | α-p, β-p, α-f, β-f | |
| 60.36 | 6 | α-p, β-p, α-f, β-f | |
| 53.08 | | | |
| 51.59 | | | C-α |
| 49.96 | | | |

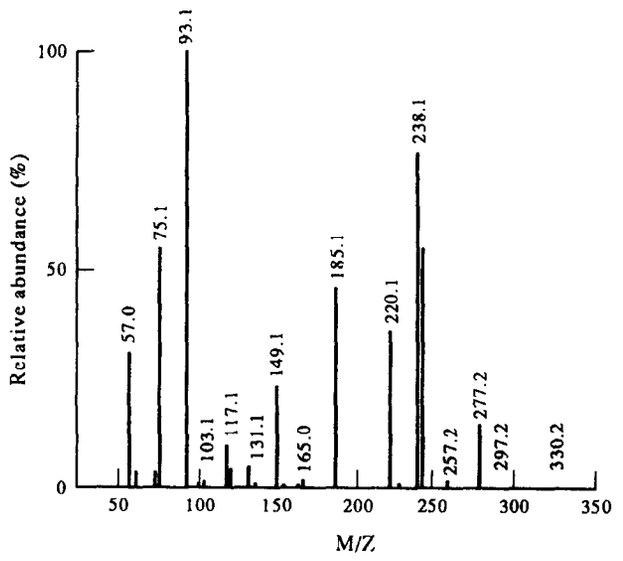


**Fig. 1.** FAB-mass spectrum of fructosyl glycine.

### Metal ion binding to crude MRP

Proton liberation as a function of pH for the crude MRP preparation is described in Fig. 2. Clearly, proton displacement is evident in the case of zinc, copper, magnesium and calcium, indicating that components of the MRP were capable of binding these metal ions. The extent of proton displacement in the pH range 3.5–10 suggests that the strength of complexation occurred in the order:

$$Mg^{2+} > Cu^{2+} = Ca^{2+} > Zn^{2+}.$$

Unfortunately, the pH electrode used did not allow accurate measurement of pH values greater than 10 and it was decided in advance that titrations would

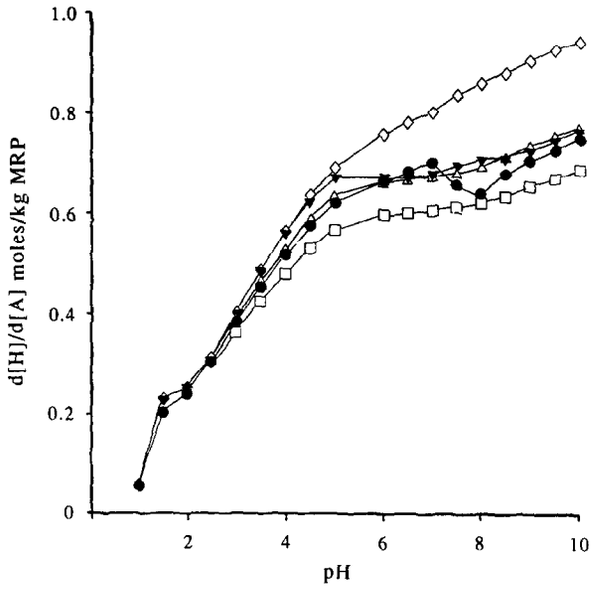


**Fig. 2.** Proton liberation expressed per g of crude MRP of glucose and glutamate as a function of pH in the absence of metal ions (– □ –), or in the presence of zinc (– • –), copper (– ▼ –), magnesium (– ◇ –) or calcium (– △ –). Values are averages for two titrations. Coefficients of variation (not displayed) had an average value of 7.5%.

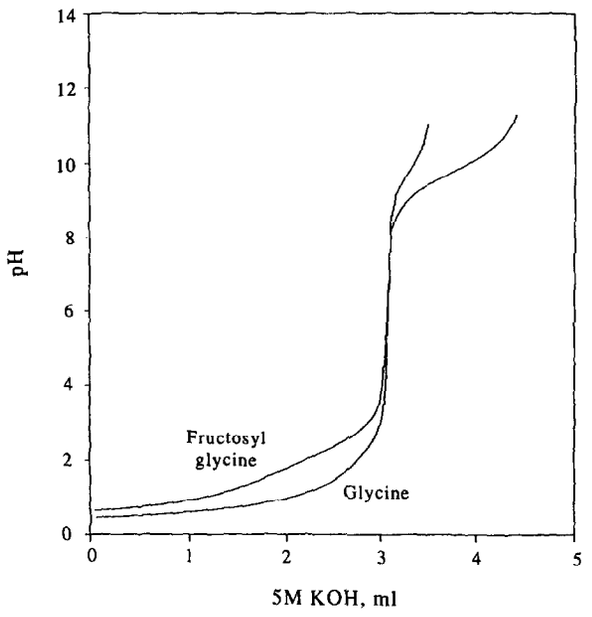


**Fig. 3.** Titration curves for glycine and fructosyl glycine in the absence of metal ions illustrating the higher p$K_a$ and the lower p$K_b$ of fructosyl glycine.

be terminated at this pH. It is of interest that turbidity developed in the MRP solutions in the presence of metal ions at high pH values (> pH 8.0). This provides additional evidence for the formation of a complex and suggests that the solubility of the complex formed is less than that of the free metal ion or ligand.

### Comparison of metal ion binding by glycine and fructosyl glycine

Titration curves for glycine and fructosyl glycine are presented in Fig. 3. Clearly, these curves suggest that fructosyl glycine had a higher p$K_a$ value and a slightly lower p$K_b$ value than glycine. Unfortunately, titration curves were terminated at pH 11.00 due to the unsuitability of the glass electrode used to measure pH accurately in the higher range (Radiometer Copenhagen, *Analytical Electrode Guide*, Publication No. 916-309).

Titration curves for fructosyl glycine in the absence of metal ions or in a zinc or calcium system are presented in Fig. 4. Proton displacement was evident in the case of zinc, indicating that binding occurred. However, the titration curve for fructosyl glycine in the presence of calcium was not significantly different from the titration curve in a metal-free system. Therefore, it appears from these data that fructosyl glycine may bind zinc but not calcium. A similar trend was observed for the titration curves for glycine.

Quantitative data for the binding of protons or zinc by glycine and fructose–glycine are presented in Table 2. While the p$K_a$ of fructosyl glycine was approximately 1 unit higher than that of glycine, its p$K_b$ value was reduced by almost the same amount.

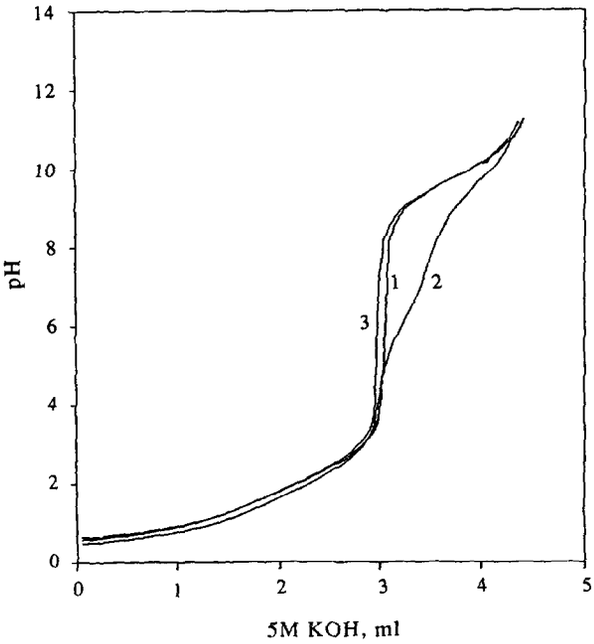


**Fig. 4.** Titration curves for fructosyl glycine in the absence of metal ion (1) or in the presence of zinc (2) or calcium (3). Proton displacement was evident only in the case of zinc.

The affinity of fructosyl glycine for zinc was also reduced compared with glycine. This reduction in binding strength was expressed as a reduction in each of the stepwise formation constants ($K_1$, $K_2$, $K_3$), where one, two or three ligands are co-ordinated to the central zinc. The reduction in binding strength of zinc to fructosyl glycine is illustrated more dramatically by the lower values of overall stability constants ($\beta_2$ and $\beta_3$) compared to glycine. The most dramatic difference in the zinc complexation of glycine and fructose–glycine was the difference between the distribution of species vs pH

**Table 2. Zinc and proton binding data for glycine and fructosyl glycine**

| | Glycine | Fructosyl glycine | *P* value* |
|---|---|---|---|
| Log $K_1$ | 5.40 ± 0.13 | 4.27 ± 0.06 | < 0.0001 |
| Log $K_2$ | 4.47 ± 0.12 | 3.83 ± 0.22 | < 0.0001 |
| Log $K_3$ | 2.73 ± 0.14 | 1.92 ± 0.02 | < 0.005 |
| Log $\beta_2$ | 9.88 ± 0.24 | 8.09 ± 0.19 | < 0.0001 |
| Log $\beta_3$ | 12.49 ± 0.34 | 9.86 ± 0.05 | < 0.005 |
| $pK_a$ | 2.23 ± 0.09 | 3.25 ± 0.15 | < 0.0001 |
| $pK_b$ | 9.73 ± 0.07 | 8.93 ± 0.17 | < 0.0001 |

Means ± standard deviations for two titrations.
*Student's *t*-test.

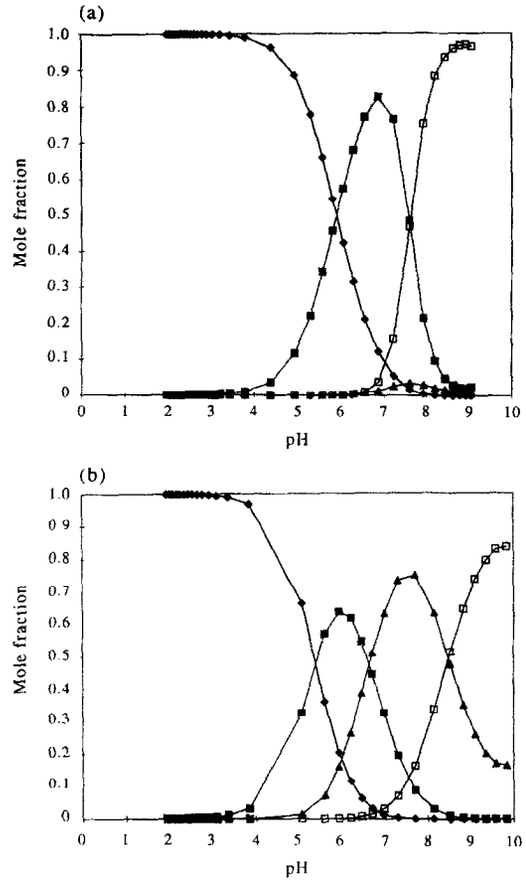


**Fig. 5.** Calculated species distribution for (a) the Zn(II)–fructosyl glycine system and (b) for the Zn (II)–glycine system as a function of pH. Legend: unbound Zn(II) (– ◆ –); $ML$ (– ■ –); $ML_2$ (– ▲ –); $ML_3$ (– □ –). $[M] = 0.02$ M; $[L] = 0.08$ M.

(Fig. 5). In the case of fructosyl glycine, the predominant species at physiological pH was $ML_1$, whereas the species $ML_1$ and $ML_2$ were approximately equally predominant in the glycine–zinc system. The most surprising difference, however, was that the $ML_3$ complex for fructosyl glycine predominated over the $ML_2$ complex at pH 8.0.

## DISCUSSION

The finding that crude MRP can complex copper and zinc is consistent with the results of Homma *et al.* (1986) who studied the brown pigments in coffee. Interestingly, Homma *et al.* (1986), who did not examine the ability of these compounds to complex calcium or magnesium, also reported that copper was bound more tightly than zinc. The stronger binding of copper compared to zinc is also consistent with that predicted by the Irving–Williams series (Irving & Williams, 1953; Phillips & Williams, 1966) for transition metal ions:

$$Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}.$$

However, the present finding that copper was bound more tightly than zinc *in vitro* by MRP appears to be at variance with the results of (O'Brien *et al.*, 1994). In rats fed MRP, it appeared that urinary zinc output was increased more than the excretion of urinary copper. However, it is not clear from the *in vitro* binding results how many compounds in the crude MRP were capable of complexing zinc and copper. Furthermore, it was not possible to predict whether such complexes would be absorbed from the intestine. Thus, even the slight differences in the pharmacokinetics and metabolism of these substances *in vivo* could lead to large differences in their effects on mineral homoeostasis.

Although the rules governing the strength of calcium and magnesium binding are less clearly defined, the order of binding affinity in a mononuclear binary system would be expected to be (Monk, 1951; Irving & Williams, 1953):

$$Cu > Zn > Mg > Ca.$$

Thus, although the relationships between Cu and Zn binding and Mg and Ca binding is consistent with what might be expected, the overall order of binding is not consistent with a simple mononuclear binary system. It is possible that some of the high molecular weight compounds in the MRP system may act as polynuclear ligands. It is also possible that more than one ligand exists in the system examined. Thus, comparisons of the present system with one-ligand systems may be of little value.

Calcium complexation may help to explain the report of O'Brien *et al.* (1988) that the glucose–glutamate MRP inhibited Ca uptake by everted gut sacs *ex vivo*. Paradoxically, such inhibition of Ca absorption does not appear to occur *in vivo* as assessed by balance experiments possibly as a consequence of metabolism or other effects.

The $pK_a$ and $pK_b$ values for glycine (Table 2) were similar to those reported by other workers (Perrin, 1979; Martell & Smith, 1982). Perrin (1979) cites values for the $pK_a$ and $pK_b$ of glycine from the literature of 2.26–3.25 and 9.27–9.94, respectively. The recent paper by Mossine *et al.* (1994) reported $pK$ values of glycine of 2.33 ($pK_a$) and 9.60 ($pK_b$). This suggests that the proton liberation system adopted was suitable for the purpose outlined here, i.e. to compare the values of stability constants of glycine and fructose–glycine for zinc

complexation. The observed increase in $pK_a$ of fructosyl glycine compared with glycine differs from that reported by Mossine *et al.* (1994). Mossine *et al.* (1994) reported a lowering of $pK_a$ for the amino acids in fructosyl glycine, and fructosyl alanine. The $pK_a$s of the Amadori products fructosyl γ-aminobutyric acid, fructosyl δ-aminovaleric acid, fructosyl ε-aminocaproic acid and fructosyl N-α-formyl lysine were unchanged. Mossine *et al.* (1994) proposed that while the inductive effects of the electronegative carbonyl atom contributed to the observed reduction in $pK_a$s, the effect of the sugar hydroxyl groups on solvation was more important.

The observed reduction in $pK_b$ compared with glycine is consistent with previous work. However, the value of 8.93 obtained in the present study was higher than the values of 8.18–8.4 (Anet, 1957; Hagan *et al.*, 1970; Mossine *et al.*, 1994) reported previously. It is possible that impurities in the preparation or inconsistencies in the methods used could account for these differences. This reduced basicity of fructosyl glycine accounts for the more rapid elution of the compound from ion-exchange columns compared with glycine (Hagan *et al.*, 1970).

The values for the formation constants for $Zn^{2+}$–glycine complexes are within the ranges of values reported in the literature (Perrin, 1979; Martell & Smith, 1982) ($K_1$, 3.70–5.92; $K_2$, 4.13– 5.09; $K_3$, 2.51–3.21). A most interesting finding was the failure of fructosyl glycine to bind significant amounts of calcium. This finding alone suggests that calcium homoeostasis *in vivo* may not be influenced by the Amadori compound *per se* which is known to be absorbed and excreted in the urine (Finot & Magnenat, 1981; Nair *et al.*, 1981; Perkins *et al.*, 1981). Thus, it is possible that calcium is bound only by high molecular weight compounds which are not absorbed. The ability of fructosyl glycine to complex zinc in the form of three possible complexes is consistent with what might be expected for a glycine derivative. Surprisingly, however, the strength of binding was reduced compared with glycine. Unfortunately, it is not possible to speculate from these data whether the strength of metal binding with other Amadori compounds would also be less than the parent amino acids. However, if this was so, then the affinity of Amadori compounds for metal ions *in vitro* would be very much at variance with the effects of Amadori compounds on mineral homoeostasis *in vivo*. Nevertheless, it can be argued that the most important differences between Amadori compounds and their parent amino acids in this respect is that Amadori products, if absorbed, are largely excreted unchanged in the urine (Finot & Magnenat, 1981; Nair *et al.*, 1981) whereas the amino acid would presumably enter intermediary metabolism. Thus, Amadori compounds, while retaining the ability to complex metal ions, may not be subject to the same stringent homoeostatic control *in vivo* as their parent amino acids. It is, therefore, likely that the activity of Amadori compounds, and possibly Maillard reaction products in general, on mineral metabolism is more a function of their metabolic transit than their capacity to bind metal ions.

The ability of fructosyl glycine to complex zinc and possibly other metal ions emphasizes the importance of ensuring metal-free preparations when performing spectroscopic experiments such as NMR. This derives from the different chemical shifts for free and co-ordinated ligands (Axtman *et al.*, 1960; Fuentes *et al.*, 1975). The present preparation of fructosyl glycine was found to contain $< 0.03$ mg/g of zinc and $< 0.1$ mg/g of copper by atomic absorption spectrophometry so that this effect was unlikely.

Complexation may have important effects on the absorption, rates of metabolism, interaction with target molecules and excretion *in vivo* (Burger, 1990). Thus, complexation reactions may have a major influence on the toxicity or other activity of MRPs. In the absence of other data, prediction of the effect of MRPs on mineral homoeostasis *in vivo* from stoichiometric stability constants is difficult. It has long been known that the formation of insoluble complexes of trace metals in the intestine may limit bioavailability (Oberleas *et al.*, 1966). However, the effect of soluble complexes on mineral bioavailability is variable (Turnbull *et al.*, 1990; Clydesdale, 1988). Some complexes are actually more bioavailable because of charge neutralization which facilitates transport through cell membranes (Clydesdale, 1988). Formation of complex species involving products of the Maillard reaction and metal ions is insufficient evidence alone to suggest that such complexes would influence mineral bioavailability *in vivo*. However, complex formation offers a plausible explanation for the deleterious effects of MRPs on mineral metabolism observed in previous studies (Johnson *et al.*, 1983; O'Brien *et al.*, 1988, 1994).

Interaction of intermediates and products of the Maillard reaction with metal ions may have a significant influence on the chemistry of the reaction. The decrease in electron density on the donor groups of ligands participating in complexation reactions may result in changes in chemical behaviour and reactivity (Burger, 1990). Changes in electron density and conformational changes resulting from complexation may offer novel opportunities to exercise control over the Maillard reaction. Indeed, Kato *et al.* (1981) reported that $Cu^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ promoted the Maillard reaction of ovalbumen (1% protein, pH 10, 0.5 mg% metal ion). Kawakishi & Uchida (1990) suggested that the autoxidative cleavage of Amadori products in a system containing cupric ions may proceed via their cupric ion complexes.

Lingnert & Eriksson (1981) suggested a mechanism for the antioxidant properties of Maillard products based on a radical chain-breaking effect of reductones and other reducing compounds formed in the Maillard reaction. Indeed, pure reductones prepared from secondary amines have been demonstrated to have antioxidative properties (Evans *et al.*, 1958). However, an alternative mechanism may be via the ability of Maillard compounds to complex heavy metal ions (Eichner, 1981). The present mineral binding results for crude MRP, which demonstrates the ability of MRP to bind

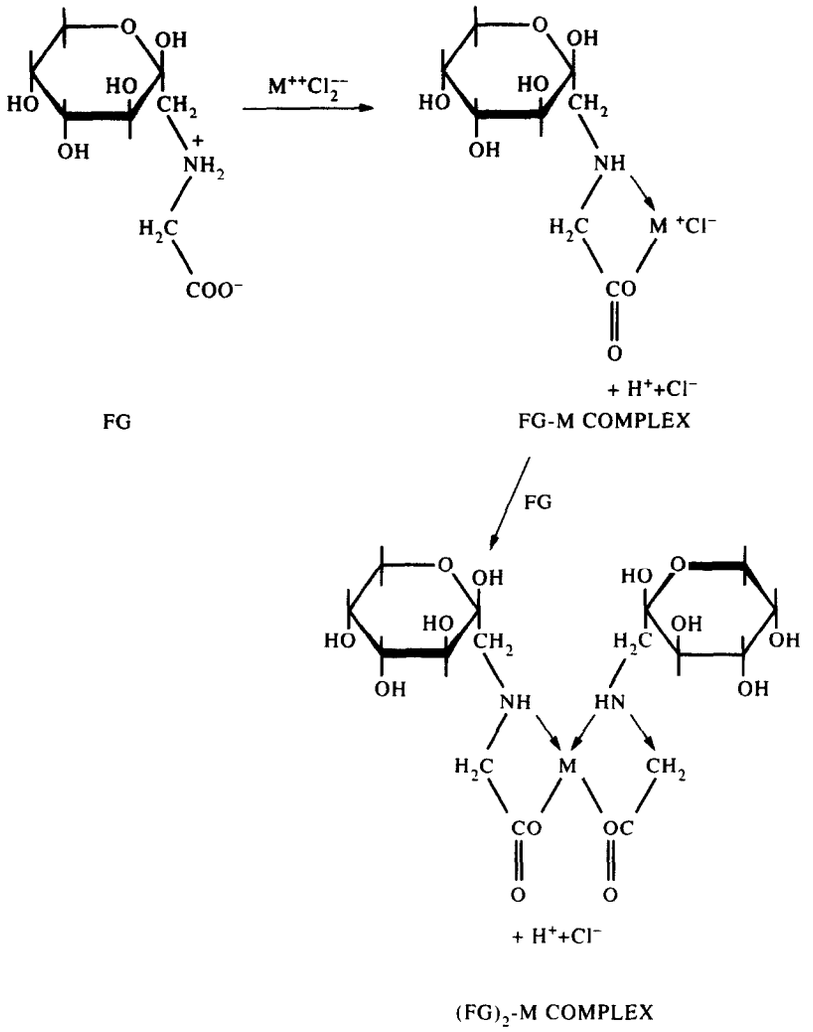


**Fig. 6.** Putative stepwise formation of complexes between fructosyl glycine (FG) and divalent metal ion (M).

zinc and copper, support this hypothesis. In contrast, the weaker binding of zinc to fructosyl glycine compared to glycine appears to contradict the hypothesis. However, glucose–glycine or glucose–glutamate Maillard products may not be very important as antioxidants in food systems in the presence of Maillard products of lysine or arginine. Furthermore, it is possible that Amadori compounds *per se* are not the most important products of the Maillard reaction with respect to antioxidant activity. Clearly, further work is required to examine the mineral binding properties of a range of browned systems for comparison with their antioxidant properties.

The present mineral binding data suggest the formation of a number of ligands for metal ions in the Maillard browning reaction. While the Amadori compound fructosyl glycine may complex zinc and probably copper, the identity of Maillard products which may complex calcium and magnesium remains unknown. The different $pK_a$ and $pK_b$ values of the Amadori compound, fructosyl glycine compared with glycine may offer one explanation for the weaker binding of zinc to the former. The putative structures of the $Zn^{2+}$ complexes of fructosyl glycine may be inferred from previously published work on glycine (Fig. 6). Clearly, steric hindrance may also have contributed to the lower stability of the fructosyl glycine–$Zn^{2+}$ system. The extent to which hydroxyl groups contribute to metal complexation by MRPs has not been examined. Sugars containing an axial–equatorial–axial configuration of hydroxyl groups may complex metal ions although the strength of binding appears to be relatively weak (Clydesdale, 1988). It is possible that such a mechanism might contribute to the activity of advanced MRPs especially in the case of $Ca^{2+}$ or $Mg^{2+}$ complexation. Further work to separate ligands for metal ions from MRPs would be useful to elucidate mechanisms of action and could help predict their potential for disturbing mineral homoeostasis *in vivo*.

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